TAp73 regulates the spindle assembly checkpoint by modulating BubR1 activity

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The role of various p73 isoforms in tumorigenesis has been controversial. However, as we have recently shown, the generation of TAp73-deficient (TAp73−/−) mice reveals that TAp73 isoforms exert tumor-suppressive functions, indicating an emerging role for Trp-73 in the maintenance of genomic stability. Unlike mice lacking all p73 isoforms, TAp73−/− mice show a high incidence of spontaneous tumors. Moreover, TAp73−/− mice are infertile and produce oocytes exhibiting spindle abnormalities. These data suggest a link between TAp73 activities and the common molecular machinery underlying meiosis and mitosis. Previous studies have indicated that the spindle assembly checkpoint (SAC) complex, whose activation leads to mitotic arrest, also regulates meiosis. In this study, we demonstrate in murine and human cells that TAp73 is able to interact directly with several partners of the SAC complex (Bub1, Bub3, and BubR1). We also show that TAp73 is involved in SAC protein localization and activities. Moreover, we show that decreased TAp73 expression correlates with increases of SAC protein expression in patients with lung cancer. Our results establish TAp73 as a regulator of SAC responses and indicate that TAp73 loss can lead to mitotic arrest defects. Our data suggest that SAC impairment in the absence of functional TAp73 could explain the genomic instability and increased aneuploidy observed in TAp73-deficient cells.

Bub1 | meiosis | mitotic arrest | p73 | spindle checkpoint

A ccurate chromosome segregation during meiosis and mitosis is critical to the preservation of euploidy in eukaryotic cells (1). Errors in the molecular mechanisms regulating segregation result in aneuploidy, a hallmark of spontaneous abortions, birth defects, and many cancers (2, 3). The spindle assembly checkpoint (SAC) is a regulatory mechanism that senses the improper attachment of sister chromatids to the mitotic or meiotic spindle and delays anaphase until all chromosomes are correctly oriented for segregation (4, 5). More than 20 proteins participate in, or are functionally associated with, the SAC, including MAD2, BUB1, BUB3, BUBR1, cyclin B, Rael1, and Aurora B (6).

The high levels of chromosome mis-segregation in cancer cells suggest that they may have undergone SAC inactivation. Several SAC-related genes have been linked to tumors in humans and mice. Human gastric cancers frequently show mutations of MAD2 (7), and bi-allelic mutations of BUB1, which encodes BUBR1 in humans, lead to the rare familial disorder known as mosaic variegated aneuploidy. This disease features development. These mice are also subject to other defects such as birth defects, and many cancers (2, 3). The spindle assembly checkpoint (SAC) is a regulatory mechanism that senses the improper attachment of sister chromatids to the mitotic or meiotic spindle and delays anaphase until all chromosomes are correctly oriented for segregation (4, 5). More than 20 proteins participate in, or are functionally associated with, the SAC, including MAD2, BUB1, BUB3, BUBR1, cyclin B, Rael1, and Aurora B (6).

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in aneuploidy, suggesting that TAp73 is playing a role in the maintenance of genomic stability. This is supported by previous studies showing a connection among p53 family members, SAC regulation, and genomic instability (30–33), a crucial player in tumorigenesis (34). In view of these data, we reasoned that the observations indicate that, in the absence of TAp73, kinetochore vesicle breakdown, Bub1 localized to chromosomes of WT oocytes and tubulin assembly was evident (Fig. 1B). Both these events were delayed in TAp73-deficient oocytes. Whereas Bub1 failed to associate with chromosomes and remained cytoplasmic in a subset of ovulated oocytes—particularly those with scattered chromosomes (Fig. 1A)—BubR1 formed distinct foci around kinetochores, which appeared diffused in TAp73-deficient cells. Quantification of BubR1 protein levels in whole oocytes or in the metaphase plate area alone revealed that whole TAp73−/− oocytes showed an overall increase in BubR1 protein (Fig. 1D), but the amount of BubR1 protein specifically located within the metaphase plate was decreased in the mutant oocytes (Fig. 1E). Immunostaining to detect BubR1 in human cells treated with control or TAp73 siRNA [Fig. 1F and supporting information (SI) Fig. S1] resulted in similarly diffused and disorganized distribution of BubR1 in the absence of TAp73. These observations indicate that, in the absence of TAp73, kinetochore scaffold formed by Bub1 is either absent or altered, causing insufficient tethering of BubR1 to the kinetochore complex.

