Tumorigenesis and Neoplastic Progression

Disabled-2 Heterozygous Mice Are Predisposed to Endometrial and Ovarian Tumorigenesis and Exhibit Sex-Biased Embryonic Lethality in a p53-Null Background

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Disabled-2 (Dab2) is a phosphoprotein involved in cellular signal transduction and endocytic trafficking. The expression of Dab2 is frequently lost or suppressed in several epithelial tumors, and studies of its cellular function and growth suppressive activity when re-expressed in cancer cells led to the suggestion that Dab2 is a tumor suppressor. A role for Dab2 in epithelial cell positioning organization was derived from study of knockout mice: homozygous deletion of *dab2* results in early embryonic lethality due to the disorganization of the primitive endoderm, the first epithelium in early embryos. We now report that *dab2* heterozygous mice develop uterine hyperplasia and ovarian preneoplastic morphological changes at a high frequency. Crossing into a *p53*^{-/-} background unexpectedly produced few female *dab2*^{+/-};*p53*^{-/-} mice, while the male *dab2*^{+/-};*p53*^{-/-} were born at the expected Mendelian frequency. The tumor-prone phenotype of *dab2*^{+/-} mice provides additional support for a role of human Dab2 as a tumor suppressor, and the sex-biased embryonic lethality suggests a genetic interaction between *p53* and *dab2* genes in female mice. (Am J Pathol 2006, 169:258–267; DOI: 10.2353/ajpath.2006.060036)

Disabled-2 (Dab2) was initially identified as a phosphoprotein in mitogenic signal transduction in murine mac-

rophages.¹ The involvement of Dab2 in human ovarian cancer was first suggested after identification of a differentially expressed polymerase chain reaction (PCR) product in ovarian carcinomas compared to nontumor ovarian surface epithelial cells.² Since then, studies of its loss of expression in tumors^{3–5} and growth suppressive activity when re-expressed^{4–7} suggest a putative tumor suppressor function for Dab2 in ovarian cancer. The involvement of Dab2 loss in other epithelial tumor types has also been suggested.^{8–10}

Studies in cultured cells provide a cellular function for Dab2: the protein binds clathrin,^{11,12} AP-2,^{11,12} myosin VI,^{13,14} and LDL family receptors.^{11,15–17} Thus, Dab2 is a cargo-specific adaptor for the endocytic trafficking of LDL-family receptors.¹² Dab2 was also reported to influence cellular mitogenic signal transduction^{7,18–20}; however, the cellular mechanism is less certain. Knockout mouse studies demonstrate the biological importance of Dab2: the mouse *dab2* homozygous knockout is early embryonic lethal.^{21,22} The early embryonic lethality is caused by the disorganization of primitive endoderm, the epithelial cell type of early embryos.²² Thus, a role for Dab2 in epithelial organization is proposed,^{5,22,23} providing a provocative explanation for the mechanism of tumor suppression. Nevertheless, a tumor suppressor role for Dab2 is still tentative, and further evidence for a causative role of Dab2 deficiency in tumor development is desirable.

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Several causative genetic and epigenetic changes for ovarian cancer have been considered and proposed,^{24,25} and some have been verified in mouse models. Mutation in the tumor suppressor p53 is common in ovarian carcinomas²⁶; however, *p53*-null mice usually do not develop ovarian epithelial tumors,²⁷ even with ovarian transplantation to wild-type host²⁸ or conditional inactivation in ovaries²⁹ that allow longer latent time. Conditional activation of *Kras2* in the ovaries leads to endometriosis, and superimposing a *Pten* deletion in the ovaries results in the development of ovarian carcinomas of the endometrioid histological subtype.³⁰ Nevertheless, known genetic changes that predispose the ovaries to either benign or malignant lesions are not abundant. Here, we report that *dab2*-haplodeficient mice develop hyperplasia and dysplasia in the uterus and papillomatosis and cystadenomas in the ovarian surface, providing support for a tumor suppressor role of *Dab2*.

Materials and Methods

Genetically Targeted Mutant Mice and Genotyping

Two lines of *dab2* knockout mice were established previously,²² and the colonies were maintained by inbreeding in the C57BL6–129/sv background in the animal facility of Fox Chase Cancer Center. The two lines have given identical phenotypes and will not be distinguished here. At ~1 month of age, DNA was extracted from tails to amplify simultaneously in PCR reactions both the wild-type (850 bp) and targeted (1100 bp) allele for *dab2*. The PCR reaction was performed by addition to the template DNA of a common master mix of PCR reagents including three primers: a sense primer to the *dab2* gene, P1 (5'-CACATATGAGAGAGAACGGGC-3'), an anti-sense primer for the wild-type *dab2* gene, P2 (5'-TCCGGTTGTCCGACGGGGC-3'), and another anti-sense primer specific for the recombinant mutant allele, P3 (5'-GAGTTTGACCGTCTACGTGCC-3').

