Mps one binder 1a (MOB1A) and MOB1B are key components of the Hippo signaling pathway and are mutated or inactivated in many human cancers. Here we show that intact Mob1a or Mob1b is essential for murine embryogenesis and that loss of the remaining WT Mob1 allele in Mob1aΔ/Δ 1btr/+ or Mob1aΔ/+ 1btr/tr mice results in tumor development. Because most of these cancers resembled trichilemmal carcinomas, we generated double-mutant mice bearing tamoxifen-inducible, keratinocyte-specific homozygous-null mutations of Mob1a and Mob1b (kDKO mice). kDKO mice showed hyperplastic keratinocyte progenitors and defective keratinocyte terminal differentiation and soon died of malnutrition. kDKO keratinocytes exhibited hyperproliferation, apoptotic resistance, impaired contact inhibition, enhanced progenitor self renewal, and increased centrosomes. Examination of Hippo pathway signaling in kDKO keratinocytes revealed that loss of Mob1a/b altered the activities of the downstream Hippo mediators LATS and YAP1. Similarly, YAP1 was activated in some human trichilemmal carcinomas, and some of these also exhibited MOB1A/1B inactivation. Our results clearly demonstrate that MOB1A and MOB1B have overlapping functions in skin homeostasis, and exert their roles as tumor suppressors by regulating downstream elements of the Hippo pathway.
Cancer susceptibility and embryonic lethality in Mob1a/1b double-mutant mice

Miki Nishio,1 Koichi Hamada,1 Kohichi Kawahara,1 Masato Sasaki,2,3 Fumihito Noguchi,4 Shuhei Chiba,5 Kensaku Mizuno,6 Satoshi O. Suzuki,6 Youi Dong,7 Masaaki Tokuda,7 Takumi Morikawa,1 Hiroki Hikasa,1 Jonathan Eggenschwiler,8 Norikazu Yabuta,9 Hiroshi Nojima,9 Kentaro Nakagawa,10 Yutaka Hata,10 Hiroshi Nishina,11 Koshi Mimori,12 Masaki Morii,12,13 Takehiko Sasaki,2,14 Tak W. Mak,3 Toru Nakano,15 Satoshi Itami,4 and Akira Suzuki1,2

1Division of Cancer Genetics, Medical Institute of Bioregulation, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan. 2Global Centers of Excellence Program, Akita University Graduate School of Medicine, Akita, Japan. 3The Campbell Family Institute for Cancer Research, University Health Network, Toronto, Ontario, Canada. 4Department of Regenerative Dermatology, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan. 5Department of Biomedical Sciences, Graduate School of Life Science, Tohoku University, Sendai, Miyagi, Japan. 6Department of Neuropathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan. 7Department of Cell Physiology, Faculty of Medicine, Kagawa University, Kagawa, Japan. 8Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA. 9Department of Molecular Genetics, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan. 10Department of Medical Biochemistry and 11Department of Developmental and Regenerative Biology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan. 12Department of Surgery, Kyushu University, Beppu Hospital, Beppu, Oita, Japan. 13Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan. 14Department of Medical Biology, Akita University Graduate School of Medicine, Akita, Japan. 15Department of Pathology, Medical School and Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka, Japan.

Mps one binder 1a (MOB1A) and MOB1B are key components of the Hippo signaling pathway and are mutated or inactivated in many human cancers. Here we show that intact Mob1a or Mob1b is essential for murine embryogenesis and that loss of the remaining WT Mob1 allele in Mob1a+/1b+/ or Mob1a+/1b+/ mice results in tumor development. Because most of these cancers resembled trichilemmal carcinomas, we generated double-mutant mice bearing tamoxifen-inducible, keratinocyte-specific homozygous-null mutations of Mob1a and Mob1b (kDKO mice). kDKO mice showed hyperplastic keratinocyte progenitors and defective keratinocyte terminal differentiation and soon died of malnutrition. kDKO keratinocytes exhibited hyperproliferation, apoptotic resistance, impaired contact inhibition, enhanced progenitor self renewal, and increased centrosomes. Examination of Hippo pathway signaling in kDKO keratinocytes revealed that loss of Mob1a/b altered the activities of the downstream Hippo mediators LATS and YAP1. Similarly, YAP1 was activated in some human trichilemmal carcinomas, and some of these also exhibited MOB1A/1B inactivation. Our results clearly demonstrate that MOB1A and MOB1B have overlapping functions in skin homeostasis, and exert their roles as tumor suppressors by regulating downstream elements of the Hippo pathway.

Introduction

Tissue homeostasis requires a balance of cell proliferation, apoptosis, and differentiation. During tissue development, progenitor cells divide for a limited number of times before exiting the cell cycle and undergoing terminal differentiation (1). When progenitor cells proliferate inappropriately and/or undergo abnormal differentiation, cancer can result (2).

Skin is a tissue that begins as a single-layered epithelium made up of keratinocytes that later stratify and differentiate into hair follicles (HFs) or interfollicular epidermis (IFE). Disruption of signals balancing growth and differentiation in keratinocytes can initiate skin cancers. The most common skin cancer is basal cell carcinoma (BCC), the nodular subtype of which originates from HFs. Trichilemmal carcinomas are also HF-derived skin cancers, but are much more rare and show characteristic abnormalities of outer hair root sheath differentiation (3). Although impairment of the Sonic Hedgehog (SHH) intracellular signaling pathway is now well established as the cause of BCC (4, 5), the molecular mechanism underlying the development of trichilemmal carcinomas is unknown.

