FAT10 Plays a Role in the Regulation of Chromosomal Stability*

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Aneuploidy is a key process in tumorigenesis. Dysfunction of the mitotic spindle checkpoint proteins has been implicated as a cause of aneuploidy in cells. We have previously reported that FAT10, a member of the ubiquitin-like modifier family of proteins, is overexpressed in several gastrointestinal and gynecological cancers. Here we show that FAT10 interacts with MAD2, a spindle checkpoint protein, during mitosis. Notably, we show that localization of MAD2 at the kinetochore during the prometaphase stage of the cell cycle was greatly reduced in FAT10-overexpressing cells. Furthermore, compared with parental HCT116 cells, fewer mitotic cells were observed after double thymidine-synchronized FAT10-overexpressing cells were released into nocodazole for more than 4 h. Nonetheless, when these double thymidine-treated cells were released into media, a similar number of G1 parental and FAT10-overexpressing HCT116 cells was observed throughout the 10-h time course. Additionally, more nocodazole-treated FAT10-overexpressing cells escape mitotic controls and are multinucleate compared with parental cells. Significantly, we observed a higher degree of variability in chromosome number in cells overexpressing FAT10. Hence, our data suggest that high levels of FAT10 protein in cells lead to increased mitotic nondisjunction and chromosome instability, and this effect is mediated by an abbreviated mitotic phase and the reduction in the kinetochore localization of MAD2 during the prometaphase stage of the cell cycle.

Genetic instability is an important phenomenon that underlies tumorigenesis. Chromosome instability (CIN)3 involving gains and loss of chromosomes has been found to occur in most malignancies, whereas microsatellite instability, which occurs at the nucleotide level, is less commonly observed in cancers (1). Two forms of CIN, namely structural instability and numerical instability (aneuploidy), can be observed in various tumors. Genes responsible for CIN in human cancers include those involved in the condensation of chromosomes, cohesion of sister chromatids, formation of microtubules, and kinetochore structure and function as well as mitotic “checkpoint” genes that monitor the proper progression through the cell cycle (1, 2).

MAD2 (mitotic arrest-deficient 2) is a key mitotic spindle checkpoint protein whose primary role is to ensure that all of the chromosomes are properly attached to the mitotic spindle before the onset of anaphase (3). It is activated by associating with unattached kinetochores. Activated MAD2 binds to Cdc20 and prevents the anaphase-promoting complex from ubiquitylating securin. As a result, anaphase is delayed until all of the kinetochores are attached by microtubules and the chromosomes are properly aligned along the metaphase plate (4–6). MAD2 is an essential gene, and MAD2−/− mice die in utero (7). Loss of one allele of MAD2 has been reported to result in premature anaphase and CIN in mammalian cells (8).

Disregulation of MAD2 has been implicated in various cancers. Reduced expression of MAD2 associated with loss of mitotic checkpoint control was observed in ovarian cancer cells (9), breast cancer cells (3), and nasopharyngeal carcinoma cells (10). Interestingly, MAD2 has been reported to be overexpressed in colorectal (11) and gastric (12) cancers. Nonetheless, mutations of MAD2 are infrequent in bladder tumors, soft tissue carcinomas, hepatocellular carcinomas (13), lung cancer, and breast cancer (14). Recently, it was demonstrated that the deregulation of the Rb pathway leads to aberrant overexpression of MAD2, which then contributes to mitotic alterations and chromosome instability (15). Aberrant interaction of MAD2 with other proteins may also deregulate the checkpoint function of MAD2. For example, overexpression of CMT2 (caught by MAD2), which is capable of binding to MAD2, induces premature entry into anaphase without chromosome segregation (16).