The mating pairs of *p53* mutant mice (B6.129S2-Trp53tm1Tyj/J) were purchased from Jackson Laboratory (Bar Harbor, ME), and an inbreeding colony was maintained in the animal facility at Fox Chase Cancer Center by crossing heterozygous mice. The mice were originally made by the laboratory of Dr. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA) and were donated to the Jackson Laboratory. Similar to the original reports,²⁷ the mice homozygous for the Trp53tm1Tyj mutation show no visible phenotype but develop lymphomas at 3 to 6 months of age. Genotyping of the *p53* locus by PCR followed the protocol from the Jackson Laboratory. A new inbreeding colony was established by crossing three pairs each of *dab2*^{+/-} with *p53*^{+/-} mice. Progeny with *dab2*^{+/-};*p53*^{+/-} genotype were further intercrossed to produce *dab2*^{+/-};*p53*^{-/-} mice for analysis.

Histology and Histochemistry

Uterine horns containing attached ovaries were harvested for analysis from female *dab2*^{+/-} mice and wild-type littermates of various ages. The tissue samples were formalin-fixed and paraffin-embedded. Sections (5 μ m) were cut and adhered to positively charged slides (Fisher, Pittsburgh, PA). Standard hematoxylin and eosin (H&E) staining was applied. Immunohistochemistry using a primary antibody to *Dab2* (BD Transduction Laboratories, Lexington, KY) diluted 1:400 was performed as previously described.^{3,22}

Rodent Tumor Grading

The *dab2* mouse colonies were maintained by crosses between *dab2*^{+/-} progenies throughout a 1-year period. *dab2*^{+/-} female mice of 3 to 6 months of age were euthanized, and the uteri removed for histological examination. In every experiment, matching *dab2* wild-type littermates (approximately one-fourth of the mutants) were used as controls for comparison. The sections were examined in a blinded manner to identify hyperplastic lesions or adenocarcinomas. Endometrial hyperplasia and adenocarcinomas were evaluated following a modification of accepted human lesion classifications.³¹ The following four categories were used: 1) hyperplasia grade I or hyperplasia without atypia, characterized by increased pseudostratification and eventual stratification of the endometrial glands, mild dilation of the lumen, and minimal papillary formations; 2) hyperplasia grade II or mild atypical hyperplasia, with increased stratification of the endometrial epithelium, increased papillary outgrowth in dilated glands, complexity of glandular structure, and occasional loss of polarity and nuclear atypia; 3) hyperplasia grade III or moderate atypical hyperplasia, having similar but accentuated features as seen in grade II, especially dilated complex glands and numerous papillary formations and an increased gland-to-stromal ratio; 4) hyperplasia grade IV or complex atypical hyperplasia, characterized by increased structural complexity and cellular atypia, stratification, and mitotic activity, and little stroma left between glandular structures. Occasional stromal or myometrial invasion seen in an otherwise grade IV endometrial hyperplasia was interpreted as early adenocarcinoma. The histopathological results were systematically reviewed by A.K.-S., an experimental pathologist with experience in rodent tumors.

Human Tissue, Tumor Samples, and Sections

All experimental protocols involving usage of human normal and tumor tissues were examined and approved by the Human Investigation Committee and were considered exempted. Normal and cancerous human endometrial tissue samples were obtained from surgeries performed at Emory University Hospital. Fresh tumor or nontumor tissues from surgery were snap-frozen in liquid nitrogen, after diagnosis and inspection by pathologists. In this study, three normal uterine tissues and 28 tumors

Table 1. Uterine Neoplastic Phenotypes in *dab2*^{+/-} Mice

Genotype	Total number	Endometrial hyperplasia				Total (%)
		Grade I	Grade II	Grade III	Grade IV	
<i>dab2</i> ^{+/+}	33	1	0	0	0	1 (3%)
<i>dab2</i> ^{+/-}	134	13	25	8	2	48 (36%)

The number and percentage of uteri with hyperplastic characteristics were determined in a total of 134 female *dab2*^{+/-} and 33 wild-type littermates. Most of the mice were ~6 months of age. A small percentage (~20%) of both the mutant and wild-type mice were 9 to 12 months old at the time of sacrifice.

all classified as high-grade endometrial carcinomas were used. The tissue samples were formalin-fixed and paraffin-embedded. Sections (5 μ m) were cut and adhered to ProbeOn Plus-charged and precleaned glass slides (Fisher) or aminoalkylsilane-coated Silane-Prep glass slides (Sigma, St. Louis, MO) for use in immunohistostaining. The human tumor tissues were provided and reviewed by C.C., a surgical pathologist.