The evolutionarily conserved Hippo signaling pathway was first identified as regulating the cell proliferation and apoptosis-controlling organ size in Drosophila (6, 7). In mammals, the canonical Hippo pathway includes the following: Neurofibromin 2 (NF2) (8), the Mammalian STE20-like protein (MST) kinases (9), large tumor suppressor homolog (LATS) and Nuclear Dbf2-related (NDR) kinases (10), the adaptor proteins SAV1 (WW45) (11) and MOB1 (12), and their downstream transcriptional coactivators YAP1 and its paralog transcriptional coactivator with PDZ-binding motif (TAZ, WWTR1) (13). All of these molecules (except YAP1 and TAZ) have been implicated as tumor suppressors. For example, mice deficient in Lats1 or Mst1/2 develop various cancers (14, 15), SAV1 and MOB are mutated in human cancer cell lines (16, 17), and Hippo components are reduced in human cancer samples (18). Homozygous-null mutant mice lacking Nf2, Mst1/2, SAV1, Lats2, or Yap1 are all embryonic lethal (15, 19–22), precluding study of these mediators in tumorigenesis. To complicate matters, multiple homologs of each mammalian Hippo element exist, and a noncanonical Hippo pathway influences the localization and activity of canonical Hippo components (15, 23). Thus, the precise physiological functions of Hippo elements in mammals remain obscure.

Authorship note: Koichi Hamada and Kohichi Kawahara contributed equally to this work. Satoshi Itami and Akira Suzuki contributed equally as co-senior authors.

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MOB1 was originally shown in yeast to regulate mitotic exit and modulate cytokinesis (24–29). In Drosophila, dMOB1 (MATS) overexpression is tumor suppressive, while loss of dMOB1 triggers tumor development (17). In humans, 7 MOB homologs (hMOB1A-B, 2A-C, 3, 4, where MOB indicates Mps one binder) have been identified (30), and hMOB1A (hMATS1, MOBKL1B) and hMOB1B (hMATS2, MOBKL1A) share 95% amino acid identity. Although no apparent functional domain has been found in hMOB1A/1B, only these 2 MOB proteins can bind to and activate LAT51/2 (31). In vitro, hMOB1A overexpression inhibits cell proliferation, while suppression of hMOB1B or hMOB1A mediated by shRNA or siRNA increases proliferation (30) or impairs mitotic exit (32), respectively. In vivo, the hMOB1A gene is expressed in melanoma and breast cancer cell lines and its expression is downregulated in human colorectal and non–small-cell lung cancers (17, 33, 34). However, the normal function of hMOB1 proteins in vivo is unknown. To assess whether MOB1 is an important functional component of mammalian Hippo signaling and to determine whether MOB1 is truly a tumor suppressor in vivo, we generated and characterized double-mutant mice lacking Mob1a and Mob1b.

**Results**

Embryonic lethality of Mob1a/1b-null mutant mice. We first generated single-mutant mice bearing a null mutation of Mob1a (Mob1aΔ/Δ) or a trapping mutation of Mob1b (Mob1bΔ/Δ) (Supplemental Figure 1, A–D; supplemental material available online with this article; doi:10.1172/JCI63735DS1) but no abnormalities were observed in morphology, body weight, histology, or life span (Supplemental Figure 1E and data not shown). We then attempted to generate double-homozygous–null mutant (Mob1aΔ/ΔMob1bΔ/Δ) mice, but no viable pups were obtained (Supplemental Table 1), indicating that complete loss of Mob1 is embryonic lethal. We next analyzed embryos from Mob1aΔ/ΔMob1bΔ/Δ intercrosses at various time points during gestation. By E6.5, although decidua were formed, 28% of embryos were absorbed too severely to genotype (Figure 1A and Table 1). Thus, MOB1A and MOB1B have overlapping functions, and MOB1 is essential for postimplantation embryogenesis.

To pinpoint the embryonic defect, we obtained individual blastocysts (E3.5) from Mob1aΔ/ΔMob1bΔ/Δ intercrosses and cultured them for 8 days. Mob1aΔ/ΔMob1bΔ/Δ blastocysts appeared normal at E3.5, but showed growth failure of the inner cell mass (ICM) by day 8 (Figure 1B). In contrast, the trophectoderm (TE) of Mob1aΔ/ΔMob1bΔ/Δ blastocysts developed normally.

To create conditional tamoxifen-inducible Mob1 mutants, we generated ERCreMob1aΔ/ΔMob1bΔ/Δ ES cells and established embryoid bodies (EBs) using the hanging drop method. Control and mutant EBs were cultured for 2 days with (or without) tamoxifen to delete Mob1 and then grown without leukemia inhibitory factor (LIF) for 3–4 days. Quantitative RT-PCR examination showed that Gata4 and Pdgfra mRNAs (essential for primitive endoderm formation) were markedly suppressed in Mob1aΔ/ΔMob1bΔ/Δ EBs, whereas Cdx2 and Eomesodermin mRNAs (essential for ICM) were only slightly inhibited (Figure 1C). Levels of Oct3/4 (Pou5f1) and Sox2 mRNAs (essential for ICM), as well as Nanog, Fgf5, Pax6, and Sox2 mRNAs (essential for primitive ectoderm), were normal in the double-mutant EBs. Immunostaining of double-mutant blastocysts with antibodies recognizing Cdx2 or Troma-1 (TE markers), Nanog or Sox2 (primitive ectoderm markers), or Oct3/4 confirmed that the levels of these proteins were also normal in the absence of Mob1. However, levels of GATA4 and PDGFRA proteins (primitive endoderm markers) were greatly reduced in the mutants (Figure 1D). Moreover, YAP1, which is usually expressed only in the nuclei of TE cells, was abnormally activated and expressed in the nuclei of ICM cells (Figure 1D). Thus, Mob1aΔ/ΔMob1bΔ/Δ embryos have a defect in primitive endoderm formation.

**Table 1**

Viability of embryos of the indicated Mob1a genotypes from Mob1aΔ/ΔMob1bΔ/Δ intercrosses at the indicated stages of embryogenesis.