As in the TAp73 selective knockout, high levels of BubR1 were detected in the nuclei of TAp73-depleted human cells (Fig. 1A and B). Ovulated oocytes from TAp73−/−/+ and TAp73−/− mice were immunostained for tubulin (green), DNA (blue), and BubR1 (red). (Magnification: 40 × 10; insets, 100 × 10.) Arrows indicate specific localization of BubR1. (D) Increased total BubR1 protein. Total BubR1 and tubulin protein levels in TAp73−/− and TAp73−/− MII oocytes were determined by using immunostaining and deconvolution imaging software. Results shown are mean ± SE for the indicated number (n) of oocytes per group (***P < 0.001, Student t test). (E) Decreased BubR1 localization at the metaphase plate area. The DNA concentration/ BubR1 protein ratio in TAp73−/− and TAp73−/− oocytes was determined by using immunostaining and deconvolution imaging software. Results shown are the mean DNA/BubR1 ratio ± SE for the indicated number (n) of oocytes per group (***P < 0.001, Student t test). (F) Mis-localization of BubR1. HeLa cells treated for 48 h with control siRNA (siCt) or TAp73 siRNA (siTAp73) were exposed for 8 h to 0.6 μM nocodazole and stained with anti-BubR1 and DAPI. Arrows indicate BubR1 staining; arrowheads indicate co-localization (siCt) and non-co-localization (siTAp73) of BubR1 with chromosomes. (Magnification: 63 × 10.)

**Fig. 1.** TAp73 deficiency causes mis-localization of SAC components. (A and B) Mis-localization of Bub1 in ovulated oocytes (A) and 3 h after initiation of in vitro maturation (B). Blue staining (arrows), chromosomes; green staining (arrowheads), Bub1 staining; red staining, tubulin. (Magnification: 100 × 10.) (C) Decreased expression of BubR1. Ovulated oocytes from TAp73−/−/+ and TAp73−/− mice were immunostained for tubulin (green), DNA (blue), and BubR1 (red). (Magnification: 40 × 10; insets, 100 × 10.) Arrows indicate specific localization of BubR1. (D) Increased total BubR1 protein. Total BubR1 and tubulin protein levels in TAp73−/− and TAp73−/− MII oocytes were determined by using immunostaining and deconvolution imaging software. Results shown are mean ± SE for the indicated number (n) of oocytes per group (***P < 0.001, Student t test). (E) Decreased BubR1 localization at the metaphase plate area. The DNA concentration/ BubR1 protein ratio in TAp73−/− and TAp73−/− oocytes was determined by using immunostaining and deconvolution imaging software. Results shown are the mean DNA/BubR1 ratio ± SE for the indicated number (n) of oocytes per group (***P < 0.001, Student t test). (F) Mis-localization of BubR1. HeLa cells treated for 48 h with control siRNA (siCt) or TAp73 siRNA (siTAp73) were exposed for 8 h to 0.6 μM nocodazole and stained with anti-BubR1 and DAPI. Arrows indicate BubR1 staining; arrowheads indicate co-localization (siCt) and non-co-localization (siTAp73) of BubR1 with chromosomes. (Magnification: 63 × 10.)

in aneuploidy, suggesting that TAp73 is playing a role in the maintenance of genomic stability. This is supported by previous studies showing a connection among p53 family members, SAC regulation, and genomic instability (30–33), a crucial player in tumorigenesis (34). In view of these data, we reasoned that the infertility and tumor-prone phenotype of TAp73−/− mice might be caused by a common cellular defect. In the present study, we investigated whether defects observed in TAp73-deficient mice could be related to defaults in SAC activity. Moreover, we analyzed whether TAp73 is able to directly interact with components of the spindle assembly checkpoint, and consequently regulate its activity.