Cell Culture and Western Blot

A panel of six uterine (AN3, HEC-1-B, KLE, MES-SA, RL95-2, SK-UT-1B) and eight cervical (C-33A, CaSKI, HeLa, HT-3, ME-180, MS751, SiHa, SW756) human cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum supplemented with 10 ng/ml epidermal growth factor, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μ g/ml streptomycin, in a 5% CO₂ incubator at 37°C. Lysates were prepared from proliferating cells that were ~80% confluent by directly collecting the cells into sodium dodecyl sulfate-lysis buffer (50 mmol/L TrisCl, pH 6.8, 100 mmol/L dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% glycerol) using a rubber cell scraper. The lysate was boiled for 5 minutes after harvesting, before loading onto 7.5% polyacrylamide-sodium dodecyl sulfate gels for electrophoresis and immunoblotting. Dynamin (125 kd), which has a similar molecular weight as the 96-kd Dab2 in human cells and which exhibits constant protein level in cells, served as a loading control. Both anti-dynamin and anti-Dab2 monoclonal antibodies were from Transduction Laboratories.

Results

Uterine Hyperplasia in *dab2*-Haplodeficient Mice

Although *dab2*^{+/-} mice develop normally, continuous breeding of the two *dab2* knockout lines uncovered an obviously reduced reproductive capacity of the females, although not of the males. In timed-mating experiments, *dab2*^{+/-} females exhibited vaginal plugs as frequently as wild-type littermates. However, *dab2*^{+/-} females had a much lower rate of pregnancy (~10%) compared to wild-type littermates (~50%). By 6 months of age, the *dab2*^{+/-} female mice were essentially sterile, whereas *dab2*^{+/+}

male or wild-type females remained reproductively competent up to 1 year of age. Dissection and histological examination of the uteri from the mice in these colonies revealed widespread uterine hyperplasia and dysplasia in *dab2*^{+/-} females (Table 1). In wild-type mice, the endometrium is composed of a single cell layer of columnar epithelium and displays characteristic morphological changes showing elongation and mild branching during the early proliferative phase of the reproductive cycle (Figure 1, A and B). In *dab2*^{+/-} females, luminal endometrial hyperplasia and dysplasia were common (Figure 1, C–E), and two cases (1.5%) showed advanced endometrial hyperplasia including the glandular components that are compatible with endometrioid adenocarcinomas (or grade IV) (Figure 1F). Of the 46 (34.3%) additional *dab2*^{+/-} uteri classified as hyperplasia (Table 1), 13 cases were classified as hyperplasia grade I or hyperplasia without atypia (Figure 1C), 25 cases were determined to be hyperplasia grade II or mild atypical hyperplasia (Figure 1D), and 8 cases were classified as hyperplasia grade III or moderate atypical hyperplasia (Figure 1E). Most changes were usually limited to the luminal epithelium. In the 33 control cases of *dab2*^{+/+} littermates, only one case of mild hyperplasia was observed. Dab2 staining was generally negative in hyperplastic and adenocarcinoma lesions of the endometrial epithelia from *dab2*^{+/-} mice (Figure 1, H and I) compared to the normal endometrial epithelia of wild-type littermates (Figure 1G). In the endometria from *dab2* wild-type littermates, Mib-1-positive cells were most often stromal with occasional to moderate number of positive cells depending on the cycle and are rarely epithelial (Figure 1, J and K). In hyperplastic endometrial epithelia from *dab2*^{+/-} mice, nearly all epithelial cells were positive for Mib-1 nuclear staining in hyperplasia of grade I (Figure 1L), grade II (Figure 1M), and grade III (Figure 1N) lesions. Curiously, the presence of Mib-1-positive cells was focally reduced in the two cases of endometrioid adenocarcinomas (Figure 1O). This is unique for the adenocarcinomas from *dab2*^{+/-} mice because in comparison, endometrioid adenocarcinomas from *pten*^{+/-} mice are Ki67-positive in a much higher percentage of cells. Thus, a reduction of *dab2* gene copy number leads to preneoplastic transformation of uterine epithelial cells. The involvement of Dab2 loss in human uterine cancer has not been investigated, although the loss of Dab2 expression and its tumor suppressor function have been extensively investigated in human ovarian cancer.^{3–5}