FAT10 is another protein that was identified using the yeast two-hybrid system to noncovalently associate with MAD2 (17). Also known as diubiquitin, FAT10 is an 18-kDa protein containing 165 amino acid residues. It comprises two tandem ubiquitin-like domains and belongs to the ubiquitin-like modifier (UBL) family of proteins (18). Its N and C termini are 29 and 36% identical to the corresponding segments of ubiquitin. Similar to ubiquitin, it contains the C terminus Gly-Gly residues that are important for conjugating to other proteins as well as the conserved Lys residue, which may serve as a potential site for polyubiquitination. Recently, it was reported that FAT10 degradation is accelerated by its interaction with NEDD8 (neural precursor cell-expressed, developmentally down-regulated 8) ultimate buster-1L (19). Additionally, the degradation of FAT10 and its conjugates were also found to be ubiquitin-independent (20). FAT10 was observed to be up-regulated in human fetal cells from pregnancies affected with Trisomy 21 (21). Its role in tumorigenesis is suggested by its ability to be up-regulated by the
inflammatory molecule, tumor necrosis factor α (22), a presumptive tumor promoter (23, 24). We recently reported that FAT10 expression is up-regulated in hepatocellular carcinoma and other gastrointestinal and gynecological cancers (25).

In this study, we demonstrate that FAT10 interacts with MAD2, but only during mitosis. We also demonstrate that the kinetochore localization of MAD2 is reduced in FAT10-overexpressing cells. Furthermore, overexpression of FAT10 results in an abbreviated mitotic phase, as evidenced by a delay of entry into mitosis but not G2. Additionally, a greater number of FAT10-overexpressing cells escape mitotic controls and are multinucleate upon prolonged nocodazole treatment. Notably, we observed a higher degree of variability in chromosome number in cells overexpressing FAT10, suggesting a more pronounced degree of mitotic nondisjunction in these cells. This is the first report to identify FAT10 as an important player in the regulation of mitosis and CIN.

MATERIALS AND METHODS

Generation of Plasmid Constructs and HCT116 Cell Lines Stably Expressing FAT10—The Gateway Cloning Technology (Invitrogen) was utilized to clone the FAT10 cDNA downstream of the N-terminal His6 tag peptide of the destination vectors pDEST26 (with CMV constitutive promoter) and pT-REx-DEST31 (with tetracycline-inducible promoter) to generate pEXPR26-HisFAT10 and pTREXPR31-HisFAT10, respectively.

HCT116 colon carcinoma cells were transfected with pEXPR26-HisFAT10 or co-transfected with pTREXPR31-HisFAT10 and pcDNA6/TR...
(containing the tetracycline repressor gene) using Superfect™ transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions to generate cells expressing FAT10 constitutively or under tetracycline induction. Forty-eight hours post-transfection, a 1:100 dilution of cells was seeded into McCoy’s 5A medium (Sigma) containing 10% fetal calf serum and 0.7 mg/ml G418-sulfate (Promega). Additionally, 1 μg/ml Blasticidin (Invitrogen) was added to cells cotransfected with pTREXPR31-HisFAT10 and pcDNA6/TR. Cells were then incubated at 37°C in 5% CO2 for ~10 days, and several colonies were selected and analyzed for FAT10 expression by Western blot analyses using anti-FAT10 antibody (25). For cells transfected with pTREXPR31-HisFAT10 and pcDNA6/TR, expression of HisFAT10 was induced for 24 h with 2 μg/ml tetracycline (Invitrogen) prior to harvesting for Western analysis. One clone stably expressing FAT10 constitutively (FAT10) and three clones stably expressing FAT10 under tetracycline induction (TetFAT116a, TetFAT116b, and TetFAT116c) were selected for further analyses.

Characterization of HCT116 Cells Stably Expressed FAT1—Morphology of cells was examined under an Olympus Research inverted microscope (IX51), and images were captured with a Qimaging Retiga 1300R digital imager (Qimaging). The growth profile of these cells was determined by seeding these cells in 24-well plates. At various time points as indicated, cells were harvested, and only viable cells were counted using trypan blue exclusion and a hemocytometer. Cell cycle profile was examined by fixing the cells in 2% paraformaldehyde, staining them with propidium iodide solution, and analyzing the stained cells using the FACScalibur™ instrument (BD Biosciences). Apoptotic profile was examined using the Annexin V-phycocerythrin kit (BD Biosciences) according to the manufacturer’s protocol and analyzed using the FACScalibur™ instrument.

