Mitotin/CENP-F in mitosis, transcriptional control, and differentiation

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Summary

Mitotin/CENP-F is a large nuclear/kinetochore protein containing multiple leucine zipper motifs potentially for protein interactions. Its expression levels and subcellular localization patterns are regulated in a cell cycle-dependent manner. Recently, accumulating lines of evidence have suggested it a multifunctional protein involved in mitotic control, microtubule dynamics, transcriptional regulation, and muscle cell differentiation. Consistently, it is shown to interact directly with a variety of proteins including CENP-E, NudE/Nudel, ATF4, and Rb. Here we review the current progress and discuss possible mechanisms through which mitotin may function.

Mitotin, also named CENP-F, was initially identified as a human autoimmune antigen [1, 2] and an *in vitro* binding protein of the tumor suppressor Rb [3, 4]. Its dynamic temporal expression and modification patterns as well as ever-changing spatial distributions following the cell cycle progression imply itself a multifunctional protein. Recently, growing lines of evidence have highlighted its roles in a wide variety of cellular activities, including mitotic control, microtubule (MT) dynamics, gene regulation, and muscle cell differentiation. New findings have also raised new questions to be addressed in the future.

Biochemical and cytological properties

Mitotin gene is located at human chromosome 1q32–41 [3]. It contains 19 exons and spans about 51 kb (Figure 1). The mRNA is more than 10 kb and encodes a large acidic protein of 3114 amino

acid residues (GenBank Accession No. CAH73032) [3]. The gene expression is cell cycle-dependent, as the mRNA levels increase following S phase progression and peak in early M phase [3]. Such patterns are regulated by the Forkhead transcription factor FoxM1 [5].

Mitotin is a nuclear protein in interphase and exhibits dynamic localizations in M phase. In primate cells, its protein levels are cell cycle-dependent as well. Mitotin/CENP-F is undetectable or weakly expressed in G1. When cells were released from early G1, bulk mitotin appeared several hours after Rb phosphorylation [3]. Therefore, mitotin expression occurs after G1 progression through the restriction point marked by phosphorylation of Rb [6, 7], suggesting a correlation with the S phase entry. Indeed, expression was seen after cells were synchronized in S phase [3]. The high level expression then persists till anaphase [1–3]. Such a property makes mitotin/CENP-F a useful proliferation marker in cancer research [8–11]. Inside cells, mitotin is transiently associated with the outer kinetochore plate and

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Figure 1. Structure of the human mitosis/CENP-F gene. Bold numbers indicate positions of the first and last nucleotides for exons according to a reference genomic DNA sequence (GenBank Accession No. NC_000001). The numbers in each bracket denote the first and last amino acids encoded by each exon. The lengths of exons are drawn to scale.

Figure 2. Schematic comparisons for mitosis orthologs. Sequences are based on GenBank accessions CAH73032 (human mitosis), XP_223060 (rat mitosis), AF194970 (LEK1), AK029517 (5' sequence for mouse mitosis), and U62026 (CMF1). The putative full-length mouse mitosis sequence was obtained by joining AK029517 and AF194970 together. Only sequences with at least three blocks of leucine heptad repeats are considered as a zipper motif. Conserved motifs are indicated by dashed lines.

the spindle apparatus in early M phase and is redistributed to the spindle midzone in anaphase and to the midbody in telophase, followed by complete degradation at the end of cell division [1–3, 12]. It is highly phosphorylated in M phase [3], but whether the phosphorylation is required for its dynamic reorganizations remains unclear. In addition, mitosis/CENP-F is farnesylated at the C-terminal CAAX motif. Such a modification that is required not only for its localization to the nuclear envelope and kinetochores, but also for timely progression of cells through G2/M and degradation of the protein after mitosis [13, 14].

Mitosis contains multiple motifs and functional domains, including a pair of highly-charged tandem repeats and eight leucine zipper motifs potentially for protein–protein interactions (Figure 2) [3, 15]. Its secondary structure is predicted

to be mostly α -helical, except for the C-terminal region of 220 residues, which is proline-rich. In addition to the CAAX motif at the extreme C-terminus [13], mitosis contains an Rb-binding domain and a bipartite nuclear localization signal (NLS) (Figure 2) [3, 12]. NCBI conserved domain search also reveals that it shares low similarities with chromosome segregation ATPase (SMC) at multiple positions (Figure 2).