Figure 1. Endometrial hyperplasia in *dab2*^{+/-} mice. Three- to six-month-old female *dab2*^{+/-} mice and control *dab2*^{+/+} littermates were euthanized, and the uteri were collected for histological examination. **A–F:** Representative examples of H&E staining. **A:** A normal luminal endometrium from a *dab2*^{+/+} mouse in nonproliferating phase (mid-estrus) showing a simple columnar epithelium of well-organized tall cylindrical cells. **B:** A proliferating phase (proestrus) luminal endometrium from a *dab2*^{+/+} mouse showing a simple columnar epithelium of well-organized tall cylindrical cells with increasing size and number of cells. Edematous changes in stroma can be noted. **C:** A hyperplasia grade I endometrium from a *dab2*^{+/-} mouse has mild dilation and branching of the endometrial luminal epithelia with pseudostratification and small papillary outgrowths (arrows). Occasional areas of cell crowding can be noted. **D:** A hyperplasia grade II luminal endometrium from a *dab2*^{+/-} mouse is characterized by increased dilation, some epithelial stratification and clear papillary formations (arrows) with mild cell atypia. **E:** A hyperplasia grade III endometrium from a *dab2*^{+/-} mouse is composed of large papillary outgrowths (arrows) with increased cell crowding and mild to moderate cellular atypia mainly in the luminal epithelia. **F:** A hyperplasia grade IV endometrium from a *dab2*^{+/-} mouse. Changes are seen in the glandular component accompanied by similar changes in the luminal endometrium. They consist of increased gland/stromal ratio, changes in polarity and cellular atypia, and apoptotic and mitotic figures are seen in these hyperplastic glands. The lesion also shows the pushing border of this grade IV lesion (arrow), a feature compatible with early invasive adenocarcinoma. Dab2 immunostaining of normal luminal endometrial epithelium (**G**) from a wild-type female compared to endometrial hyperplasia (**H**) and adenocarcinoma (**I**) from *dab2*^{+/-} mice. Dab2 staining is reduced/absent in hyperplastic endometrial epithelia and adenocarcinoma cells. **J–O:** Mib-1 (Ki67) immunohistochemical staining was performed to estimate the proliferating activity of the endometrium. **J:** Normal endometrium of a wild-type mouse in nonproliferating phase (mid-estrus) showing very few proliferating cells. Proliferating cells were noted in the stroma. **K:** Normal endometrium from a wild-type control in proliferating phase (proestrus) showing increased proliferating cells both in the luminal endometrium and uterine gland. Only a small number of epithelial cells are positive for Mib-1 nuclear staining. Hyperplastic endometrium from *dab2*^{+/-} mice show positive Mib-1 staining in nearly all epithelial cells in hyperplasia grade I (**L**), grade II (**M**), and grade III (**N**). Mib-1 staining is reduced in endometrioid adenocarcinomas (grade IV) from *dab2*^{+/-} mice (**O**).

Ovarian Phenotypes in *dab2*-Haplodeficient Mice

Since *Dab2* was previously studied mostly for its role in ovarian cancer, we examined the morphology of ovaries from heterozygous *dab2* mutant mice compared to wild-type littermates. Frequently, the morphology of the ovarian surface epithelium was altered in *dab2*^{+/-} mice compared to wild-type littermates in mice older than 6 months. None of the 20 control *dab2*^{+/+} littermates showed noticeable

changes in ovarian morphology (Figure 2A). Ovaries from 18-month-old *dab2*^{+/-} mice typically exhibited surface epithelial dysplasia and papillomatosis (Figure 2B), which are considered by some as precursors of ovarian cancer.^{32–35} At 1 year of age, the ovaries of 100% of the 50 *dab2*^{+/-} female mice examined showed ovarian lesions with various degrees of change (Figure 2, C and D). Such ovarian lesions can be observed in some 6-month-old mice, in which significant number of follicles are still present in the ovaries (Figure 2, E and F).

Figure 2. Ovarian phenotypes of *dab2*^{+/-} mice. **A:** A representative ovary from an 18-month-old wild-type littermate is shown as control. **B:** Morphological dysplasia and papillomatosis of ovarian surface epithelia in a representative example of an 18-month-old *dab2*^{+/-} mouse. An **arrow** indicates area of severe surface papillomatosis. **C:** Ovarian surface epithelial dysplasia and papillomatosis were observed in a *dab2*^{+/-} mouse at 12 months of age. An **arrow** indicates area of severe surface papillomatosis, and the **boxed area** is shown at a higher magnification in **D**. **E:** An example of an ovary from a 6-month-old *dab2*^{+/-} mouse is shown. An **arrow** indicates area of surface papillomatosis, and the **boxed area** is shown at a higher magnification in **F**. **G–I:** Three examples of ovarian cystic adenomas in *dab2*^{+/-} mice. Three ovaries from 6-month-old *dab2*^{+/-} mice contain ovarian cystic adenomas showing hyperplastic and papilla epithelia. The slides were H&E stained (**top**) or immunostained with pan cytokeratin (**bottom**, brown color). Magnified details of the area indicated by **asterisks** are shown at the **right**.

In a smaller percentage of *dab2*^{+/-} mice (5 to 6%, or 8 of 143 mice at 6 to 12 months of age), the ovaries exhibited large cysts to be considered ovarian cystadenomas. The cysts were lined with hyperplastic epithelial cells, which were positive for cytokeratin, and showed papilla projections and epithelial stratifications (Figure 2, G–I). These morphological changes resemble human ovarian epithelial lesions, considered by some to be preneoplastic,^{32–35} and to some extent early serous borderline tumors and usually do not occur spontaneously in mice.

