Centromere-localized breaks indicate the generation of DNA damage by the mitotic spindle

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Most carcinomas present some form of chromosome instability in combination with spindle defects. Numerical instability is likely caused by spindle aberrations, but the origin of breaks and translocations remains elusive. To determine whether one mechanism can bring about both types of instability, we studied the relationship between DNA damage and spindle defects. Although lacking apparent repair defects, primary *Dido* mutant cells formed micronuclei containing damaged DNA. The presence of centromeres showed that micronuclei were caused by spindle defects, and cell cycle markers showed that DNA damage was generated during mitosis. Although the micronuclei themselves persisted, the DNA damage within was repaired during S and G2 phases. DNA breaks in *Dido* mutant cells regularly colocalized with centromeres, which were occasionally distorted. Comparable defects were found in *APC* mutant cell lines, an independent system for spindle defects. On the basis of these results, we propose a model for break formation in which spindle defects lead to centromere shearing.

centrosome | chromosome instability | double-strand break | H2A.X

Most solid tumors show changes in chromosome number, a phenomenon termed aneuploidy, which is caused by chromosome segregation errors and rarely observed in normal cells (1). Many tumors not only gain or lose whole chromosomes, but also accumulate intragenic mutations, thought to suppress negative control on proliferation. Mathematical models predict that chromosomal instability initiates tumorigenesis before the mutation of tumor suppressor genes, underlining the importance of aneuploidy (2). Other studies indicate that aneuploidy is required for sporadic carcinogenesis and collaborates with intragenic mutations by generating multiple copies of mutated chromosomes (3).

Because of their capacity to initiate and propagate intragenic mutations, DNA breaks are considered important in carcinogenesis (3). To examine the origin of breaks, several studies have analyzed the link between cancer and the repair of double-strand DNA breaks (DSBs) (4). But even though mutation of DSB repair genes results in DNA damage after irradiation, little effect was found in untreated cells (5, 6). In addition, sporadic carcinomas bear few if any mutations in DSB repair genes, and many tumor samples show augmented instead of reduced repair activity (7, 8). The role of DSB repair in de novo generation of structural defects thus remains uncertain.

Another potential route for DSB generation involves the mitotic spindle. Spindle defects, an important cause of aneuploidy (1), are frequent in solid tumors, and background γ H2A.X levels correlate well with aneuploidy (9). γ H2A.X has also been observed after the induction of mitotic arrest by spindle disruption (10, 11). When combined with chromosome end-to-end fusions, the mitotic spindle can damage chromosomes by a mechanism termed the breakage–fusion–bridge (BFB) cycle (12, 13). Still, it remains unclear whether chromosome breakage in the BFB cycle is caused by the spindle itself or by contraction of the midbody actin ring. Inactivation of genes that control the metaphase checkpoint causes severe spindle defects and generally is lethal due to extensive aneuploidy (14). In this study, we used primary cells bearing a

mutated *death inducer obliterator* (*Dido*) gene. The main product of the *Dido* gene, Dido3, is a structural protein that localizes to the spindle pole in mitosis (15) and to the synaptonemal complex in meiosis (16). The *Dido* mutation compromises the spindle metaphase checkpoint, leading to an increased frequency of aberrant anaphases (17). Although they suffer from myelodysplastic/myeloproliferative diseases (18), *Dido* mutant mice are viable and can thus provide a cellular model in which to study downstream effects. For a second model, we used colon cancer cell lines lacking a functional *adenomatous polyposis coli* (*APC*) gene. APC is a microtubule-associated protein that regulates spindle dynamics and microtubule–chromosome attachments (19).

We encountered DSBs in micronuclei in *Dido* mutant mouse primary embryonic fibroblasts (MEFs) without signs of DNA damage in the main nucleus. DSBs were found in centromere-containing micronuclei, probably formed by uncorrected merotelic attachments. This shows an aneuploid origin combined with clastogenic effects. Most DSBs in *Dido* mutant MEFs were found adjacent to centromeres, which were occasionally distorted. *APC* mutant cell lines showed similar defects. Thus, two independent models of spindle defects show comparable centromere-localized DSBs. Although mitosis was thought to contribute to chromosomal instability only by chromosome missegregation, our results show that spindle defects can directly generate DSBs. We propose a model in which spindle defects promote tumor formation through several pathways.