<table>
<thead>
<tr>
<th>Genotype of Mob1a</th>
<th>Total</th>
<th>Viable</th>
<th>+/+</th>
<th>Δ/+</th>
<th>ΔΔ</th>
<th>Absorbed</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E16.5</td>
<td>108</td>
<td>39</td>
<td>69</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E14.5</td>
<td>15</td>
<td>4</td>
<td>11</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E10.5</td>
<td>19</td>
<td>8</td>
<td>9</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E9.5</td>
<td>27</td>
<td>11</td>
<td>13</td>
<td>0</td>
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<td></td>
</tr>
<tr>
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<td>3</td>
<td>12</td>
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<tr>
<td>E6.5</td>
<td>47</td>
<td>11</td>
<td>33</td>
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<td></td>
</tr>
<tr>
<td>E3.5</td>
<td>33</td>
<td>9</td>
<td>24</td>
<td>0</td>
<td></td>
<td></td>
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</tbody>
</table>

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Figure 1

Loss of Mob1a/1b impairs mouse embryo survival. (A) Morphologies of representative E6.5 embryos of the indicated genotypes from Mob1aΔ/ΔMob1bΔ/Δ intercrosses. Mob1aΔ/ΔMob1bΔ/Δ embryos were absorbed after implantation. (B) Left: photographs of E3.5 blastocysts of the indicated genotypes on day 0 and after culture for 8 days. The ICM is surrounded by TE giant cells. Right: mean ICM ratio (ICM area/TE area) ± SEM plotted for blastocysts of the indicated genotypes (n = 8/group); *P < 0.01. (C) Quantitative PCR determination of mRNAs for the indicated genes in EBs generated from ERCreMob1aΔ/ΔMob1bΔ/Δ ES cells. ES cells were cultured with/witout tamoxifen for 2 days, and control and mutant EBs were generated using the hanging drop method after culture for 3 days (Cdx2, Eomesoderm, Oct3/4, Sox2, Pdgfra, Gata4). mRNA levels in the mutant are expressed as the percentage increase over control values; *P < 0.05. (D) Immunostaining to detect the indicated proteins in blastocysts of the indicated genotypes. Nuclei were visualized with DAPI. Results show are representative of at least 3 independent trials and at least 3 mice/group. Data are presented as the mean ± SEM, and P values were determined using the 2-tailed Student’s t test.
Figure 2
Loss of Mob1a/1b promotes tumorigenesis. (A and B) Tumor types arising in Mob1aΔΔ1btr/ or Mob1aΔ+/1btr/tr mice. (A) Macroscopic or microscopic photographs of the indicated gross or H&E-stained tumors in mutant mice. Yellow arrows, tumor masses. Scale bars: 100 μm. (B) Kaplan-Meier analysis of tumor onset and survival for the Mob1aΔΔ1btr/ (n = 31), Mob1aΔ+/1btr/tr (n = 48), and control Mob1aΔ+/1btr/+ (n = 22) mice in A. (C) Southern blots of tumor DNA showing loss of the WT Mob1 allele. Top (hybridized to probe A of Supplemental Figure 1A): lanes 1–3, control thymic DNA from Mob1aΔΔ, Mob1aΔ+/, and Mob1aΔ+/ mice, respectively; lanes 4–5, osteosarcomas; 6–7, fibrosarcomas; 8–9, skin cancers; 10–11, breast cancers; 12–13, salivary gland cancers of Mob1aΔ+/1btr/tr mice. Bottom (probe C): 1–3, control tail DNA of Mob1bΔΔ, Mob1bΔ+/, and Mob1bΔ+/ mice, respectively; 4–5, osteosarcomas; 6–7, fibromyosarcomas; 8–9, skin cancers; 10–11, liver cancers of Mob1aΔ+/1btr/tr mice. Results shown are representative of at least 3 independent trials and at least 3 mice/group. Data are presented as the mean ± SEM, and P values were determined using the 2-tailed Student’s t test.
tosis in calvaria occurred in 92% of animals with just 1 WT Mob1 allele as well as extraskeletal osteosarcomas (24%), subcutaneous fibrosarcomas (or myofibrosarcomas) (22%), breast cancers (16%), lung cancers (5%), and salivary gland cancers (5%). Liver cancers were found only in Mob1aΔ/Δ Mob1b+/+ mice (50%). Interestingly, none of our Mob1 deficient mice developed colon tumors or melanomas, unlike Mob1a deficient humans (17, 33). Nevertheless, Mob1 is a powerful tumor suppressor in multiple tissues in vivo.

Keratinocyte-specific Mob1a/1b-deficient mice show gross skin abnormalities and die during lactation. Because the dominant tumor in Mob1aΔ/Δ Mob1b+/+ and Mob1aΔ/Δ Mob1bΔ/Δ mice arose in the skin, we generated tamoxifen-inducible, keratinocyte-specific Mob1a/1b double-homozygous mutant mice (Krt14CreERMob1aΔ/Δ Mob1bΔ/Δ). The Keratin-14 (Krt14) promoter directs gene expression in the basal layer of epidermal and follicular keratinocytes, including stem cells in the HF bulge. Thus, tamoxifen-induced, Krt14-controlled Cre-mediated deletion of a floxed gene disrupts expression of that gene throughout the IFE and HFFs (35). When we administered tamoxifen to Krt14CreERMob1aΔ/Δ Mob1bΔ/Δ mice at P1, we created double-homozygous conditional mutant mice (Kdko(P1)) that initially appeared healthy. Single-mutant mice (Krt14CreERMob1aΔ/Δ Mob1bΔ/Δ) without tamoxifen or Krt14CreERMob1aΔ/Δ Mob1bΔ/Δ mice with tamoxifen were indistinguishable from the WT in gross appearance, histology, and survival (data not shown), allowing us to choose Krt14CreERMob1aΔ/Δ Mob1bΔ/Δ mice without tamoxifen as controls. Southern blotting confirmed that tamoxifen disrupted the Mob1aΔ/Δ allele in almost all keratinoxytes of Kdko(P1) mice, leaving the Mob1bΔ allele (Supplemental Figure 3A). In addition, immunostaining using an Ab recognizing both MOB1A and MOB1B (Supplemental Figure 3B) confirmed that the MOB1A and MOB1B proteins are expressed both in IFE cells (especially in the granular layer) and in HF cells (especially in matrix cells and outer root sheath cells) in normal mice and humans (Supplemental Figure 3, C and D). In contrast, MOB1A and MOB1B proteins were not detected in the epidermis of Kdko(P1) mice (Supplemental Figure 3C).