However, no malignant or large ovarian tumors were ever observed in any *dab2*^{+/-} mice produced in our colony throughout a 2-year period. In a total of ~200 female and 100 male mice analyzed at 6 to 12 months of age, we occasionally found hyperplastic lesions in other organs, including the prostate (two cases), mammary ductal ectasia (three cases), meningiomas in cerebral ventricles (three cases), skin squamous cell carcinoma (two cases), and lymphoma (five cases). Such cases were relatively uncommon, and these lesions were not controlled stringently with wild-

type littermates to demonstrate significance. We conclude that *dab2* haploinsufficiency does not commonly lead to widespread tissue hyperplasia in other organ sites. Thus, *dab2* haploinsufficiency predisposes the ovarian surface epithelia for preneoplastic morphological changes. Nevertheless, additional genetic or epigenetic factors are required for tumor development.

Sex-Biased Embryonic Lethality of *dab2*-Haploinsufficient Mice in a *p53*-Null Background

Human ovarian and endometrial carcinomas commonly contain *p53* mutations,^{26,36} but a *p53* deletion does not generally lead to tumor development in ovaries or uteri nor is it sufficient to induce preneoplastic changes in mouse models.^{27–29} We reasoned that mice with *dab2* inactivation might develop more malignant ovarian and endometrioid tumors in a *p53* mutant genetic background. To examine the potential synergy in tumor devel-

Table 2. Genotype Distribution of Live Mice Born from Crossing Between Male and Female *dab2*^{+/-}:*p53*^{+/-}

<i>dab2</i>	<i>p53</i>	Observed			Predicted or (assuming <i>dab2</i> ^{-/-} lethal)	
		Number	%	Fraction	%	Fraction
Male						
(+/+)	(+/+)	15	7.4	1.2/16	8.3	1/16 or (1.33/16)
(+/+)	(+/-)	39	19.3	3.1/16	16.6	2/16 (2.66/16)
(+/+)	(-/-)	17	8.4	1.3/16	8.3	1/16 (1.33/16)
(+/-)	(+/+)	22	10.9	1.7/16	16.6	2/16 (2.66/16)
(+/-)	(+/-)	65	32.2	5.1/16	33.3	4/16 (5.32/16)
(+/-)	(-/-)	44	21.8	3.5/16	16.6	2/16 (2.66/16)
(-/-)	(+/+)	0			0	1/16 (0/16)
(-/-)	(+/-)	0			0	2/16 (0/16)
(-/-)	(-/-)	0			0	1/16 (0/16)
		Total 202				
Female						
(+/+)	(+/+)	14	7.5	1.2/16	8.3	1/16 or (1.33/16)
(+/+)	(+/-)	44	23.6	3.8/16	16.6	2/16 (2.66/16)
(+/+)	(-/-)	15	8.1	1.3/16	8.3	1/16 (1.33/16)
(+/-)	(+/+)	26	14.0	2.2/16	16.6	2/16 (2.66/16)
(+/-)	(+/-)	80	43.0	6.9/16	33.3	4/16 (5.32/16)
(+/-)	(-/-)	7*	3.8	0.6/16	16.6	2/16 (2.66/16)
(-/-)	(+/+)	0			0	1/16 (0/16)
(-/-)	(+/-)	0			0	2/16 (0/16)
(-/-)	(-/-)	0			0	1/16 (0/16)
		Total 186				

Matings of young (2 to 4 months of age) siblings in an inbreeding colony of *dab2*^{+/-}:*p53*^{-/-} were followed throughout a 1-year period in an attempt to produce mice with *dab2* and *p53* compound mutations. The pups born were genotyped at ~1 month of age.

*Of the seven *dab2*^{+/-}:*p53*^{-/-} females produced, four died of unexplained reasons at 1 to 2 months of age. The percentage and ratio of male and female progenies with *dab2*^{+/-}:*p53*^{-/-} genotype are highlighted in bold.

opment of *dab2*^{+/-} with additional tumor-prone genotypes, we crossed *dab2*^{+/-} with *p53* knockout mice. We found that the *dab2*^{+/-}:*p53*^{-/-} mutation was female-biased embryonic lethal (Table 2). In the breeding experiments, 7 female pups of the *dab2*^{+/-}:*p53*^{-/-} genotype were obtained compared to 44 male littermates of the same genotype, which approximates the expected Mendelian ratio. Of the seven *dab2*^{+/-}:*p53*^{-/-} females produced, four died of unexplained reasons at 1 to 2 months of age. No discernable pathology was found from histopathological analysis of the three remaining female *dab2*^{+/-}:*p53*^{-/-} mice at ~2 months of age. The theoretically expected *dab2*^{+/-}:*p53*^{-/-} genotype from the 186 total female progenies should be 31; thus, we observed a 4.4-fold reduction in the number of the combined *p53*-null and *dab2* haplotype in females but not in males. The sex-biased embryonic lethality of *dab2*^{+/-}:*p53*^{-/-} mice indicates a genetic interaction between *p53* and *dab2* genes in females. Thus, the potential synergistic effect of *p53* and *dab2* deficiencies in tumor predisposition may be investigated using tissue-specific mutation techniques in future experiments.