**Results**

**Spindle Assembly Checkpoint Components Are Mis-Localized in TAp73−/− Mice.** The aneuploidy of p53−/− cells (33) and the spindle defects observed in TAp73−/− cells (28) prompted us to examine the expression and localization of Bub1 and BubR1 in TAp73−/+ and TAp73−/− oocytes. The localization of Bub1 (Fig. 1A and B) and BubR1 (Fig. 1C–E) to the meiotic spindle and its associated chromosomes was diminished in TAp73−/− oocytes resuming meiosis I in vitro as well as in ovulated oocytes arrested in prophase of meiosis II (Fig. 1A and C). Shortly after germinarial
levels of BubR1 protein were also found in TAp73−/− mouse embryonic fibroblasts (MEFs) that had been synchronized in culture and treated with the microtubule-disrupting agent nocodazole for 24 h (Fig. S2A), as well as in H1299 human lung carcinoma cells engineered to over-express various TAp73 isoforms (Fig. S2B). Overexpression of the ΔNp73β isoform did not have any impact on Bub1 or BubR1 protein levels (Fig. S2B). Taken together, these results show that an imbalance of p73 isoforms induces a modification in the expression and localization of SAC components such as Bub1 and BubR1.

To determine whether TAp73 plays a direct role in controlling SAC protein localization, we immunostained TAp73+/+ and TAp73−/− oocytes to detect TAp73. In TAp73+/+ oocytes, TAp73 was present in the cytoplasm and associated specifically with the meiotic spindle at metaphase II (Fig. S3). Immunostaining to detect ΔNp73 with a specific antibody (35) showed that this isoform did not co-localize with either the kinetochore or the spindle (data not shown). These data suggest that TAp73 is required for correct SAC protein expression and localization.

**TAp73 Physically Interacts with the SAC Components BubR1 and Bub1.** We next investigated whether TAp73 might physically interact with the SAC proteins BubR1 and Bub1. In parallel, we analyzed whether ΔNp73 could disrupt TAp73-SAC protein interaction by competing with TAp73 for SAC protein binding. We carried out co-immunoprecipitation experiments using HeLa cells engineered to over-express isoforms of TAp73 or ΔNp73 and determined whether any of these proteins bound directly to the SAC components BubR1 or Mad2. Only TAp73 (not ΔNp73) was found to bind BubR1 endogenously in two different human cancer cell lines—MDA-MB-231 (breast) and SW480 (colon)—that had been synchronized and treated with nocodazole before immunoprecipitation with anti-BubR1 and blotting with the TAp73-specific antibody (35) H-79 (Fig. 2A, Center) or IMG-246 Imgenex antibody (data not shown). Similarly, when MDA-MB-231 cells were immunoblotted using an anti-p73 antibody that recognizes both TAp73 and ΔNp73, only the binding of endogenous TAp73 to endogenous BubR1 was detected; no interaction between ΔNp73 and BubR1 was observed (Fig. 2A Right). We found that BubR1/p55 binding was impaired that it was the C-terminal part of TAp73 (which is also present in ΔNp73) that interacts with Bub1 (Fig. 2B) and Bub3 (Fig. S4A). However, during overexpression, both TAp73 and ΔNp73 interacted in vitro with BubR1 (but not with Mad2; Fig. S4B). We conclude that, in vivo, only TAp73 is able to physically interact with SAC components.