By 13 days of age, Kdko(P1) mice were significantly smaller than control littermates and readily identified by their “wrinkled-bear” facial skin (Figure 3A) and ruffled hair (Figure 3A). These mutants’ front paws (Figure 3A), ears, and lips were hyperplastic and enlarged. All Kdko(P1) mice died of malnutrition within 10–30 days of birth (Figure 3), likely due to dysphagia caused by hyperplasia of the oral cavity epithelium.

We next analyzed the architecture of Kdko(P1) skin at various time points after birth. In WT mice, dividing keratinocytes are restricted to the basal epidermal layer. As these cells exit the cell cycle, they differentiate to form the spinous and granular skin layers as well as the dead, enucleated stratum corneum layers. The anagen phase of hair development thickens the skin until day 17, when the first catagen phase is triggered and skin thickness regresses until day 19. Hair remodeling then begins its lifelong cycle of spontaneous regrowth and regression (36). We saw this pattern clearly in our biopsy series from control mice (Supplemental Figure 4). However, the IFE and HFs of Kdko(P1) mice showed hyperplastic multilayered epithelium at day 16 (Figure 3B), and impaired epidermal regression during catagen (Supplemental Figure 4; day 19). In addition, parakeratosis (reduced enucleation) was evident in the stratum corneum layer of Kdko(P1) mice (Figure 3C).

To investigate keratinocyte differentiation, we used immunostaining to detect keratin markers in the fat pad epidermis, which allows easy analysis of epidermal layers. Keratin-15+ (KRT15+) cells, the most primitive in IFE, normally form a single basal layer attached to the IFE basement membrane (BM). This configuration was clearly visible in our controls, whereas the KRT15+ cells of Kdko(P1) mice, although nearly normal in number, were scattered inside the IFE without attachment to the BM (Figure 3D). In control mice, Krt14+ and Krt15+ cells were present in 1 or 2 IFE layers (as expected), but appeared in multiple layers in Kdko(P1) mice (Figure 3D). Moreover, there were increased numbers of irregularly organized KRT10+ cells in the suprabasal layers of Kdko(P1) epidermis (Figure 3D). Filaggrin+ cells, the most highly differentiated keratinocytes, were present in Kdko(P1) epidermis but at a reduced percentage (Figure 3D). Similar IFE alterations were observed in the back skin of Kdko(P1) mice (Figure 3E and Supplemental Figure 5). KRT17+ outer root sheath cells were significantly increased in back skin HFs of Kdko(P1) mice, and Trichohyalin+ inner root sheath cells were elevated slightly (Figure 3E). Thus, the phenotype of Kdko(P1) mice may be largely due to abnormal expansion of immature stem/progenitor skin cells.

To try to overcome the early lethality of Kdko(P1) mice, we waited until Kdko mice reached 28 days of age before treating them with tamoxifen for 7 days. However, like Kdko(P1) mice, all of these Kdko(P28) mice showed keratinocyte hyperplasia (Figure 3F) and died within 15–55 days after treatment (Supplemental Figure 6). Thus, Mob1 deficiency in epidermal cells severely disrupts normal IFE and HF development and homeostasis.

Tumorigenic anomalies in Mob1a/1b double-homozygous mutant keratinocytes. To determine whether loss of MOB1 conferred tumorigenic properties on keratinocytes, we first immunostained Kdko(P1) skin with anti-Ki67 Ab and found increased numbers of Ki67+ proliferating epithelial cells (Figure 4A). In control mice at P13, a few Ki67+ cells were located among basal IFE cells and outer root sheath cells, with more scattered among HF matrix cells. However, the incidence of Ki67+ cells in Kdko(P1) skin at P13 was 1.5 times higher than in controls, and Ki67+ cells were also found in the suprabasal layers normally quiescent in control epidermis. In addition, TUNEL-positive apoptotic cells were reduced in Kdko(P1) epidermis (Figure 4B). Thus, increased proliferation and repressed apoptosis may contribute to Kdko(P1) epidermal hyperplasia. Cell-plating studies revealed that the saturation density of Kdko(P1) keratinocytes was also increased (Figure 4C). Because the cell size (forward scatter) of control and mutant keratinocytes showed no difference by FACS analysis (data not shown), the increased saturation density of the mutant suggests impaired contact inhibition. Histological analysis confirmed that the mutant basal epidermal layer showed significantly increased cell density.

<p>| Table 2 |
| Total tumor numbers (incidence) for the mice in Figure 2A |</p>
<table>
<thead>
<tr>
<th><strong>Mob1aΔ/Δ Mob1bΔ/Δ</strong></th>
<th><strong>Mob1aΔ/Δ Mob1bΔ/Δ</strong></th>
<th><strong>Overall</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin cancer</td>
<td>23 (100%)</td>
<td>14 (100%)</td>
</tr>
<tr>
<td>Exostosis</td>
<td>20 (87%)</td>
<td>14 (100%)</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>7 (30%)</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>6 (26%)</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>0 (0%)</td>
<td>7 (50%)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>5 (22%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>0 (0%)</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>Salivary gland cancer</td>
<td>1 (4%)</td>
<td>1 (7%)</td>
</tr>
</tbody>
</table>
Similar defects were observed in and Mob1b aberrant mitosis, were also increased in clei, which are cytoplasmic chromosomal fragments generated by keratinocytes exhibited multi-polar spindles (Figure 4F). Microtubulin-associated with multipolar spindle formation that impairs chromosome segregation to furrow completion (late anaphase/telophase); and part IV, from furrow completion to cell division (cytokinesis). We used time-lapse video microscopy to measure the duration of each of parts I–IV in control and kDKO(P1) keratinocytes. Part IV was significantly accelerated in MOB1-deficient cells compared with controls, while part I was slightly faster in the mutant (Supplemental Figure 8, A and B). No significant differences were detected for parts II and III. Thus, loss of MOB1 accelerates a cell’s exit from mitosis.