Loss of Dab2 Expression Closely Correlates with Epithelial Morphological Transformation in Human Ovarian Cancer

It has been well established that Dab2 expression is lost in ~90% of ovarian tumors.³⁻⁵ The knockout mouse study suggests that Dab2 functions in maintaining epithelial organization in the embryos.²² Here we further investigated the relationship between cell organization and morphology and the expression pattern of Dab2 in hu-

man ovarian tumors. The goal was to verify if histological data are consistent and supportive of the hypothesis for a function of Dab2 in the maintenance of epithelial organization.

The majority of ovarian cancer is derived from the ovarian surface epithelium, a flat to columnar simple epithelial cell type.³² Dab2 is expressed strongly in the surface epithelial layer^{3,5} and in a few cells (believed to be macrophages) scattered in the stroma. The loss of Dab2 expression is thought to be an early step in ovarian tumorigenesis.³ In a nontumor area of an ovary adjacent to neoplastic tissues, some epithelia with minor morphological changes can be detected, which presumably represent early alterations that occur in neoplastic transformation. In the example of an early stage of epithelial morphological transformation, cells pile up and start to stratify (Figure 3A, arrowheads). In the epithelium, most cells were positive for Dab2 expression but a few cells showed no Dab2 staining. Without exception, the cells that were negative for Dab2 expression (Figure 3A, arrowheads) were located in the basal layer and covered by neighboring cells, with no apical surface exposed. In histological examination of slides of portions of ovarian tumors that are confined to ovaries, areas of contiguous epithelia linking morphological normal to neoplastic lesions often can be found. In more than 50 cases of such epithelial transitional zones examined, we consistently found that Dab2 staining was positive in the monolayer region of the ovarian surface epithelium, and Dab2 expression was lost in the multilayer neoplastic lesions. Two representative examples of Dab2 immunostaining of transition zones found in a low-grade ovarian ade-

Figure 3. Loss of Dab2 expression associates with morphological transformation in human ovarian cancer. Paraffin-embedded ovarian tumor tissues were sectioned and analyzed after H&E staining and immunostaining. **A:** An example of Dab2 immunostaining of an ovarian surface epithelium that is starting to form a double cell layer (arrow) in several regions. **B:** After examination of a paraffin block that consists of both tumor and nontumor ovarian tissues, an epithelial transition zone was identified (asterisk) in this stage II serous carcinoma of the ovary. Sections from this tissue were immunostained for Dab2. **Left:** Panels ($\times 100$) show a simple (monolayer) epithelium colliding with transformed cells. **Right:** Transition junctions (asterisk) where simple epithelium (arrowhead) is positive for cytoplasmic Dab2 staining, and morphologically dysplastic cells (arrow) have lost Dab2 expression. **C:** An example of an epithelial transition zone linking the simple (monolayer) ovarian surface epithelium (arrowhead) with an invasive malignant ovarian carcinoma (arrow). A close-up of the monolayer (**D**) and tumor area (**E**) of this tumor is shown. A potential mitotic figure is indicated by an arrow.

nocarcinoma (Figure 3B) and a more malignant, invasive ovarian carcinoma (Figure 3C), respectively, are shown. Simple epithelia (monolayer epithelial cells) were Dab2-positive, Dab2 expression was absent in the stratified epithelial areas (multilayered cells) (Figure 3, B and C), and the tumor cells were either detached from the surface or invaded into the stroma (Figure 3C, arrow). Monolayer Dab2-positive cells were generally negative of the Ki67 proliferative marker (Figure 3D, arrow), and loss of Dab2 and monolayer organization in tumor cells correlated with an increased Ki67 staining indicative of proliferation (Figure 3E). Hence, loss of Dab2 expression correlates closely with the morphological transformation of the ovarian surface from a monolayer to multilayered proliferative epithelium. Thus, we confirmed that Dab2 expression closely correlates with morphological transformation, consistent with the phenotype of endoderm epithelial disorganization of the *dab2* knockout mouse embryos.²²