Immunofluorescence Analysis—HCT116 or FAT116 cells were grown on coverslips and fixed in 4% paraformaldehyde solution. These cells were then permeabilized in 0.2% Triton solution and co-stained with rabbit anti-FAT10 polyclonal antibody (25) and mouse anti-MAD2 monoclonal IgG (BD Biosciences). Alexa Fluor® 488 chicken anti-mouse or Alexa Fluor® 647 chicken anti-mouse or Alexa Fluor® 488 anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) as the secondary antibodies. Cells were also incubated with DAPI to distinguish the cell cycle stages. Cellular localization and cell cycle stage observations were performed on the LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Immunoprecipitation—HCT 116 cells were infected with either control vector adenoviruses (control) or adenoviruses expressing the FAT10 gene (25). Forty-eight hours later, these cells were incubated in 300 ng/ml nocodazole-containing media or left untreated. Twenty hours later, the cells were pelleted and lysed. Cell debris was removed through centrifugation, and the protein concentration of the protein lysate was adjusted to 3 mg/ml. Immunoprecipitation was carried out using the protein G-immunoprecipitation kit (Roche Applied Science) on 600 μg of the lysate with 2 μg of either of the following antibodies: p16 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which served as a nonspecific rabbit IgG control; p53 antibody (Santa Cruz Biotechnology), which served as a nonspecific mouse IgG control; monoclonal MAD2 antibody (BD Biosciences); and FAT10 antibodies generated by our laboratory in rabbits. The immunoprecipitated proteins were electrophoresed in 18% SDS-polyacrylamide gel, and Western blot analyses was performed as described previously (25) using either FAT10 or MAD2 antibodies.

Generation of MAD2-EGFP Fusion Construct—To determine the subcellular localization of the MAD2 protein, a MAD2-EGFP fusion construct was generated via a two-step PCR as described previously using the FACScalibur™ instrument.

**DNA Content, Mitotic Index, and Apoptosis Determination—**HCT116, FAT116, uninduced TetFAT116c, and tetracycline-induced TetFAT116c were synchronized to G1/S phase using double thymidine treatment. Briefly, cells were incubated in media containing 3 mM thymidine (Sigma) for 17 h, followed by 12 h in media without thymidine and finally another 15 h in media containing 3 mM thymidine. To determine the mitotic index, synchronized cells were released into 300 ng/ml nocodazole-supplemented media to arrest cells at mitosis, harvested at the indicated time points in PBS containing 4 mM EDTA, and fixed in 2% paraformaldehyde. Fixed cells were permeabilized with 0.2% Triton X-100 solution and probed with an antibody (Upstate Biotechnology, Inc., Lake Placid, NY) as the primary antibody and Alexa Fluor® 488 chicken anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) as the secondary antibody. Stained cells were analyzed using the FACScalibur™ flow sorter (BD Biosciences). To determine DNA content, synchronized cells were released into fresh working media without nocodazole, harvested at the indicated time points, and fixed in 2% paraformaldehyde. These cells were then stained with propidium iodide solution and analyzed using the FACScalibur™ instrument.

**FIGURE 2. FAT10 interacts with MAD2 during mitosis.** Untreated (upper two panels) or nocodazole-treated (lower two panels) HCT116 (Control) or FAT10-expressing HCT116 (FAT10) cells are immunoprecipitated with anti-FAT10, anti-MAD2, or nonspecific control antibodies (anti-p16 was used as a nonspecific rabbit IgG control, whereas anti-p53 was used as a nonspecific mouse IgG control) and probed with anti-MAD2 or anti-FAT10 antibodies on Western blots. The leftmost panel shows blots before immunoprecipitation (IP), whereas the middle and right panels show blots after immunoprecipitation. The topmost panel and third panel from the top show proteins probed with the anti-FAT10 antibody, whereas the second panel from the top and the bottom panel represent the Western blot of the same proteins probed with anti-MAD2 antibody.
FIGURE 3. MAD2 localization during prometaphase is altered in FAT10-overexpressing cells. A–C, pcMVEGFP or pcMVMAD2EGFP constructs were transfected into either HCT116 or FAT116 cells. A, Western blot of these cells probed with a 1:5,000 dilution of either anti-EGFP, anti-MAD2, anti-FAT10, or anti-actin primary antibodies (Ab) and a 1:30,000 dilution of horseradish peroxidase-goat anti-rabbit secondary antibody. Advanced ECL reagent (Amersham Biosciences) was used to visualize the blot, and the film was exposed for 3 min. **D**, Representative images of transfected cells stained with DAPI and viewed by confocal microscopy. **E**, Quantification of MAD2 localization at kinetochores as determined by confocal microscopy. The normalized relative intensity of MAD2 fluorescence at kinetochores was determined for HCT116 and FAT116 cells transfected with pcMVMAD2EGFP constructs. **n** represents the number of independent experiments performed for each cell line. **Bars**, Standard error of the mean.
A paired two-tailed t test was utilized to evaluate the significance of the difference between control/uninduced and FAT10-induced cells.