**TAp73 Is Involved in BubR1 Function.** We hypothesized that the loss of TAp73 in TAp73−/− mice might lead to a weakened SAC response. We therefore analyzed BubR1 activity after the induction of mitotic arrest in the presence or absence of TAp73. BubR1 was originally characterized as a protein kinase that controls the activation of the anaphase-promoting complex by binding and inhibiting p55cdc20 (p55), the major anaphase-promoting complex regulatory protein (36). Recently, activated BubR1 was shown to phosphorylate specific targets, including itself, and to induce mitotic cell death (31, 37). When we examined the effect of TAp73 deficiency on the ability of BubR1 to bind p55, we found that BubR1/p55 binding was impaired in TAp73−/− MEFs (Fig. 3A) and HeLa cells treated with TAp73 siRNA (Fig. 3B). Moreover, the binding of BubR1 with p55 was increased when TAp73 isoforms (mainly the TAp73β isoform) were overexpressed in H1299 cells, with or without nocodazole (Fig. 3C Left). Conversely, when TAp73 protein was reduced in HeLa cells via siRNA treatment, a decrease in SAC activity was observed, as evidenced by reduced phosphorylation of BubR1 and phospho-histone H3 (Fig. 3D Upper). Moreover, the increase in p55 level after nocodazole treatment (Fig. 3D Right) was lost in the absence of TAp73 (Fig. 3D Left), reflecting the absence of p55 binding with BubR1. Finally, when TAp73 was overexpressed, BubR1-mediated phosphorylation of histone H1 was increased (Fig. 3E, lanes 1–3). Thus, TAp73 is able to potentiate BubR1 activity, and TAp73 deficiency compromises BubR1 functions. Moreover, although overexpressed ΔNp73 increased BubR1/p55 binding to some extent, no modification of BubR1 kinase activity was observed after overexpression (Fig. 3F, lanes 1–5). These data indicate that TAp73, and not ΔNp73, plays a role in the SAC response in vivo.

**Correlation of TAp73, ΔNp73, and hBUBR1 Expression in Human Lung Cancer.** hBUBR1 expression is up-regulated in numerous lung cancer cell lines (38), and mitotic checkpoint defects are a feature of human lung cancers exhibiting chromosomal instability (39). When we assessed the expression of TAp73, ΔNp73, and hBUBR1 in matched normal and tumoral lung tissue samples from 18 patients with lung cancer, we found that TAp73 down-regulation, ΔNp73 up-regulation, a decreased TAp73/ΔNp73 ratio, and hBUBR1 up-regulation were all correlated in these samples (Fig. 4A; Pearson score, P = 0.001). The large increases in hBUBR1 mRNA levels seen in this experiment were consistent with the increased BubR1 protein observed in TAp73−/− oocytes and MEFs. However, these increases in BubR1 protein were not associated with increased BubR1 activity. Instead, the increase in total hBUBR1 protein may be a hallmark of the loss of hBUBR1 activity, which may be associated in some cases with protein mis-localization. These results suggest that a decrease in the TAp73/ΔNp73 ratio indicates a weakened SAC response
frequently associated in human tumors with a chromosome gation. Alterations in genes encoding mitotic regulators are fects might result in tumorigenesis is subject to intense investi- regulation of the SAC might lead to infertility and tumorigenesis. (31), and p73 is necessary for the mitotic death elicited by Bub1 mutation display increased aneuploidy and infertility (9). BubR1 has been implicated in the control of p53 functions (31), and p73 is necessary for the mitotic death elicited by Bub1 deregulation (45). The interaction between Bub1 and p73 was not investigated in the latter study but our work provides direct evidence of a molecular link between p73 and Bub1. The impact of aberrant chromosomal segregation and aneuploidy on tumorigenesis is slowly becoming better understood (40, 46–48), and our data have shed more light on this issue by linking TAp73 to SAC components.

Our findings provide a potential molecular mechanism for the association, at least in some tumors, of tumorigenicity and chemo-resistance with TAp73 loss of function and a reduced TAp73/ΔNp73 ratio. A relative lack of TAp73 not only predisposes a cell to aneuploidy and a CIN phenotype, but also more accurately than the determination of TAp73 or ΔNp73 expression alone.