Mitosis in other species has also been characterized. Its orthologs are termed LEK1 in mouse or CMF1 in chicken [16–18], though the reported sequences do not appear to be full-length (Figure 2). They share overall structural characteristics with mitosis, except that CMF1 does not contain the duplicated region (Figure 2). Divergence analysis suggests that the duplication event appears to occur after separation of bird and mammal from

their common ancestor during evolution [19]. LEK1 targets to kinetochores [19], while such a localization has not been documented for CMF1. Moreover, their interactions with Rb have also been confirmed [20, 21]. Interestingly, both CMF1 and LEK1 manifest distinct expression patterns during development. CMF1 is highly expressed in differentiating chicken heart from stages 9 to 18; after this, the expression levels drop dramatically [16, 18]. Its subcellular localization is also variable. In differentiating cardiac myocytes, CMF1 locates in the cytoplasm [18]. In differentiating skeletal myocytes, however, its distribution is initially in the nucleus in early myoblasts, and then in the cytoplasm [22]. LEK1, in contrast, is ubiquitously expressed in early mouse embryo. However, its expression is dramatically declined throughout the embryo as the overall cell proliferation slows down [17]. Furthermore, LEK1 does not seem to be cell cycle-dependent [17]. How the differences affect their physiological functions, however, still remains unclear.

Mitotin is critical for proper M phase progression

The kinetochore is a three-layer organelle located at the primary constriction, or centromere, of a chromosome. Kinetochore provide sites of spindle MT attachment for proper M phase progression. There dwells a group of proteins to manage MT-related activities such as MT-kinetochore interaction, kinetochore MT dynamics, and MT-based chromosome motility [23–25]. In addition, another group of proteins, for instance, Mad2, functions in the so-called “spindle checkpoint” by monitoring the status of MT attachment to guarantee proper anaphase onset, thus avoiding the disaster of genomic instability [23, 26, 27].

Kinetochore localization of mitotin is affected in several aspects. Its kinetochore targeting is conferred mainly through cooperation between an essential “core” region and the internal repeat, which appears to stabilize the interaction of the core region with kinetochores [28]. Silencing Bub1 [29], CENP-I [30], RanBP2/Nup358 [29, 31], Zwint-1 [32], or Sgt1 [33] also abolishes the kinetochore targeting of mitotin/CENP-F. Based on available information, genetic links for these proteins can be speculated (Figure 3). Which

Figure 3. Possible molecular links for kinetochore targeting of mitotin/CENP-F. Bub1 appears to lie most upstream and is required for kinetochore targeting of all the indicated proteins [29–31, 33, 34]. Zwint-1 is downstream of Bub1 and upstream of mitotin [32], but its relationship with other proteins is not clear. Direct interactions have not been reported among these proteins.

protein(s) directly tethers mitotin, however, is not clear. On the other hand, like many other kinetochore proteins, mitotin is also constitutively transported from kinetochores to spindle poles along MTs by the cytoplasmic dynein/dynactin motor complex [19, 35]. Such a translocation may potentially reduce mitotin levels at the kinetochore after MT attachment [19].

Mitotin is important for proper M phase progression. Silencing mitotin/CENP-F in HeLa cells expressing GFP or YFP-tagged histone 2B (H2B) results in pre-anaphase delay [36–38]. Some chromosomes experience difficulties to reach the spindle midzone. After prolonged mitotic block, cells may exit M phase by either anaphase initiation or chromosome decondensation. Chromosome misalignment is also observed in U2OS cells knocking down mitotin/CENP-F, though anaphase is usually initiated much earlier than in HeLa cells [5]. Nevertheless, variations in mitotic behaviors exist among reports [5, 36–38]. This may be due to different repression efficiencies or incomplete depletion of the entire mitotin protein, considering the large size of mitotin mRNA. Bomont and colleagues find that the MT-kinetochore interface in CENP-F-depleted cells appears

unstable. Mad1, which is essential for kinetochore targeting of Mad2, undergoes repeated association–dissociation cycles at the kinetochore [36]. Holt and colleagues report that silencing CENP-F weakens the adhesion between sister chromatids [38]. Therefore, in addition to efficiencies of RNAi, different cell lines or sublines might exhibit different sensitivity to these defects, accordingly manifesting variations in behavior.

Mitosin appears to play a role in correction of false MT–kinetochore connections. During prometaphase/metaphase transition, there are chances for MTs from the same pole to associate with both sister kinetochores in a chromosome to form syntelic attachment and for those from opposing poles to bind the same kinetochore to form merotelic attachment [39]. These incorrect attachments are destabilized or compromised during M phase progression in intact cells [40–42] but appear to persist in mitosis-depleted HeLa cells [37, 38]. Among key factors involved in the correction of false MT attachments are Aurora B kinase and the kinesin motor MCAK [42–44]. Whether mitosis is linked to these molecules remains unclear.