Loss of Dab2 Expression in Human Endometrioid Adenocarcinomas and Cancer Cells

The predisposition to uterine hyperplasia in *dab2*^{+/-} mice was unexpected since a role for Dab2 in uterine cancer has not been suggested. Thus, we investigated the expression of Dab2 in human endometrium and cancer. In three blocks of nontumor human uterine tissue containing normal endometrium, Dab2 was observed in endometrial

epithelial cells (Figure 4A). Twenty-eight paraffin-embedded endometrioid adenocarcinomas were processed and investigated for the expression of Dab2 protein by immunostaining. Dab2 expression was found to be absent in cancer cells in all of the carcinomas. As shown in the three examples (Figure 4, B–D), Dab2 staining was absent in cancer cells, but positive cells (likely fibroblasts or macrophages) are scattered in the stroma, serving as an internal control for the staining. In some cases, epi-

Figure 4. Loss of Dab2 expression in endometrioid adenocarcinomas. Paraffin-embedded endometrial tumor tissues were sectioned and analyzed by immunostaining for Dab2. **A:** An example of Dab2 immunostaining in a human normal endometrium. **B–D:** Three examples of Dab2 staining in human endometrioid carcinomas. Note in **B** that the normal endometrial gland stains positively whereas the surrounding carcinoma cells are negative for Dab2. Original magnifications: $\times 40$ (**A**, left); $\times 200$ (**A**, right).

Figure 5. Dab2 expression in a panel of gynecological cancer cell lines. Western blot analysis for the expression of Dab2 in total cell lysate: HEC-1-B, SK-UT-1B, RL95-2, KLE, CaSki, C-33A, HT-3, ME-180, SW756, SiHa, AN3, MES-SA, MS751, and HeLa cells. Ova, ovarian surface epithelial cells, serve as a positive control for Dab2. Dynamin (125 kD) was used as a loading control.

thelial cells in morphologically normal endometrium located inside tumor masses were still positive for Dab2 staining (Figure 4B).

We also investigated an available panel of six endometrial and eight cervical cancer cell lines by Western blot analysis (Figure 5). Of these, eight cell lines including HEC-1-B, SK-UT-1B, RL95-2, KLE, CaSki, C-33A, HT-3, and ME-180 showed the complete loss of Dab2 protein. Dab2 was weakly expressed in SW756 and SiHa cells. The other four cell lines (AN3, MES-SA, MS751, and HeLa) showed strong Dab2 protein expression. For the six endometrioid cancer cell lines, Dab2 was positive in two (AN3 and MES-SA) and lost in four cell lines (HEC-1-B, SK-UT-1B, RL95-2, and KLE). Of cervical cancer cells, SiHa and HeLa were Dab2-positive; MS751 and SW756 were weak; and the other four lines, C-33A, HT-3, CaSki, and ME-180, were negative for Dab2. Thus, the loss and aberrant expression of Dab2 in endometrioid tumors and cancer cells suggests that the loss of Dab2 may also be involved in the development of human endometrial cancer. Further, approximately half of cervical cancer cells were negative for Dab2 expression, which may be suggestive for additional investigation of Dab2 loss in cervical cancer.

Discussion

Our finding that *dab2* heterozygous mice develop pre-neoplastic changes in ovaries and hyperplastic lesions in uteri provides support for the previous speculation that Dab2 has a tumor suppressor function. That *dab2*^{+/-} mice developed only a small percentage of microscopic endometrioid carcinomas and did not develop significant tumor masses indicates that the loss of Dab2 is a contributing factor to the development of ovarian and uterine tumors. Presumably, the loss of Dab2 may cooperate with additional genetic alterations, such as a p53 mutation, to promote tumor development. p53 mutations are frequent in high-grade ovarian and uterine cancer,^{26,36} providing a rationale for our examination of tumor development in combined p53 mutation and *dab2* haploinsufficiency.

Unexpectedly, we found that the *p53*^{-/-}:*dab2*^{+/-} genotype exhibits sex-biased embryonic lethality. Male *p53*^{-/-}:*dab2*^{+/-} mice were born at a normal Mendelian ratio whereas the number of living adult female *p53*^{-/-}:*dab2*^{+/-} mice were greatly reduced compared to the expected normal proportion. The female embryonic lethality of *dab2*^{+/-} mice in the *p53*-null background pro-

hibits us from analyzing the potential synergistic effect of a combined mutation of *dab2* and *p53* in the development of ovarian or uterine tumors using the current mouse models. Thus, further analysis will require conditional and tissue-specific *p53* and/or *dab2* deletion in future experiments using mouse models.³⁷ A sex-biased embryonic lethality has been observed previously in the *p53*^{-/-}:*msh2*^{-/-} genotype,³⁸ suggesting the *p53* gene and the *msh2*-mediated DNA repair pathway exhibit interaction in the female genetic background. Similarly, it may be speculated that *p53* and *dab2* genes interact in the XX but not XY genetic background. The phenomenon may be interesting, but the lack of understanding for genetic interaction in the whole organism prevents us from postulating a credible molecular explanation. It is tempting, however, to speculate that the sex-specific *p53/dab2* interaction may underlie the involvement of *dab2* inactivation in neoplasms specific to females, such as ovarian, breast, and uterine tumors.

