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Identification of Fat4 as a candidate tumor suppressor gene in breast cancers

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Abstract

Fat, a candidate tumor suppressor in *Drosophila*, is a component of Hippo signaling pathway involved in controlling organ size. We found that a ~3Mbp deletion in mouse chromosome 3 caused tumorigenesis of a non-tumorigenic mammary epithelial cell line. The expression of Fat4 gene, one member of the Fat family, in the deleted region was inactivated, which resulted from promoter methylation of another Fat4 allele following the deletion of one Fat4 allele. Re-expression of Fat4 in Fat4-deficient tumor cells suppressed the tumorigenicity while suppression of Fat4 expression in the non-tumorigenic mammary epithelial cell line induced tumorigenesis. We also found that Fat4 expression was lost in a large fraction of human breast tumor cell lines and primary tumors. Loss of Fat4 expression in breast tumors was associated with human Fat4 promoter methylation. Together, these findings suggest that Fat4 is a strong candidate for a breast tumor suppressor gene.

INTRODUCTION

Loss-of-function mutations of the Fat gene in *Drosophila* causes hyperplasia of the pupal imaginal disks,¹ suggesting that Fat is a candidate tumor suppressor gene. Excessive cell proliferation occurs with normal epithelial organization and differentiation potential following the loss of Fat expression.² Fat belongs to the cadherin family that is involved in cell adhesion and consists of more than 80 members in mammalian species.^{3–5} The classic cadherins are Ca²⁺-dependent cell-cell adhesion proteins characterized by five repeated cadherin-specific motifs in their extracellular domain.⁶ This motif is an approximately 110-amino-acid peptide that mediates homophilic interactions with other cadherin molecules, forming dimers which then interact with dimers on neighboring cells.⁶ Fat contains 34 cadherin motifs, four EGF-like repeats, a transmembrane domain, and a cytoplasmic region.

Recently, Fat was found to be a component of Hippo signaling pathway, which plays an important role in controlling organ size.^{7–8} Core components of Hippo signaling pathway include Salvador (SAV), warts (Wts), hippo and yorkie(YKI).^{8–12} Hippo phosphorylates and activate Wts. SAV potentiates the phosphorylation reaction. Tumor suppressor Wts encodes a kinase which phosphorylates YKI for inactivation. YKI is transferred into nucleus from cytoplasm after activation to act as a transcriptional coactivator. Fat is likely to function at the apical point of the Hippo signaling as a potential transmembrane receptor in *Drosophila*.^{13–14}

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In addition to the control of cell proliferation, Fat is also involved in the planar polarity formation, which refers to the asymmetry of a cell within the plane of the epithelium.¹⁵ The cytoplasmic domain of *Drosophila* Fat was found to interact with atrophin,¹⁶ which is a transcriptional co-repressor.¹⁷ This interaction contributes to the function of Fat in planar polarity.¹⁶

There are four members of the Fat family in mice and humans (Fat1, MEGF1/Fat2, Fat3, and Fat4), which structurally resemble *Drosophila* Fat.^{18–21} The tumor suppressor function is not observed with Fat1 as the null mutation of Fat1 in mice did not exhibit overgrowth of any organs.²² The knock out experiment did reveal novel roles of Fat1 in adhesion and cell-cell signaling. The function of the other three Fat genes remains to be investigated.

The identification of tumor suppressor genes has greatly advanced our understanding of breast cancer biology.²³ Despite the progress, the total number of known tumor suppressor genes account for only a small fraction of familial breast cancer cases.²³ In addition, the target genes for the vast majority of the loss of heterozygosity in breast cancers have not been revealed.^{24–25} Therefore, many tumor suppressor genes must exist and remain to be discovered in breast cancers. Recently, we developed a method based on LoxP/Cre mediated homologous recombination to obtain random chromosome deletions in the genome.²⁶ By applying this method to a mammary epithelial cell line, we identified Fat4, one member of the Fat family, as a strong candidate for a breast tumor suppressor gene. Furthermore, we found that Fat4 expression was lost in a large fraction of human breast tumors.

MATERIALS AND METHODS

Chromosome deletion and tumor development

5×10^6 of Phoenix-Eco packaging cells were transfected with 15 μ g of LacZ/Neo-5'Pur-LoxP-TK or Hyg-3'Pur-LoxP through the calcium phosphate precipitation method. Forty eight hours after the transfection, LacZ/Neo-5'Pur-LoxP-TK viral supernatant was added to 5×10^5 NOG8 cells with 8 μ g/ml of polybrene. After 5 hours of incubation, the medium with retrovirus was replaced with fresh medium. Hyg-3'Pur-LoxP retrovirus was used for infection on the second day. At 48 h after retroviral infection, the cells were subjected to G418 and hygromycin selection. A total of 20 plates of NOG8 cells were used.