Mitosis-depleted HeLa cells show reduced tension across sister kinetochores in fully aligned chromosomes [36–38]. This can be just a consequence of merotelic MT attachment [37, 38]. Nevertheless, as kinetochore pairs oriented roughly in parallel with the spindle axis do not differ from the unparallel ones in their interkinetochore distances [37], attenuated functions of kinetochore motors, such as cytoplasmic dynein and CENP-E, may contribute to the tension reduction as well [23]. Mitosis/CENP-F has been shown to directly bind CENP-E, whose depletion also results in misaligned chromosomes [45–48]. Dynein is linked to mitosis through other proteins (Nudel and NudE) as well (see below). Both CENP-E and dynein are sensitive to MT attachment, probably due to their poleward transport, with CENP-E as a dynein cargo [35, 49]. Upon mitosis depletion, dynein and CENP-E are further reduced at kinetochores in MT-dependent manner, suggesting that mitosis stabilizes them against MT-dependent shedding [37]. Decreased steady-state levels of kinetochore dynein and CENP-E may thus result in reduced tension across bi-attached kinetochores.

Figure 4. Interaction between mitosis and Nudel in yeast two-hybrid system. (a) The kinetochore-binding regions of mitosis interact with Nudel. Schematic diagrams of different mitosis mutants are shown. The number above each diagram represents amino acid position. Mitosis^{ATK} is a mutant capable of efficient kinetochore localization, while mitosis^{ATC} contains a point mutation that disrupts the localization [28]. Nudel^{P1} (containing residues 142–345) is a partial polypeptide identified from a two-hybrid screen for mitosis-associated proteins. The β -galactosidase activity of each combination is shown as Mean $\pm$ SD. (b) Mapping for the mitosis-binding domain of Nudel. Different Nudel mutants were tested for their abilities to interact with mitosis^{ATK}.

Mitotin appears to function in MT dynamics as well. Mitotin-depleted cells exhibit higher incidence of spindle abnormality including multi-astral and aberrant bipolar spindles [37, 38]. Recently, overexpressing a cytoplasmic form of LEK1 (cytLEK1) is shown to disorganize MT arrays in interphase 3T3 cells through interaction with murine NudE [50]. NudE and its paralog Nudel (NudE-like) are dynein regulators that bind both dynein and another dynein regulator Lis1 [51–55]. Lis1 can alter MT dynamics by reducing the catastrophe events of the MT plus ends [56, 57, 58]. Dynein also regulates disassembly of MT minus end in M phase [59]. We cloned Nudel in a yeast two-hybrid screen using mitotin kinetochore binding domains as bait (Figure 4) [53]. Mitotin binds Nudel at a region similar to the LEK1-binding domain of NudE (Figure 4) [50]. However, as the NudE-binding domain of LEK1 is equivalent to residues 2598–2675 of mitotin, NudE and Nudel apparently bind different regions on mitotin (Figure 4). Human mitotin is mainly a nuclear protein in interphase [1–3, 19]. Given the activity of cytLEK1 on MTs in interphase, however, mitotin may modulate MT dynamics through Nudel and NudE in mitosis after nuclear envelope breakdown and thus contributes to spindle organizations and functions. On the other hand, more lines of solid evidence are still required to support the existence of an endogenous cytoplasmic form of mouse mitotin in interphase. At least, the documented exogenous cytLEK1 [50] appears to still lack ~500 amino acids at its N-terminus (Figure 2) and may thus behave merely as a dominant-negative mutant.

Mitotin may have a role in maintenance of chromosome condensation

An intriguing phenotype of mitotin/CENP-F-depleted mitotic cells is the chromosome decondensation before anaphase onset [37, 38]. This occurs unlikely as a result of excess illumination during live cell microscopy since calibration has been done for the system [37]. Moreover, in other cases, mitotic cells arrested in M phase can be monitored in our system for hours without losing viability. In intact cells, mitotin/CENP-F is rapidly degraded at the end of mitosis [2, 12], coincident with the timing for nucleus reassembly

which requires chromosome decondensation [60]. Therefore, mitotin may play a role in maintenance of chromosome condensation till its degradation at late M phase. Depleting mitotin by RNAi thus results in premature chromosome decondensation.