**Analysis of Chromosomal Numbers—G-banded chromosome analysis was performed on HCT116 parental cells, FAT116 cells, uninduced parental HCT116, TetFAT116a, TetFAT116b, and TetFAT116c cells, and tetracycline-induced parental HCT116, TetFAT116a, TetFAT116b, and TetFAT116c cells. The parental HCT116 and FAT116 cells had been in continuous culture for ~11 months with ~80 subcultures (i.e. these cells had undergone ~250 cell doublings). Tetracycline-inducible TetFAT116a, TetFAT116b, and TetFAT116c cells and parental HCT116 cells were grown in media with or without 3 µg/ml tetracycline supplementation (Sigma) for 33 subcultures (or ~100 doublings). These cells were reselected in 5 µg/ml Blasticidin and 0.7 mg/ml G418 before karyotype determination.

Briefly, cells were first synchronized for 17 h in media containing 3 mM thymidine, replaced with fresh medium without thymidine for 7 h, and then treated with 0.1 µg/ml colcemid (Invitrogen) for 1.5 h. Cells were detached in phosphate-buffered saline containing 4 mM EDTA and rinsed with phosphate-buffered saline without EDTA, swelled in 0.06 M KCl solution, and fixed in a 3:1 methanol/glacial acetic acid mix. Fixed cells were dropped onto microscope slides, partially digested with trypsin, and stained with Giemsa solution (Invitrogen). Chromosome numbers were counted using BandView (Applied Spectral Imaging GmbH, Mannheim, Germany).

**RESULTS**

**Cells Stably Overexpressing FAT10 Have Growth, Cell Cycle, and Apoptotic Profiles Similar to Those of Parental Cells—**We generated and characterized the HCT116 cell line stably overexpressing FAT10 named FAT116. FAT116 cells were found to express high levels of the FAT10 protein, as shown by Western blot analyses (Fig. 1A). The FAT10 protein was found to be expressed in the nucleus of these FAT116 cells (Fig. 1B) through confocal microscopy.

Morphology (Fig. 1C) and growth properties (Fig. 1D) of FAT116 cells were found to be similar to their parental HCT116 cells. Additionally, expression of FAT10 in FAT116 cells also did not alter the general cell cycle (Fig. 1E, top two panels) or apoptotic profiles (Fig. 1E, bottom two panels) significantly.

**FAT10 Interacts with MAD2 during Mitosis—**As FAT10 was reported to interact with MAD2 (17), it may play a role in the regulation of mitosis. We thus evaluated this interaction between FAT10 and MAD2 occurs during the mitotic phase of the cell cycle using co-immunoprecipitation assays. We were unable to detect the FAT10 protein in the anti-MAD2 immunoprecipitate or vice versa in unsynchronized cells, where a majority of the cells are in the G1 phase (Fig. 2, untreated). Notably, when these cells were arrested at mitosis with nocodazole, MAD2 was detected in the anti-FAT10 or anti-MAD2 immunoprecipitate in FAT10-expressing HCT116 cells and only in anti-MAD2 immunoprecipitate in control cells (Fig. 2, Nocodazole treated). FAT10 was detected in anti-MAD2 or anti-FAT10 immunoprecipitate of only nocodazole-treated FAT10-expressing HCT116 cells (Fig. 2). Neither FAT10 nor MAD2 was detected when cells were co-immunoprecipitated with nonspecific control antibodies (Fig. 2). These results confirm the previous observations (17) that MAD2 and FAT10 are capable of interaction, and we demonstrate that the interaction occurs during mitosis.