It is somewhat unexpected that *dab2*^{+/-} mice developed endometrial lesions since Dab2 loss has not been investigated or reported in uterine tumors previously. We now provide data indicating Dab2 expression is commonly lost in human endometrioid carcinomas and cancer cell lines, suggesting that Dab2 loss may also be involved in the development of human endometrial cancer. Why a genetic inactivation of a specific gene predisposes humans or rodents to certain types of neoplasm is a challenging question in tumor biology. Uterine neoplasm in mice appears to be the primary target of mutations that may cause epithelial neoplasm (instead of lymphomas or sarcomas). *Pten*^{+/-} mice also frequently develop uterine lesions.^{39,40} Possibly, endometrial epithelia exhibit active tissue remodeling when mice reach reproductive stages, and the vigorous tissue remodeling may promote the further selection of predisposed cells to develop neoplastic lesions.

Our observation of a close correlation between Dab2 loss and ovarian surface epithelial morphological transformation is consistent with the endoderm disorganization of the early embryonic phenotype of *dab2* knockout mouse embryos.²³ A mechanistic explanation for the epithelial disorganization caused by Dab2 deficiency has not been established. In mammalian cells, the cellular function of Dab2 is well understood: Dab2 is an endocytic adaptor protein linking specific endocytic cargos to the myosin motor.¹² The N-terminal PID/PTB (phosphotyrosine-interacting domain/phosphotyrosine binding) domain does not bind a phosphotyrosine residue but binds to sequences present in many cell surface glycoproteins containing an NPXY motif.^{16,17} Lipoprotein receptor family member proteins (LRPs)^{11,15,16} associate with Dab2 in cells, and megalin, also known as LRP-2, appears to bind Dab2 with the highest affinity. The Dab2 protein also contains multiple motifs and domains for binding to clathrin and α -adaptin and was shown to associate with clathrin-coated endocytic vesicles *in vivo*.^{11,12} Additionally, the C-terminal portion of the proline-rich domain of Dab2 was found to bind *in vitro* to the globular or cargo-binding domain of myosin VI and associate *in vivo* with myosin VI.^{13,14} Thus, Dab2, as an endocytic adaptor, couples

selective endocytic cargos to motors (such as myosin VI) attached to microfilaments in unidirectional trafficking.¹² Dab2-mediated directional trafficking of specific clathrin-coated cargos is required for the restricted distribution of glycoproteins to the apical surface,²³ which is by definition apical-basal polarity, in mammalian epithelial cells. Consequently, we propose that Dab2-mediated directional transport of cellular membrane proteins to the apical cell surface domains generates apical and basal polarity. We also reason that the establishment and maintenance of polarity are critical for surface positioning and organization of epithelial cells, and epithelial disorganization, the characteristic phenotype of Dab2 deficiency, is the consequence of the loss of polarity. Once the ability to generate polarity is lost (such as by the loss of Dab2) in an epithelial cell located in a monolayer epithelium, neighboring cells may invade and occupy the apical surface of the cell. Ultimately, cell organization is compromised, and the epithelium is disrupted.

Epithelial cell disorganization is a hallmark of malignancy.⁴¹ The association of carcinomas, malignancies of epithelia, with the loss of epithelial polarity and organization is well known, although the underlying mechanism is not clear. Recently, gene knockout studies have suggested that loss of polarity may be causative for neoplastic transformation. Lethal giant larvae (Lgl) has a role in maintenance of cell polarity, and Lgl1 knockout mice develop severe brain dysplasia.⁴² Also, the Peutz-Jeghers cancer syndrome gene LKB1 was shown to regulate epithelial polarity, although the mechanism is not yet clear.^{43,44} LKB1 knockout mice die at midgestation, and LKB1^{+/-} mice develop gastrointestinal polyps,⁴⁵⁻⁴⁷ confirming the tumor suppressor function of LKB1 and suggesting the causative effect of epithelial polarity loss in cancer. Our finding of the uterine tumor-prone phenotype of *dab2*^{+/-} mice adds to the accumulating evidence between the link of epithelial polarity and cancer. The causative effect of loss of polarity in knockout mice may be explained as the morphological transformation accelerates the selection of compromised cells with genetic and epigenetic changes favoring the formation of neoplastic lesions.

In summary, the current characterization of the tumor-prone phenotype of *dab2*^{+/-} mice provides support for the tumor suppressor activity of Dab2 and indicates that loss of Dab2 may be a causative factor in the promotion of epithelial disorganization and a contributing factor promoting the development of cancer precursor lesions and neoplastic transformation.

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