Results

Localized DNA Damage in *Dido* Mutant Cells. Primary MEFs from *Dido* mutants show a high frequency of spindle defects and lagging chromosomes (15, 17). When lagging chromosomes persist until mitotic exit, they form micronuclei in the subsequent interphase (20). Aside from spindle defects, faulty repair can generate micronuclei through the loss of centromere sequences (21). To compare micronucleus formation under different conditions, primary MEFs from *Dido*, *Ku80*, and *ATM* mutants were labeled with an antibody to the DSB marker γ H2A.X (22), and the frequency of micronuclei was determined (Fig. 1). *Ku80* has a critical role in nonhomologous end joining (NHEJ) and *ATM* is a kinase that phosphorylates several proteins involved in NHEJ and homologous recombination repair (HRR) (23, 24).

A small proportion of wild-type (WT) MEFs contained micronuclei, and this proportion increased in *Dido*, *Ku80*, and *ATM* mutants (Fig. 1A). γ H2A.X labeling revealed small constitutive γ H2A.X foci (6, 25), not related to DSB, in all cell lines. The number

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Fig. 1. Localized DNA damage in mutant cells. MEFs were seeded on coverslips, labeled with anti- γ H2A.X antibody (red), and studied by fluorescence microscopy. DNA was DAPI stained (blue). Quantitation (A) showed an increased proportion of γ H2A.X-positive micronuclei in *Dido* mutant MEFs. Error bars indicate standard deviation. (B–E) Representative images of WT (B) cells and *Dido* (C), *Ku80* (D), and *ATM* (E) mutants are shown. Micronuclei are indicated with arrowheads. Strong γ H2A.X labeling was found in micronuclei (C), although *Dido* mutant MEFs show no further DNA damage. The weaker signals correspond to constitutive foci not related to DSBs. (Scale bar, 5 μ m.)

and localization of constitutive γ H2A.X foci is specific for each cell line. Although *Ku80* mutant MEFs show an exceptionally high number of constitutive foci and undue H2A.X phosphorylation in heterochromatic regions, these phenomena are not associated with widespread DSBs. However, untreated WT, *Ku80* mutant, or *ATM* mutant MEFs showed hardly any of the high-intensity γ H2A.X label characteristic for DSBs (Fig. 1 B, D, and E). The *Dido* mutation caused a marked (fourfold) increase in micronuclei, many of which with high-intensity γ H2A.X labeling (Fig. 1C). *Ku80* or *ATM* inactivation, too, increased the frequency of micronuclei, but few of these were γ H2A.X positive (Fig. 1 D and E). γ H2A.X-positive micronuclei were also found in *Dido* mutant embryos (Fig. S1), showing in vivo DSB formation. In addition, a subset of *Dido* mutant MEFs showed DNA damage in the main nucleus (Fig. S1). In contrast to radiation-induced damage, which gave rise to more or fewer globular regions of γ H2A.X (Fig. 2), nuclear γ H2A.X regions in *Dido* mutant MEFs were jagged. In conclusion, the *Dido* mutation gives rise to localized DSBs, but repair mutants do not show this kind of “spontaneous” DNA damage.

Normal DSB Repair and H2A.X Phosphorylation in *Dido* Mutant Cells. Even though repair gene mutations cause retention of γ H2A.X after DSB induction, no effect is found in untreated cells (5, 6). Untreated *Dido* mutant MEFs, however, formed micronuclei containing damaged DNA. We therefore analyzed the DSB repair efficiency of the *Dido* mutant. Because the *Dido* mutation had no significant effect on overall radiation sensitivity (Fig. S2), DSB kinetics were assayed. MEFs were treated with 2 Gray γ -radiation and labeled with anti- γ H2A.X antibodies at different time points (Fig. S3). High-intensity γ H2A.X foci were counted to quantify DSBs (Fig. 2A). Both WT and *Dido* mutant MEFs showed the rapid appearance of γ H2A.X foci after irradiation and gradually lost these as DSB repair took place. After 8 h, few γ H2A.X foci remained in WT and *Dido* mutant MEFs. The number of γ H2A.X foci did not differ significantly between WT and *Dido* mutant MEFs at any of the time points. Thus, although *Dido* mutants generated more γ H2A.X-positive micronuclei than repair-deficient controls, their DSB repair capacity was comparable to that of WT animals.