**Localization of MAD2 at the Kinetochore Is Greatly Reduced in FAT10-overexpressing Cells—**To determine the fate of MAD2 in FAT10-overexpressing cells, we generated a construct, pCMVMAD2EGFP, whereby the MAD2 gene was fused with the EGFP gene. We then transfected this construct as well as the vector control construct not containing MAD2, pCMVEGFP, into either the parental HCT116 or the FAT116 cells and demonstrated through Western analyses that the EGFP and MAD2-EGFP fusion proteins could be expressed (Fig. 3A). As shown in Fig. 3B, when pCMVEGFP, the vector control plasmid, was introduced into either the parental HCT116 or FAT116 cells, the EGFP fluorescence signal was diffused throughout the cell (Fig. 3B, top two panels). When the construct, pCMVMAD2EGFP, was transfected into the parental HCT116 cells, the MAD2-EGFP fusion protein seemed to accumulate as bright punctate signals at the kinetochores (Fig. 3B, bottom left panels) during the prometaphase but not the metaphase stage of the cell cycle. This observation is consistent with the reported localization of MAD2 during mitosis (26).

Interestingly, in FAT10-overexpressing FAT116 cells, the MAD2-EGFP fusion protein appeared diffuse whether the cells were in the prometaphase or metaphase stage of the cell cycle (Fig. 3B, bottom right panels). Statistically significant (p < 0.01) reductions in the punctate signals at the kinetochores was observed in the prometaphase stage in FAT10-expressing cells compared with parental HCT116 cells (Fig. 3C). In cells that did not receive the pCMVEGFP or pCMVMAD2EGFP construct, no fluorescence was observed (Fig. 3B, top row of each panel). Taken together, these results suggest that MAD2 localization at the kinetochore is greatly reduced in FAT10-overexpressing cells during the critical prometaphase stage of the cell cycle.

These observations are consistent with the results obtained when HCT116 and FAT116 cells were stained with anti-FAT10, anti-MAD2 antibodies, and DAPI (Fig. 3D and E). As shown in Fig. 3D, in parental HCT116 cells where FAT10 expression is negligible, MAD2 aggregated at the kinetochores during the prometaphase stage of the cell cycle. However, when the FAT10 gene was overexpressed in FAT116 cells, MAD2 aggregation at the kinetochores was significantly reduced (p < 0.01).

Hence, FAT10 interaction with MAD2 during mitosis may decrease the efficiency of binding of MAD2 to the unattached kinetochore, although it does not seem to completely block the function of MAD2. These results are consistent with the previous observation that MAD2 haploinsufficiency is sufficient to cause premature anaphase and chromosome instability (8).

**FAT10 Overexpression Results in an Abbreviated Mitotic Phase—**Since FAT10 interacts and reduces the kinetochore localization of MAD2 during mitosis and since MAD2 is a mitotic checkpoint protein (5), we examined the effect of overexpression of FAT10 on cell cycle regulation and especially on mitosis. HCT116 parental cells, HCT116 cells stably expressing FAT10 constitutively (TetFAT116), and uninduced and induced HCT116 cells stably expressing FAT10 under the tetracycline-inducible promoter (TetFAT116c) were synchronized at G1/S phase using double thymidine treatment (Fig. 4, A, C, and G, 0 h). These cells were released into either media containing nocodazole or fresh media without nocodazole and at various time points were stained with the mitosis-specific antibody, PM2 (Fig. 4B) or propidium iodide to monitor their progress into mitosis (Fig. 4, A, D, and E) or the next G1 phase (Fig. 4, C, F, and G), respectively.
FAT10 Overexpression Induces CIN