We noted that kDKO(P1) mice had an apparent increase in the number of skin progenitors in the IFE (Figure 3D). We therefore performed RT-PCR to evaluate stem/progenitor cells in the HFs. CD34+ and LGR6+ cells are the most immature HF stem cells located in the bulge and isthmus. We found that CD34 and Lgr6 mRNAs were normal or reduced in kDKO(P1) keratinocytes, but that markers of activated stem/progenitor cells, such as Sox9 and Lgr5, were elevated (Figure 4H). These findings were confirmed by FACS analysis and immunohistochemistry (Figure 4H). However, despite their normal numbers, CD34+ cells in mutant HFs could not localize to the proper bulge area (Supplemental Figure 9). This result mirrors the failure of HF stem cells to localize to the basal cell layer in MOB1-deficient IFE and HFs (Figure 3D). In addition, SOX9+ cells (which are normally located in the bulge at P21) were markedly increased among kDKO(P1) keratinocytes and scattered throughout the HFs (Figure 4H). To determine how MOB1 inactivation affected HF stem cell self-renewal, we quantified the ability of the total kDKO(P1) keratinocyte population to form colonies in culture. A lack of MOB1 induced a 2.1-fold increase in colony-forming efficiency (Figure 4I). When these primary colonies were replated to test their ability to form secondary colonies, a 3.0-fold increase in secondary colony-forming efficiency was observed in the absence of MOB1 (Figure 4I). Thus, MOB1 inactivation enhances the self-renewal of activated stem/progenitor keratinocytes. This property, along with hyperproliferation, apoptotic resistance, impaired contact inhibition, increased centrosome number, and accelerated mitotic exit, may account for the frequent cancers observed in Mob1-deficient mice.

MOB1-mediated regulation of the LATS1/2-YAP1 pathway controls skin homeostasis. To characterize the mechanisms driving the kDKO(P1) phenotype, we investigated the effects of MOB1 loss on signaling mediated by YAP1 and its upstream kinases LATS1/2. In vitro, MOB1 is important for the full activation of LATS kinases (37), and activated LATS1/2 phosphorylate YAP1 and inhibit its nuclear localization (38). Using immunohistochemistry, we found that, in control mice, YAPI was localized in the nuclei of most outer root sheath cells, in some HF matrix cells, and in some IFE basal cells (Figure 5A). However, kDKO(P1) mice exhibited not only greater numbers of nuclear YAP1+ cells in these sites but also some nuclear YAP1+ cells in the upper IFE layer (Figure 5A). Immunoblotting of keratinocytes confirmed that the phosphorylation of YAP1 on Ser127 was reduced, but that total YAPI was increased in the absence of MOB1 (Figure 5B). Immunoblotting of keratinocytes confirmed that the phosphorylation of YAP1 on Ser127 was reduced, but that total YAPI was increased in the absence of MOB1 (Figure 5B), especially in the nucleus (Figure 5C). When we cultured keratinocytes under high cell density conditions, YAP1 was inactivated and localized in the cytoplasm in control cells (Figure 5D). In contrast, in kDKO(P1) keratinocytes, YAP1 remained activated and positioned in the nucleus even at high-cell density (Figure 5D). Thus, MOB1 is required for density-induced subcellular localization of YAP1.

We next investigated LATS1/2 phosphorylation in control and mutant keratinocytes. Immunoblotting demonstrated that, in the absence of any stimulation, levels of phospho-LATS1(T1079) and phospho-LATS2(T1041) were below the assay detection limit even in control keratinocytes (Figure 5E). We then stimulated control and kDKO(P1) keratinocytes with okadaic acid (OA), which activates the MST kinases acting upstream of LATS1/2 (39). While OA-stimulated control keratinocytes showed vigorous phosphorylation of LATS1/2, OA-stimulated kDKO(P1) keratinocytes did not. Strikingly, total LATS1/2 protein levels were also reduced in the mutant. This inhibition of LATS1/2 phosphorylation was not due solely to the reduction in total LATS1/2 proteins because levels of phospho-LATS1/2 were also markedly decreased when total LATS1/2 protein levels in each sample were adjusted to equality prior to electropho-
Figure 4
Tumorigenic anomalies in Mob1a/1b double-homozygous mutant keratinocytes. (A) Anti-Ki67 immunostaining of IFE (left) and HF (middle) of control and kDKO(P1) mice at P13. Scale bars: 50 μm. Quantitation of Ki67+ cells (right); *P < 0.01. (B) Histology (left) and quantitation (right) of TUNEL-stained cells in epidermis from control and kDKO(P1) mice at P16. Scale bar: 20 μm. Right: quantitation (cell number/50 μm BM); n = 5/group; *P < 0.05. (C) Keratinocytes from control and kDKO(P1) mice at P4 were cultured for the indicated number of days, and total cell numbers were counted. kDKO(P1) keratinocytes achieved higher saturation plating density; *P < 0.01. (D) Left: H&E-stained epidermal basal layer of control and kDKO(P1) mice at P19. Scale bar: 20 μm. Right: quantitation (cell number/50 μm BM); n = 5/group; *P < 0.05. (E-G) Immunostaining to detect γ-Tubulin (green) and α-Tubulin (red) in control and kDKO(P1) keratinocytes. DAPI, nuclei. Mutant keratinocytes showed excess centrosomes (E), multi-polar spindles (F), and micronuclei (G). Scale bars: 20 μm; *P < 0.05. (H) Identification of keratinocyte stem cells in HFs of control and kDKO(P1) mice at P19 (n = 4/group) using quantitative RT-PCR (left), *P < 0.01; flow cytometry (middle) to detect CD34; immunostaining (right) to detect SOX9. Scale bar: 100 μm. (I) freshly isolated control and kDKO(P1) keratinocytes were plated to generate primary colonies (left) and secondary colonies (right). Giemsa staining (top) and colony counts (bottom) were performed on day 14 after plating; n = 4/group; *P < 0.02. Results shown are representative of at least 3 independent trials and at least 3 mice/group. Data are presented as the mean ± SEM, and P values were determined using the 2-tailed Student’s t test.