Repair proteins are recruited to the actual break sites and thus colocalize with γ H2A.X foci after induction of DSBs. To test whether *Dido* proteins are recruited to DSBs, MEFs were irradiated and labeled with antibodies to *Dido* and γ H2A.X (Fig. 2B). *Dido* distributed heterogeneously in the nucleus but did not colocalize with γ H2A.X foci, indicating that *Dido* has no overt role in DSB repair.

Although γ H2A.X is used as a marker of DSB formation (22), γ H2A.X can be formed without DSBs, such as a normal cell cycle (6, 25) or XY body formation in meiosis (26). To determine whether the γ H2A.X in micronuclei is associated with DNA damage, we detected phosphorylation of the HRR protein ATM (27) and terminal transferase dUTP nick end labeling (TUNEL) (28) as supplementary markers. Whenever γ H2A.X-positive micronuclei were found in untreated *Dido* mutant MEFs, these always yielded corresponding phospho-ATM and TUNEL signals (Fig. 2 C and D), confirming γ H2A.X as a valid DSB marker. In addition, individual micronuclei were found positive for TUNEL, γ H2A.X, and distorted centromeres (Fig. 2D). Micronuclei, centromere distortion, and DNA damage may thus share a common origin. Taken together, these data indicate that only de novo DSB generation, but not diminished repair or aberrant H2A.X phosphorylation, can explain the γ H2A.X-positive micronuclei.

Micronuclei in *Dido* Mutants Are Caused by Spindle Defects. Micronuclei can have two origins: loss of centromeres due to DNA damage, generating chromosomes no longer recognized by the mitotic spindle and defective kinetochore capture due to anomalies of the spindle itself. Because previous studies showed spindle defects in *Dido* mutants (15), we labeled *Dido* mutant MEFs with antibodies to centromeres and α -tubulin and studied these by confocal fluorescence microscopy (Fig. 3). We detected individual centromeres captured by microtubules from opposite poles (Fig. 3A and B), which shows that merotelic attachments cause lagging chromosomes in the *Dido* mutant (29).

Uncorrected merotelic attachments lead to the exclusion of lagging chromosomes from the main nucleus during mitotic exit (21). In such an event, a centromere-positive micronucleus is generated. Centromeres in micronuclei thus are reliable indicators of spindle

Fig. 4. Intramitotic DNA damage in *Dido* mutant cells. (A) MEFs were seeded on coverslips, labeled with anti- γ H2A.X and anti-cyclin B1 antibodies, and studied by confocal microscopy. Whereas γ H2A.X-positive micronuclei (arrowhead) were found in cyclin B1-negative cells, no γ H2A.X was found in cells with a high cyclin B1 content. (B) MEFs were pulse labeled with BrdU for 1 h and chased in medium without BrdU for the time indicated. Subsequently, cells were labeled with anti-BrdU (green) and anti- γ H2A.X antibodies (red) and studied by confocal microscopy. Micronuclei containing bright γ H2A.X labeling were found in early-to-mid S phase cells. Late S phase cells showed micronuclei with diminishing or no γ H2A.X. No G2 phase cells were found γ H2A.X-positive, but cells that were chased until the next G1 phase again showed γ H2A.X in micronuclei. Representative images are shown. (Scale bars, 5 μ m.)

γ H2A.X and distorted centromeres, corroborated this hypothesis. To further analyze the hypothesis of spindle-generated DNA damage, we evaluated colocalization of DSBs and spindle attachment sites. *Dido* mutant MEFs were labeled with antibodies to γ H2A.X and centromeres and studied by confocal fluorescence microscopy. Whereas one subpopulation of cells showed extensive DSBs in micronuclei (Figs. 1–3), the DNA damage in another subset was restricted to smaller areas (Fig. 5A). In nearly all cases, however, γ H2A.X surrounded or was adjacent to centromeres. DSBs thus typically localized to the spindle attachment sites on the chromosomes, in agreement with centromere distortion. Mitotic figures showed comparable centromeric γ H2A.X labeling (Fig. 5B and Fig. S4), consistent with a mitotic origin of DNA damage. DSB detection by TUNEL confirmed centromere association (Fig. 5C). In contrast to these spontaneous DSBs, few radiation-induced breaks localized to centromeres in *Dido* mutant MEFs (Fig. 5D). To quantify the frequency of centromere-associated DSBs under different conditions, we scored the number of γ H2A.X foci that colocalized with centromeres. Whereas over 75% of DSBs localized to centromeres in untreated cells, less than 10% of radiation-induced DSBs showed this localization (Fig. 5E). Taken together, these results show clear differences between spindle- and radiation-induced γ H2A.X and corroborate the role of the mitotic spindle in DSB formation.