As shown in Fig. 4D, ~5% of both HCT116 and FAT116 cells entered mitosis at 4 h after release from G1/S. However, at each time point after 4 h, ~10% more parental cells entered mitosis than FAT10-overexpressing cells. Similar observations were made when uninduced and tetracycline-induced TetFAT116 cells were compared (Fig. 4E). When these experiments were repeated on different occasions, similar trends were observed. These results suggest that there may be a delay in entrance into mitosis in FAT10-overexpressing cells. However, it is also possible that the observed reduction in mitotic FAT10-expressing cells may be a consequence of these cells escaping M-phase and reverting to interphase with a tetraploid G1 DNA content.

Interestingly, reentry of cells into G1 phase from their arrest at G1/S was similar between HCT116 and FAT116 cells (Fig. 4F) as well as between uninduced and tetracycline-induced TetFAT116 cells, despite the delayed entry into mitosis of FAT1016 and tetracycline-induced TetFAT116 cells (Fig. 4G). Similar trends were observed when these experiments were repeated.

When data from the different experiments were combined and analyzed together, we found that the difference between the HCT116 and FAT116 cells (Fig. 4H) or uninduced and induced TetFAT116 cells (Fig. 4I) in time of entry into mitosis was significantly greater (p < 0.05) than their corresponding difference in time of reentry into G1. Taken together, these data suggest both a delayed and an abbreviated mitotic phase in cells overexpressing FAT10. An alternative explanation is that FAT10 overexpression results in shortened mitotic arrest in cells with spindle damage.

**FAT10 Overexpression Results in Greater Escape from Mitotic Arrest, More Multinucleate Cells upon Prolonged Mitotic Arrest, and Chromosome Instability**—To address the effect of FAT10 overexpression on mitosis, parental HCT116 and FAT116 cells were treated with 200 ng/ml nocodazole for 8 h. Mitotic cells were then shaken off and replated in media containing 200 ng/ml nocodazole for another 15 h. The doubling time for both HCT116 and FAT116 cells is ~15 h. We found more adherent FAT116 (>20%) than HCT116 (6.5%) cells (Fig. 5, A and B), suggesting that more FAT116 cells either failed to arrest or escaped mitotic arrest and continued to cycle. To rule out the possibility that the observed increase in adherent FAT116 cells was due to increased contamination of interphase FAT116 cells, the mitotic index of the reattached HCT116 and FAT116 cells after shake-off was determined. As shown in Fig. 5C, greater than 95% of the reattached cells were mitotic cells, and the percentage of interphase cells was small and similar between the HCT116 and FAT116 cells. Upon prolonged exposure to nocodazole, most of the HCT116 and FAT116 cells died. Of the cells that reattached, more FAT116 cells (~40%) showed abnormal nuclear morphology and were multinucleated (Fig. 5D) compared with the attached parental HCT116 cells (~5%). Hence, overexpression of FAT10 directly affects mitosis in cells.
We next examined if overexpression of FAT10 affects chromosome stability. Parental HCT116 cells were reported to have a relatively stable karyotype (45,X), with aneuploid cells occurring at only 6.8% (ATCC, Manassas, VA) (8). HCT116 and FAT116 cells were grown in culture for 11 months (~80 passages), which is equivalent to ~250 cell doublings, before they were harvested for chromosome analyses (Fig. 6A).

As shown in the table in Fig. 6C, the parental HCT116 cell line was itself aneuploid, with a modal chromosome number of 45 and the overwhelming majority of cells (82%) having 40–49 chromosomes/cell. None of the parental HCT116 cells contained more than 100 chromosomes. On the other hand, a majority of FAT116 cells (70%) carried 80–89 chromosomes, with 5% of these cells having more than 100 chromosomes. Thus, constitutive FAT10 overexpression increases aneuploidy in HCT116 cells. It is possible that the increased chromosome numbers observed in the FAT116 cell line were due to a clonal artifact, whereby in that particular clone, the pEXPR26-HisFAT10 plasmid was introduced into a HCT116 cell already containing 80–89 chromosomes, which was the case in ~4.2% of parental cells.