**Discussion**

Here we show that a *p53* family member, TAp73, has a role in regulating SAC functions during meiosis and mitosis. As previously described, TAp73-deficient mice are sterile and TAp73−/− oocytes show defects in spindle organization that lead to im- paired early embryogenesis. Moreover, TAp73−/− mice develop spontaneous tumors, particularly lung adenocarcinomas, and are more sensitive to chemical carcinogens. The phenotype of TAp73-deficient mice and cells suggested that TAp73 could be involved in the formation or maintenance of the proper mitotic and meiotic spindles required for chromosome alignment and genomic stability. In the absence of TAp73, MEFs and human cells have a reduced ability to initiate and maintain proper mitotic arrest as a result of insufficient BubR1 and Bub1-mediated SAC functions, leading to genomic instability. The influence of TAp73 on the SAC response may underlie the tumor suppressor activity of TAp73 and account for the infertility and the spontaneous malignancies observed in TAp73−/− mice. Fig. S5 illustrates how abrogation of TAp73-mediated regulation of the SAC might lead to infertility and tumorigenesis.

The precise molecular mechanisms through which SAC de- fects might result in tumorigenesis is subject to intense investigation. Alterations in genes encoding mitotic regulators (40) are frequently associated in human tumors with a chromosome instability (CIN) “signature” (34). Conversely, 29 of the 70 genes identified in a cancer CIN signature function as mitotic regulators (41). Gene-targeted mice lacking mitotic regulators readily develop tumors (42), and mice heterozygous for a deficiency of a mitotic regulator show enhanced sensitivity to induced tumorigenesis (12). Thus, subtle changes in expression levels of specific mitotic regulators may have important consequences for genomic stability and tumorigenicity (43). Our findings support this hypothesis by demonstrating that TAp73 is one of these regulators whose loss leads to a weakened SAC response.

Our results are also consistent with studies showing that premature exit from mitosis occurs when essential mitotic checkpoint regulators are deleted, non-functional, or only partially functional (44). Indeed, cells from mice with a hypomor- phic *BubR1* mutation display increased aneuploidy and infertility (9). TAp73 has been implicated in the control of p53 functions (31), and p73 is necessary for the mitotic death elicited by Bub1 deregulation (45). The interaction between Bub1 and p73 was not investigated in the latter study but our work provides direct evidence of a molecular link between p73 and Bub1. The impact of aberrant chromosomal segregation and aneuploidy on tumorigenesis is slowly becoming better understood (40, 46–48), and our data have shed more light on this issue by linking TAp73 to SAC components.

Our findings provide a potential molecular mechanism for the association, at least in some tumors, of tumorigenicity and chemo-resistance with TAp73 loss of function and a reduced TAp73/ΔNp73 ratio. A relative lack of TAp73 not only predisposes a cell to aneuploidy and a CIN phenotype, but also...
compromises the ability of a tumor cell to die, either spontaneously or in response to chemotherapy. Although a relative TAp73 loss is not necessarily the only mechanism responsible for tumorigenesis in this context, it would be interesting to evaluate the karyotypes and chemo-sensitivity of tumor cells in which the TAp73 loss is not necessarily the only mechanism responsible for tumorigenesis in this context, it would be interesting to evaluate the karyotypes and chemo-sensitivity of tumor cells in which the normal TAp73/Np73 balance is lost.

**Materials and Methods**

**Immunohistochemical Analysis of Oocyte Sections.** Female mice (3–7 weeks of age) were superovulated, and germinal vesicle or ovulated oocytes were collected as previously described (49). Oocyte fixation and immunocytochemistry were performed as previously described (50). Samples were viewed on a deconvolution fluorescence microscope. Optical sections each were analyzed using DeltaVision software (Applied Precision) as previously described (51).

**Statistical Analysis.** Data were analyzed by Student t test or Pearson test. For Student t test, data are presented as mean ± SD, and P values less than 0.05 were considered statistically significant.

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**Fig. 4.** Correlation of TAp73, Np73, and BubR1 expression in human lung cancer. Correlation of expression levels of TAp73, Np73, and hBubR1 in human lung cancers. (Top) Orange bars indicate that the expression of the indicated mRNA was decreased in the tumor sample compared with the paired normal tissue. Green bars indicate that the expression of the indicated mRNA was increased in the tumor sample compared with the paired normal tissue. Pearson R, P = 0.001.
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