To determine whether a deregulated spindle causes DNA damage in an independent model, we confirmed these data in cell lines with a chromosomal instability (CIN) phenotype (Fig. 6 and Fig. S5). These cell lines, bearing a mutated *APC* gene, suffer from aberrant regulation of the cytoskeleton and chromosome segregation defects (19). The CIN cell lines, too, showed γ H2A.X adjacent

Fig. 5. DSBs in untreated *Dido* mutant MEFs are centromere associated. MEFs were seeded on coverslips, labeled with anti- γ H2A.X antibodies (red, A, B, and D) or by TUNEL (C), and counter labeled with centromere antibodies (green). Cells were studied by confocal scanning laser microscopy. DNA was DAPI stained (blue). *Insets* show threefold magnification of the centromeric areas (arrowheads). An interphase (A) and anaphase (B) are shown. (C) TUNEL confirmed centromere association of DSBs. Representative images are shown. (D) In contrast to spindle-induced breaks, radiation-induced γ H2A.X foci do not colocalize with centromeres. (E) Quantitation of DSB localization in untreated and irradiated MEFs. Error bars indicate standard deviation. (Scale bar, 5 μ m.)

to centromeres, showing that shearing of centromeric chromatin is a common effect of spindle anomalies.

Discussion

Several lines of evidence suggest that aneuploidy is required for sporadic carcinogenesis in mice and collaborates with intragenic mutations during tumorigenesis (2, 3). Aneuploidy is caused by a dysfunctional mitotic spindle (33), but the origins of DNA breaks, which are thought to initiate intragenic mutations, remain poorly understood. Although repair mutants show persistent lesions after DSB-inducing treatments, the same mutations seem to have little or no effect in untreated cells, and mutations in repair genes are

Fig. 6. Centromere-associated DSBs in CIN cell lines. Caco2 (A) and HT29 (B) cells were seeded on glass coverslips, labeled with antibodies to γ H2A.X (red) and centromeres (green), and then analyzed by confocal scanning microscopy. DNA was counterstained with DAPI (blue). Areas indicated with arrowheads are shown magnified twofold in *Insets*. Representative images are shown. (Scale bar, 5 μ m.)

rarely found in sporadic tumors (5, 7, 8). In contrast, several lines of evidence implicate the mitotic spindle in DSB generation; genetic or drug-induced spindle abnormalities cause γ H2A.X accumulation (10, 11), γ H2A.X levels in untreated cells correlate with numerical chromosome instability (9), and cancer cell lines with a CIN phenotype show numerous translocations close to centromeric regions (34).

Here we show that cells with a *Dido* mutation, which causes spindle defects and an increased frequency of lagging chromosomes (15, 17), show localized DSBs in micronuclei, subnuclear regions, and nuclear protrusions. Because these DSBs were found in cells without apparent repair defects, they can only be explained by de novo generation. The micronuclei in *Dido* mutant primary MEFs contained centromeres, indicating an aneuploid (spindle) origin. In addition, we found DSBs adjacent to the centromeres, which form the spindle attachment points in mitosis. In agreement with a role of the spindle, breaks were generated in mitosis and again repaired later in the cell cycle. The *APC* mutants provide an alternative model in which chromosome segregation defects and spindle defects cause aneuploidy (19), confirming DSBs adjacent to centromeres.

The force generated by a single microtubule, ≈ 50 pN, is not considered enough to overcome the integrity of a DNA helix (35), which breaks at forces around 250 pN in vitro (36). During mitosis, however, microtubules form bundles termed K-fibers to amplify their strength (37). The combination of K-fiber formation and loss of spindle control brings about a potentially dangerous situation, in which the chromosomes are exposed to excessive force. Whereas dicentric chromosomes, the source of the breakage–fusion–bridge cycle, potentially experience the combined force of two K-fibers, lagging chromosomes typically result from a merotelic attachment. Even though merotelic attachments generally do not combine more microtubules than a single K-fiber, uncorrected merotelic attachments are able to overcome the structural integrity of the kinetochore (29). Approximately 15 microtubules connect to a single kinetochore in a merotelic attachment, which can generate a combined force three times the tensile strength of chromosomes (750 pN). Two K-fibers stretch the chromatin between centromeres in the BFB cycle, resulting in anaphase bridges (38). In a merotelic attachment, however, the microtubules act on a single centromere, which lacks the flexibility of intermediate chromatin. Thus, even though merotelic attachments do not generate as much force as two K-fibers, the small dimensions of individual kinetochores favor DNA rupture. Although the merotelic attachments themselves are relatively difficult to detect, distorted centromeres (Fig. 3*F*) point at merotelic attachments as the origin of micronuclei and nuclear protrusions in the *Dido* mutant. Also the isolated centromeric chromosome fragments (Fig. S6), the nuclear protrusions (Fig. S1), and the jagged form of damaged regions (Fig. S1) are consistent with a persistent physical force that shears the chromatin.