To rule out the clonal artifact, we also analyzed the parental HCT116 as well as three different clones of HCT116 cells expressing FAT10 under a tetracycline-inducible promoter (TetFAT116a, TetFAT116b, and TetFAT116c). Upon tetracycline induction, FAT10 expression was greatly increased (Fig. 6B). Uninduced and tetracycline-induced parental HCT116 and TetFAT116a–TetFAT116c cells were grown for ~100 population doublings (33 passages) with medium changes every 3 days, after which chromosomal analysis was performed. Approximately 84% of parental HCT116 cells contained relatively normal chromosome numbers of 40–49 whether they were treated with tetracycline or not. In contrast, when uninduced, 87–96% of Tet116a–Tet116c cells were shown to contain relatively normal chromosome numbers of 40–49 in all three clones (Fig. 6, C and D). However, in the same three clones induced with tetracycline, only between 43 and 56% of cells carried 40–49 chromosomes, with the remaining cells carrying either more or fewer chromosomes (Fig. 6, C and D). Notably, for all three clones, between 2.5 and 4% of tetracycline-induced cells were observed to contain more than 100 chromosomes (Fig. 6, C and D). This was not observed in uninduced TetFAT116 cells or uninduced or tetracycline-induced parental HCT116 cells.

**DISCUSSION**

FAT10 belongs to the UBL family of proteins that have been implicated in the regulation of diverse processes including cell cycle and the maintenance of genome integrity (18, 27). In this report, we present evidence to show that, like its other family members (e.g. SUMO (small ubiquitin-like modifier) (28)), FAT10 plays a role in the regulation of mitosis and chromosome stability.
We generated an HCT116 cell line stably expressing FAT10 and showed that the overexpression of FAT10 did not affect the morphology of the cells, growth properties, general cell cycle, or apoptotic profiles (Fig. 1). Curiously, whereas we were able to generate stable HCT116 cell lines expressing FAT10, earlier attempts by Raasi et al. (22, 29) to generate stable HeLa cell lines expressing FAT10 failed. A possible explanation could be that the HCT116 cell line may be better able to tolerate aneuploidy than the HeLa cell line. Additionally, our observation that FAT10 expression did not sensitize HCT116 cells to apoptosis seems to contradict the same report that found that FAT10 induces apoptosis in mouse fibroblast cells (29). It is possible that species-specific differences could account for this difference in the property of cells expressing FAT10, since we examined human FAT10 expression in a human cell line (HCT116), whereas the other report examined murine FAT10 expression in a mouse cell line.

Using FAT10-specific antibodies that we generated (25), we found that during interphase, FAT10 expression is primarily nuclear in HCT116 cells stably expressing FAT10 (Fig. 1B). These observations are consistent with our earlier report, where we found that FAT10 was localized in the nucleus of WRL68 cells and the tumor of a hepatocellular carcinoma patient (25), but contradicts other reports (17, 19, 29). A possible reason for this seeming discrepancy is that whereas we directly observed FAT10 expression in the cells using FAT10-specific antibodies and fluorescence microscopy, the other reports primarily detected FAT10 expression through indirect approaches including subcellular fractionation and Western blot analyses using FAT10-specific antibodies (17) or immunofluorescence microscopy using either anti-HA (29) or anti-X-press™ antibodies (19) of HA- or His-tagged FAT10.

FAT10 Interacts with MAD2 and Reduces the Kinetochore Localization of MAD2 during the Prometaphase of the Cell Cycle—Since FAT10 was reported to interact with MAD2 (17), we proceeded to elucidate the relationship between FAT10 and MAD2. Using co-immunoprecipitation assays, we found that the reported interaction of FAT10 with MAD2 occurred only during the mitotic phase of the cell cycle. We were unable to immunoprecipitate FAT10 with anti-MAD2 antibodies or vice versa using unsynchronized cells that were primarily G1 phase cells (Fig. 2). Nonetheless, we found that in mitotic cells, FAT10 can be immunoprecipitated with anti-MAD2 antibodies and vice versa (Fig. 2). Interestingly, using MAD2-EGFP fusion constructs, we found that whereas MAD2 localizes at the kinetochores in the parental HCT116 cells, in FAT10-expressing HCT116 cells, MAD2 localization at the kinetochores is greatly reduced (Fig. 3, B and C). Similar observations were made when HCT116 and FAT116 cells were stained with DAPI, anti-MAD2, and anti-FAT10 antibodies. In the prometaphase stage of the cell cycle, MAD2 was found to aggregate at the kinetochores. However, localization of MAD2 at the kinetochores was significantly reduced in prometaphase FAT116 cells (Fig. 3, D and E). This has important implications, since MAD2 is a spindle checkpoint protein that helps to ensure the fidelity of the mitotic process by delaying the