Discussion
We have demonstrated that mice completely deficient for Mob1 have the most severe phenotype among strains lacking a Hippo signaling component. Mob1a/1b double-homozygous–null mutant mice die at gastrulation, much earlier than mice lacking Mst1/2 (15), Lats2 (21), Lats1 (14), Sar1 (20), or Nf2 (19). Our data further indicate that Mob1 is essential for embryogenesis and that functions of Mob1A and Mob1B overlap. In addition, the tumor spectrum observed in heterozygous Mob1-deficient mice is the broadest among mutants lacking Hippo components. These findings suggest both that Mob1 is the key molecule in the Hippo signaling pathway and that Mob1 may have molecular target(s) other than the Hippo pathway. This notion is consistent with the reduced expression or mutation of Mob1 frequently observed in a variety of human cancers (17, 33, 34), and with Mob1’s reported binding to a range of molecules, including TSSC1, NUP98, HDAC3, and DIPA (CCDD85B) (41, 42). Our results also imply that Mob1A may be more important than Mob1B, at least for embryogenesis and liver homeostasis, because Mob1b heterozygotes lacking Mob1A show partial embryonic lethality (Supplemental Table 1) and develop liver cancers (Table 2), whereas Mob1a heterozygotes lacking Mob1b are all viable and free of liver tumors.

The lethality of Mob1-deficient mice may stem from their failure to form primitive endoderm. The endodermal markers Pdx4 and Gata4 were markedly reduced in Mob1-deficient cells, whereas the primitive ectoderm markers Nanog, Sox2, Fgf5, and Pax6 were normal. Our mutant showed abnormal YAP1 activation, and activated YAP1 normally activates the transcription factor TEAD2. TEAD2 regulates primitive endoderm-specific genes such that sustained inhibition of TEAD2 enhances primitive endoderm-specific gene expression (43). YAP1 and TEAD5 reportedly increase the expression of pluripotency genes such as Oct3/4 and Sox2 (43, 44), as well as the trophoblast gene Cdx2 (45, 46). However, levels of Oct3/4, Sox2, and CDX2 proteins were normal in our Mob1-deficient embryos. Thus, although we found YAP1 to be activated in the...
MOB1-mediated regulation of the LATS1/2-YAP1 pathway controls skin homeostasis. (A) Immunostaining of keratinocytes from control and kDKO(P1) mice at P19 to detect YAP1 in the IFE (top) and HF (bottom). Scale bar: 50 μm. (B) Immunoblot of total extracts of control and kDKO(P1) keratinocytes to detect the indicated proteins. α-Tubulin, loading control. (C) Immunoblot of cytoplasmic and nuclear fractions of control and kDKO(P1) keratinocytes to detect the indicated proteins. α-Tubulin and Lamin, cytoplasmic and nuclear loading controls, respectively. (D) Immunostaining to detect YAP1 in keratinocytes from control and kDKO(P1) mice plated at low or high cell density. YAP1 is localized in the nucleus in mutant keratinocytes even at high cell density. (E) Immunoblot to detect the indicated proteins in total extracts of control (Cont) and kDKO(P1) keratinocytes that were left untreated (OA−) or treated with OA (OA+). Left: unadjusted lysates. Right: levels of LATS1 and LATS2 proteins in each sample were adjusted to equality before electrophoresis. Results shown are representative of at least 3 independent trials and at least 3 mice/group.

Figure 5
MOB1-mediated regulation of the LATS1/2-YAP1 pathway controls skin homeostasis. (A) Immunostaining of keratinocytes from control and kDKO(P1) mice at P19 to detect YAP1 in the IFE (top) and HF (bottom). Scale bar: 50 μm. (B) Immunoblot of total extracts of control and kDKO(P1) keratinocytes to detect the indicated proteins. α-Tubulin, loading control. (C) Immunoblot of cytoplasmic and nuclear fractions of control and kDKO(P1) keratinocytes to detect the indicated proteins. α-Tubulin and Lamin, cytoplasmic and nuclear loading controls, respectively. (D) Immunostaining to detect YAP1 in keratinocytes from control and kDKO(P1) mice plated at low or high cell density. YAP1 is localized in the nucleus in mutant keratinocytes even at high cell density. (E) Immunoblot to detect the indicated proteins in total extracts of control (Cont) and kDKO(P1) keratinocytes that were left untreated (OA−) or treated with OA (OA+). Left: unadjusted lysates. Right: levels of LATS1 and LATS2 proteins in each sample were adjusted to equality before electrophoresis. Results shown are representative of at least 3 independent trials and at least 3 mice/group.

In our Mob1aΔ/+/Mob1bΔ/Δ mice, the most frequent tumors were malignant outer root sheath tumors resembling trichilemmal carcinomas (Figure 6A). Histologically, these malignancies were not BCCs because they lacked the cellular palisading typical of BCCs (4). Moreover, cultured Mob1a/1b double-mutant keratinocytes did not show the SHH pathway activation important for BCC onset (Figure 6C). Benign trichilemmomas are frequently observed in Cowden disease patients with hereditary PTEN mutations (47), but these tumors seldom become malignant. The trichilemmal growths in our Mob1-deficient mutants were clearly cancerous but showed no activation of the PTEN effector AKT (Figure 6C). Importantly, like our mutant mouse tissues, our human trichilemmal carcinoma samples exhibited frequent MOB1A/1B inactivation and YAP1 activation (Figure 6G and Table 3). These findings suggest that impaired Hippo signaling may drive trichilemmal carcinoma onset in humans.