In agreement with the appearance of DSBs in arrested cells (11), we found no DNA damage in G2 phase, but detected localized DNA damage as early as prometaphase in mitosis (Fig. S4). These DSBs apparently persisted into the next interphase. Although intramitotic DSBs apparently cause H2A.X phosphorylation (11), they allow for completion of mitosis despite extensive lesions (39). The metaphase–anaphase transition thus seems to be a critical step, as neither spindle symmetry nor chromatin integrity is monitored at this moment (39, 40). This could explain why intra-

mitotic DNA damage does not cause metaphase arrest but allows the cell cycle to continue.

The apparent randomness of structural changes may not reflect random breakpoint positioning, but could result from random transposition of related chromosome fragments (3); small pieces of chromatin consisting almost exclusively of centromeric material could be randomly transposed into noncentromeric sites. Another recurrent genetic defect in carcinomas is the loss or gain of complete chromosome arms (41). Colon cancer cell lines with a CIN phenotype, but not those with other mutations, frequently show this defect (33). The loss of whole chromosome arms is easily explained by centromeric DSBs, as this positions the break exactly at the point that separates the two chromosome arms. After chromosome segregation, free DNA ends with or without centromere identity can react with different partner substrates (Fig. S7). A single event, spindle-induced centromere shearing, may thus underlie a variety of genetic defects found in carcinomas.

Materials and Methods

Cell Culture and Immunofluorescence. *Dido*, *ATM*, and *Ku80* mutant mice have been described (18, 42, 43). All experiments were performed in compliance with the European Union and National Bioethics Committee directives. MEFs were seeded on poly-L-lysine-coated glass coverslips for immunofluorescence. For γ H2A.X detection, cells were rinsed briefly in PBS, fixed in PBS containing 4% formaldehyde, and permeabilized in PBS with 0.5% Triton X-100. For tubulin detection, cells were rinsed briefly in PBS and fixed in methanol. After blocking in PBS containing 5% goat serum (1 h, room temperature), cells were incubated with primary antibodies (1 h, room temperature). Cells were then washed, incubated with secondary antibodies (Jackson ImmunoResearch), washed again, and mounted in Prolong Gold antifade (Invitrogen). Images were captured on an IX81 laser scanning confocal microscope (Olympus), with sequential fluorophore excitation. Final images were obtained through maximum projection by ImageJ software.

Antibodies. The CREST antiserum, recognizing CENP-A, and antibodies to *Dido* have been described (16, 44). We used commercial antibodies to γ H2A.X (Millipore and Abcam), phospho-ATM (Rockland), cyclin B1 (BD Biosciences), or α -tubulin (Upstate). DNA strand breaks were determined by TUNEL assay, using a fluorescein-dUTP-based in situ cell death detection kit (Roche Diagnostics).

Cell Irradiation. To assay DSB repair, MEFs were seeded on glass coverslips and irradiated with a 2-Gray dose from a calibrated cesium source 24 h after seeding. At the times indicated, coverslips were fixed and pooled for further processing. Cells were labeled with anti- γ H2A.X antibodies, counterstained with DAPI, and studied by fluorescence microscopy. The number of γ H2A.X foci in 50 cells was counted for each time point.

Bromodeoxyuridine Labeling. To determine cell cycle phase, MEFs were pulse labeled for 1 h with 10 μ M BrdU, washed three times in medium without BrdU, and then chased for the times indicated. Cells were fixed, DNA was partially denatured in 50 mM NaOH for 5 min, and BrdU was detected with fluorescein-coupled antibodies (BD Biosciences). DSBs and small constitutive γ H2A.X foci were visualized with anti- γ H2A.X antibodies, allowing precise S phase determination (32).

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