![Figure 6](image-url)
onset of anaphase until all of the chromosomes are properly aligned at the spindle (see Ref. 30). Normally, MAD2 localizes to unattached kinetochores during the prometaphase stage of mitosis (Fig. 3B) (3, 31). We thus hypothesized that the interaction of FAT10 with MAD2 decreases the ability of MAD2 to localize to unattached kinetochores. This is likely to disrupt the role of MAD2 as a checkpoint protein, resulting in dysregulated mitosis and aneuploidy.

**FAT10 Overexpression Results in Dysregulated Mitosis and Chromosome Instability**—We proceeded to explore the effect that FAT10 overexpression has on HCT116 cells. As shown in Fig. 4, D and E, compared with parental or uninduced HCT116 cells, fewer mitotic cells were observed after double thymidine-synchronized FAT10-overexpressing cells were released into nocodazole for more than 4 h. This may be due to a delay in the entrance into mitosis or an escape from mitotic arrest. Nonetheless, when these double thymidine-treated cells were released into media, similar numbers of G1 parental and FAT10-overexpressing HCT116 cells were observed throughout the 10-h time course (Fig. 4, F and G). Taken together, these results suggested that FAT10-overexpressing cells experienced a delayed and abbreviated mitotic phase or an abbreviated mitotic arrest phase upon spindle damage.

An abbreviated mitotic phase in FAT10-overexpressing cells could potentially have adverse consequences. For example, there may be insufficient time for proper alignment of the sister chromatids at the equator, leading to premature separation of these sister chromatids and an increased rate of chromosome missegregation. This possibility is pertinent, given the observation that dysfunctional MAD2 also causes premature sister chromatid separation and chromosome instability (8). We therefore examined the effect of FAT10 overexpression on mitosis and chromosomal instability.

Upon treatment with nocodazole, more FAT10-overexpressing cells either failed to arrest or escaped mitotic arrest (Fig. 5, A and B). Prolonged exposure to nocodazole resulted in more FAT10-overexpressing cells exhibiting abnormal and multinuclear morphology than the parental controls (Fig. 5D). These results suggest dysregulation of mitosis in FAT10-overexpressing cells. Significantly, we demonstrate that FAT10 overexpression results in enhanced CIN (15). We have shown that FAT10 not only colocalizes with MAD2 during mitosis; its overexpression also results in the reduced localization of MAD2 at the kinetochore during the prometaphase stage of the cell cycle. Significantly, we demonstrate that overexpression of FAT10 in HCT116 cells results in an abbreviated mitotic phase; greater escape from mitotic controls, more multinucleate cells upon prolonged mitotic arrest, and CIN, suggesting that proper FAT10 and MAD2 stoichiometry may be essential in maintaining chromosome stability in HCT116 cells. This result is consistent with the recent reports that abberant overexpression (15) or underexpression (8) of the MAD2 gene can also lead to mitotic defects and CIN. The mechanism by which FAT10 reduced the kinetochore localization of MAD2 during the prometaphase stage of the cell cycle remains unclear and awaits further investigation. Nonetheless, our observations suggest that FAT10, like other members of the UBL family (e.g. SUMO) (28), plays a role in the maintenance of genocom stability.

Since FAT10 has been found to be overexpressed in several cancers (25), we propose that dysregulation of FAT10 expression may contribute to tumorigenesis through its interaction with MAD2 to cause CIN by deregulating mitosis.

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