Although how mitotin exerts such a role still needs further investigation, there are several supporting clues. First, the homology to SMC class of chromosome segregation ATPase (Figure 2) supports a role of mitotin in chromosome structure. SMC family proteins are chromosomal ATPases involved in chromosome condensation and/or sister chromatid cohesion [61, 62]. Mitotin and its orthologs contain a conserved P-loop motif potentially for ATP-binding [17] and thus might represent a novel class of SMC proteins. Second, a mitotin deletion mutant lacking amino acid residues 2489–2901 exhibits chromosome localization as speckles of various sizes and numbers in mitotic CHO cells [28]. In interphase, overexpressing this mutant alters nuclear chromatin organization as well [28]. As endogenous mitotin is not seen on chromosomes, its association with chromosomes might be highly dynamic. Alternatively, it might regulate chromosome morphology through other SMC proteins.

Mitotin is a negative regulator for transcription factor ATF4

Mitotin may serve as a scaffold protein in interphase to sequester and/or activate certain transcription factors. The S- and G2-dependent expression of mitotin as a nuclear matrix protein implies roles in interphase [2, 3]. The nuclear matrix is actively involved in DNA replication, higher-order chromatin organization, and gene regulation. Some nuclear matrix proteins are implicated in transcriptional regulations [63–66]. Leucine zipper motifs usually mediate protein–protein interactions in transcription factors [15, 67]. Our identification of ATF4 as a mitotin interactor is consistent with these speculations [68]. ATF4, also termed CREB2, is a member of the ATF/CREB family of basic leucine zipper (bZip) transcription factors and functions in cell proliferation, bone and lens differentiation, and stress response [69–76]. Through leucine zippers, ATF4 can dimerize with members either in the ATF/CREB family including itself or in other bZip

transcription factor families, such as c-Fos and c-Jun in AP-1 family [77–79].

Mitotin can downregulate ATF4 [68]. Overexpressing full-length mitotin suppresses the transactivation activity of ATF4, while depleting mitotin by RNAi strongly augments such activity. Mitotin interacts with a C-terminal region of ATF4 including the DNA-binding bZip domain in two-hybrid assays, implying a possibility to modulate the DNA-binding activity of ATF4. Consistently, in the presence of a mitotin mutant capable of binding ATF4 through a leucine zipper, ATF4 fails to bind DNA. In addition, mitotin also contains another ATF4-binding region whose association does not influence the DNA-binding of ATF4.

The mitotin–ATF4 interaction may have several physiological significances. First, the interaction may have a role in cell cycle progression. Cyclin A is essential for S phase progression and its gene transcription is largely stimulated by ATF/CREB family members such as ATF2 and ATF3 in S phase [80, 81]. Moreover, association of ATF2 with Jun family transcription factors can further markedly stimulate the activities of Cyclin A promoter [80]. ATF4, however, inhibits the ATF2 activity on the promoter. Furthermore, ATF4 levels increase following the cell cycle progression into S phase while ATF2 levels remain relatively constant [80]. Therefore, the increasing amount of mitotin in S phase may sequester ATF4 from the promoter, thereby allowing the more potent ATF2 and ATF3 to function. Consistent with this idea, mitotin does not interact with other ATF/CREB family members [68]. Second, mitotin may regulate ATF4 in other cellular activities such as stress response and differentiation [71–74, 76]. These speculations, however, still need experimental supports.

Mitotin has a role in differentiation through interaction with Rb

Mitotin orthologs LEK1 and CMF1 function in muscle differentiation through interaction with Rb [18, 82]. Mitotin, LEK1, and CMF1 bind Rb *in vitro* through a region homologous to that of E2F1 but different from the typical Rb-binding motif (“LXCXE”) [3, 20, 21]. Coimmunoprecipitation of LEK1 with all three members of the pocket protein family, Rb, p107, and p130, has been

reported [20, 82]. Recently, the interaction between LEK1 and Rb has been shown to be critical for embryonic stem (ES) cell differentiation toward cardiac muscles [82]. Rb-deficient ES cells exhibit delays in cardiac differentiation similar to those with repressed LEK1 expression by RNAi. Moreover, disrupting the Rb–LEK1 interaction specifically by overexpressing a nuclear form of the Rb-binding domain of LEK1 also causes similar retardation. As a result, transcription levels for genes downstream of the bone morphogenetic protein (BMP) pathway and critical for cardiac differentiation [83, 84], for instances, Nkx2.5 and Mef2c, are significantly down-regulated [82]. Activation of the BMP-dependent pathway rescues the defects in both gene expression and cardiac differentiation, placing Rb and LEK1 in a BMP-independent pathway [82]. It is known that Rb binds many transcription factors and modulates their activities either positively or negatively in cell cycle or differentiation [6, 7, 85–87]. Mitotin orthologs, on the other hand, may bind and regulate multiple transcription factors as well, with ATF4 as an example [68]. Interaction of mitotin with Rb may exert mutual influences on activities of their associated transcription factors and thus modulate cell fates.

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