Mob1-deficient keratinocytes exhibit enhanced proliferation, apoptotic resistance, impaired contact inhibition, increased centrosomes, accelerated mitotic exit, and enhanced progenitor self-renewal. In addition, polarity must be defective without MOB1 because (a) KRT15+ cells were scattered inside the IFE and not localized to the basal layer; (b) CD34+ bulge stem cells were not localized in the bulge; and (c) hair bundles in the organ of Corti were disorganized (Supplemental Figure 2B). To date, 2 transgenic mouse strains overexpressing Yap1 in the skin have been described (23, 48). Like our Mob1 mutants, Yap1 transgenic mice show hyperplastic IFE. However, these latter animals also have a severe defect in HF
**Figure 6**
Characterization of skin cancers of Mob1-deficient mice and human trichilemmal carcinomas. (A) H&E-stained sections of representative tumors from Mob1a<sup>Δ<sub>Δ</sub>1b<sup>tr/+</sup></sup> or Mob1a<sup>Δ<sub>Δ</sub>1b<sup>tr/tr</sup></sup> mice showing: (top left) characteristic trichilemmal keratinization (yellow arrows; also in right); (bottom left) atypical and highly mitotic cells; and (right) continuity with the epidermis. Scale bars: 50 μm (left); 500 μm (right). (B) Immunostaining of tumors from Mob1a<sup>Δ<sub>Δ</sub>1b<sup>tr/</sup></sup> or Mob1a<sup>Δ<sub>Δ</sub>1b<sup>tr/tr</sup></sup> mice to detect the indicated skin markers. White arrows, cancerous lesions; yellow arrow, nontumorous HF. Scale bar: 200 μm. (C) Immunoblot of total extracts of control and kDKO(P1) keratinocytes to detect ERK and AKT activation as well as the HF morphogenesis proteins GLI2 (FL; full length), LEF1 and HES1. β-Actin, loading control. (D) Immunostaining to detect GLI2 in HF s from control or kDKO(P1) mice. Scale bar: 25 μm. (E) Immunoblot to detect GLI2 in total extracts of control keratinocytes transfected with scramble siRNA (Control siSc) or Gli2 siRNA (Control siGli2), as well as in samples of 4 trichilemmal carcinomas (Tumor 1, 2, 3, 4) from kDKO(P1) mice. (F) H&E staining of a human trichilemmal carcinoma viewed at low (scale bar: 200 μm) or high magnification (scale bar: 50 μm). (G) Immunostaining to detect YAP1 and MOB1A/1B in human trichilemmal carcinomas and nearby nontumorous tissues. Top: Both nontumorous and trichilemmal carcinoma (T) tissues can be seen in low-magnification images. Bottom: High-magnification images of human trichilemmal carcinomas. Scale bars: 100 μm. Results shown are representative of at least 3 independent trials and at least 3 mice/group.
formation. This difference may account for the development of IFE-derived squamous cell carcinomas in Yap1 transgenic mice, but HF-derived trichilemmal carcinomas in Mob1-deficient mutants. At the biochemical level, Mob1-deficient keratinocytes exhibited reductions in not only phospho-LATS1/2, but also total LATS1/2 proteins. Lats1/2 mRNAs were comparable in control and mutant keratinocytes (data not shown), indicating that this surprising decrease in LATS1/2 proteins occurs posttranscriptionally. Like Mob1, Sav1 is a scaffolding protein in the Hippo pathway, but unlike Mob1 deficiency, Sav1 deficiency decreases phospho-MST, but does not affect total MST or LATS1/2 proteins (20). Another interesting biochemical observation was the activation of ERK in our Mob1-deficient keratinocytes: no connection between MOB1 and ERK has been reported to date. With respect to Hippo signaling, ERK can be activated by Yap1 (49) or suppressed by NF2 (50) or MST2 (51). Since NF2 and phospho-MST1/2 were not decreased in our Mob1-deficient keratinocytes (data not shown), we speculate that the increased Yap1 in these cells may have triggered their abnormal ERK activation.

Because all of our Mob1a/1b-deficient mice spontaneously developed tumors (and especially trichilemmal carcinomas), we believe that the loss of MOB1A/1B helps to both initiate and promote carcinoma onset. With respect to tumor initiation, the observed increase in the number of centrosomes and/or micronuclei in our mutant cells may have introduced detrimental alterations into their DNA. With respect to cancer promotion, the enhanced proliferative, apoptotic resistance, impaired contact inhibition, and increased progenitor self-renewal associated with loss of MOB1A/1B may support the growth and progression of cells that have undergone tumor initiation events.

The very rare occurrence of trichilemmal carcinomas in humans has slowed the identification of genes involved in their development. Expression levels of Mob1A, LATS1/2, and Sav1 are more frequently reduced in the advanced stages of colon (33), breast (52), and renal cancers (53) than in the early stages of these malignancies. In lung cancers, the reverse is true, since MOB1A levels are frequently lower in the early pT1 stage of non-small-cell lung cancer as compared with later stages (34). These observations suggest that the loss of Hippo signaling molecules can be an important driver of cancer progression in humans. Further study of alterations to gene or protein expression or functions of Hippo signaling components in a broad range of human malignancies may increase our understanding of their involvement in tumorigenesis.

In conclusion, our results demonstrate that (a) MOB1 is a broadly-acting tumor suppressor in mice and (b) Hippo signaling drives trichilemmal carcinoma onset in the skin. Therapeutic strategies to control Hippo signaling or MOB1 expression might therefore benefit many cancer patients, particularly those with HF-derived cancers.

### Table 3

<table>
<thead>
<tr>
<th>Trichilemmal carcinoma</th>
<th>Increased Yap1</th>
<th>Nuclear accumulation of Yap1</th>
<th>Decreased MOB1A/1B</th>
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<td></td>
<td>11/14 (79%)</td>
<td>10/14 (71%)</td>
<td>5/10 (50%)</td>
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### Generation of Mob1a<sup>ΔloxP</sup> and Mob1b<sup>ΔloxP</sup> mice. A conditional targeting vector based on the Cre-loxP system was constructed to delete a genomic fragment containing exon 2 of the murine Mob1a gene (Supplemental Figure 1). This deletion generates a frame shift and early stop codon in exon 3. We introduced 1 loxP site into Mob1a intron 1 and 2 loxP sites into intron 2 to flank Mob1a exon 2. The PGK-Hyg resistance cassette was inserted in antisense orientation between the 2 loxP sites in intron 2. The resulting targeting vector was electroporated into E14K ES cells, and homologous recombinants were confirmed by Southern blotting using 5′ flanking probe A and a Hyg-specific probe. Correctly targeted Mob1a mutant clones were transiently transfected with pMCI-Cre (54) to delete the loxP-flanked Hyg gene. Progeny clones sensitive to hygromycin were subjected to Southern blotting to identify those retaining exon 2 flanked by 2 loxP sites (the Mob1a<sup>ΔloxP</sup> allele) and those lacking exon 2 ( Mob1a<sup>ΔloxP</sup> allele; equivalent to a knockout mutation). For Mob1b-deficient mice, an ES cell line bearing a gene trap mutation of intron 2 of the Mob1b gene was obtained from the Sanger Institute (Hinxton, United Kingdom). The single integration of the trapped site was confirmed by PCR and genomic Southern blotting using 5′ flanking probe C and a neospecific probe. Mob1a<sup>ΔloxP</sup>, Mob1a<sup>ΔloxP</sup> and Mob1b<sup>ΔloxP</sup> mice were generated from ES cell clones using standard procedures and backcrossed to C57BL/6 mice 3 times before intercrossing to generate Mob1a<sup>ΔloxP</sup><sup>/ΔloxP</sup>, Mob1a<sup>ΔloxP</sup> and Mob1b<sup>ΔloxP</sup> progeny. Primers used for genotyping are listed in Supplemental Table 2.

**In vitro culture of preimplantation embryos.** E3.5 embryos derived from the intercrossing of Mob1a<sup>−/−</sup> males and females were analyzed as described (55). Briefly, blastocysts were individually cultured in 24-well plates in ES cell medium without LIF and photographed every 24 hours. On day 8, the morphology of each embryo was recorded and its genotype determined by PCR.

**Embryo immunostaining.** Immunofluorescent staining of embryos was performed as described (45). Briefly, embryos (E3.5) were cultured overnight prior to fixation in 4% paraformaldehyde. Fixed tissues were permeabilized with 0.2% Triton X-100, blocked with 2% goat serum, and incubated overnight at 4°C with Abs recognizing CDX2 (Biocare Medical), Oct3/4 (gift of H. Niwa, Riken), PDGFRα (eBiosciences), GATA4/6 (Santa Cruz Biotechnology Inc.), NANOG (ReproCELL), Troma-1 (DSHB), SOX2 (Abcam), or YAP1 (Sigma-Aldrich). Secondary Abs were Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 568 goat anti-mouse (Molecular Probes). Nuclei were visualized using DAPI (Dinjado). Stained embryos were examined by confocal microscopy.

**Generation and differentiation of Mob1a/1b double-mutant ES cells.** ES cells established from embryos derived from Mob1a<sup>ΔloxP</sup><sup>/ΔloxP</sup> mice were transfected with linearized CMV-Mer-Cre-Mer plasmid DNA (56) plus pApuR2 (57). To generate ERCreMob1a<sup>ΔloxP</sup><sup>/ΔloxP</sup> ES clones, EES cells were selected for culture for 12 days in complete ES cell medium containing 1 μg/ml puromycin (Invivogen) on an irradiation-inactivated Puro MEF feeder layer. Tamoxifen (3.3 μM; Toronto Research Chemicals) was added (or not) for 2 days to completely delete the Mob1a gene (data not shown). Control and mutant EBs were generated from hanging drops of approximately 1,000 ES cells/20 μl medium as described (58).

**Generation of keratinocyte-specific Mob1a/Mob1b double-mutant mice.** Mob1a<sup>ΔloxP</sup><sup>/ΔloxP</sup> and Mob1b<sup>ΔloxP</sup><sup>/ΔloxP</sup> mice were mated to Krt14CreER transgenic mice (35) in which Cre can be activated by tamoxifen under the control of the Krt14 promoter. Offspring carrying Krt14CreER plus 2 copies of the floxed Mob1a allele (Mob1a<sup>ΔloxP</sup>) and 2 copies of the trapped Mob1b allele (Mob1b<sup>ΔloxP</sup>) were administered tamoxifen and used in the analysis as homozygous double mutant (Krt14CreERMob1a<sup>ΔloxP</sup><sup>/ΔloxP</sup> +tamoxifen; KdKO) mice.
Krt14CreERMob1a+/+Mob1btr/tr mice without tamoxifen and Mob1a+/+Mob1btr/tr or Mob1a+/+Mob1btr/tr mice with tamoxifen but without Krt14CreER were indistinguishable in pilot experiments examining histology and MOB1 protein levels. Krt14CreERMob1a+/+Mob1btr/tr mice without tamoxifen were arbitrarily chosen to serve as controls. To induce Cre in keratinocyte stem/progenitor cells, P1 mice were intraperitoneally administered 0.2 mg tamoxifen on that one day, or P28 mice received 0.5 mg tamoxifen each day for 7 days starting on P28.

Characterization of primary keratinocytes. Primary keratinocytes isolated from control and δDKO mice were cultured as described (59). For cell saturation density assays, keratinocytes at passage 2 were seeded in type I collagen–coated 24-well plates (0.3 × 10^5/well) and cultured for up to 12 days before counting cell numbers. For colony-forming assays, keratinocytes at passage 1 were seeded in 6-well plates (0.4 × 10^5/well). After 2 weeks, half the wells were fixed and stained with Giemsa (Muto Pure Chemicals), and the number of primary colonies was counted. Keratinocytes of an unfixed well were then trypsinized and reseeded so that each well of a 6-well plate received cells from 1 primary colony. Reseeded cells were cultured for 2 weeks to generate secondary colonies.

Clinical samples. Surgically obtained trichilemmal carcinoma samples containing noncancerous tissues were acquired from the Department of Regenerative Dermatology (Osaka University). Resected cancer tissues were fixed in formalin and stained with anti-YAP1 Ab (Cell Signaling) and anti-MOB1A/1B Ab (AP7031b; Abgent) using a standard protocol.

Statistics. KaleidaGraph software was used for statistical analyses. Data are shown as the mean ± SEM, and P values were determined using the 2-tailed Student’s t test unless otherwise stated. P < 0.05 was considered statistically significant.

Study approval. The clinical sample study design was approved by the Institutional Review Board of Osaka University, and written informed consent was obtained from all patients. All animal experiments were approved by the Animal Experiment Review Boards of Kyushu University and Akita University. See Supplemental Methods for additional details.

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Address correspondence to: Akira Suzuki, Division of Cancer Genetics, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Higashiku, Higashiku, Fukuoka, Fukuoka 812-8582, Japan. Phone: 81.92.642.6838; Fax: 81.92.632.1499; E-mail: suzuki@biore.kyushu-u.ac.jp.