One week after the selection, each plate was infected with 10^8 adenovirus expressing Cre recombinase (provided by Dr. Graham from NIH). After 12 hours, the cells were washed with PBS three times and fresh medium was added. The following day, 2 μ g/ml of puromycin was added to the medium and the cells were selected with puromycin for five days. Eight-week-old BALB/c athymic nude mice were injected subcutaneously with 1×10^6 cells from each plate. Two months later, the tumor was isolated. Half of the tumor was used for DNA preparation and half of the cells were used to establish the tumor cell line.

Inverse-PCR and sequencing

To determine the retroviral integration site, 5 μ g of genomic DNA from the tumor was digested with BamHI plus ApoI for localization of the 3'LTR or SSPI plus Afl III for localization of the 5'LTR. The digested DNA was then blunted with T4 polymerase, circularized by dilution and ligation using T4 DNA ligase in a total volume of 500 μ l at 16°C for 18 hours. Circular DNA was purified and used in the primary PCR reaction using primers derived from the retroviral vectors. 0.1 μ l of primary PCR product was used as template for secondary PCR with nested primers. The secondary PCR product was sequenced at the Northwestern University Biotech facility. The primary and nested primers used were as follows:

Localization of the 5'LTR: 5'-CATGGTCAGGTCATGGATGA-3' and 5'-AGGAACAGCGAGACCACGATT-3'; its nested primers: 5'-ACGATGGTGCAGGATATCCT-3' and 5'-GATGCAAACAGCAAGAGGCT-3'.

Localization of the 3' LTR: 5'-CCGCTAAAGCGCATGCTCCA-3' and 5'-TGCAAGAACTCTTCCTCACG-3'; its nested primers: 5'-CTGCCTTGGGAAAAGCGCCT-3' and 5'-CTCGACATCGGCAAGGTGT-3'.

Quantitative real-time RT-PCR (qPCR)

qPCR was performed on ABI 7300 (Applied Biosystems) by using the SYBR Green Supermix (Applied Biosystems) according to the manufacturer's protocol. The expression levels of Fat4 were normalized against β -actin RNA. The primers used were as follows:

β -actin: 5'-CCATCTACGAGGGCTATGCT-3' and 5'-GCAAGTTAGGTTTTGTCAAAGA-3'.

mFat4: 5'-CCAACGCTCTGGTCACGTAT-3' and 5'-CTCCATTACACCAGAGTCA-3'.

hFat4: 5'-TATCACAAAACGCCCTTGCT-3' and 5'-TGGATTGTCATTGATATCCTG-3'.

Methylation assay

For sodium bisulfite DNA treatment, 1 μ g of genomic DNA (10 μ l) was denatured by adding an equal volume of 0.6 N NaOH for 5 min, followed by the addition of 208 μ l of 3.6 M sodium bisulfite and 12 μ l of 10 mM hydroxyquinone. This mixture was incubated at 55°C for 16 h to convert cytosine to uracil. Treated genomic DNA was subsequently purified using the Wizard clean up system (Promega), precipitated with ethanol, and resuspended in 100 μ l of distilled H₂O. PCR was performed in a 50- μ l reaction using 5 μ l of sodium bisulfite-treated DNA. After the PCR, a nested PCR was performed and the final PCR product was sequenced directly. The primers for PCR and corresponding nested PCR were as follows:

Mouse Fat4 promoter: 5'-GGTATGGTGAGGGGAGGGGA-3' and 5'-CTAAATTTTCGAAAATCCGAAAAAC-3'; its nested primers: 5'-GGCGTTGAGGAGGAAGGGAAA-3' and 5'-CAAAAAACTTTAAACTTACCCC-3'.

Human Fat4 promoter: 5'-AATAAATTCTAAAATTTCTAAAAAC-3' and 5'-GTTAGTAGTTTTGTTTGGTGTTA-3'; its nested primers: 5'-ACTTCTCCCAACTCTCATCC-3' and 5'-GATAAAGAGAAGGAAGGGGTG-3'.

Methylation-specific PCR was performed with bisulfite-treated DNA. The primer for both methylated and unmethylated : 5'-CCTATATCTAAAATATATAAAAAATC-3'; the primer for methylated template: 5'-GTTTTAGCGGTTATTGTCGGC-3'; the primer for unmethylated template: 5'-GTTTTAGTGGTTATTGTTGGT-3'.

Transfection

The BAC clone DNA (RPCI-23 MM BAC 194E5 from Invitrogen) was linealized with Not I. Tumor cells (3×10^5) were seeded on 10cm plates 24 h before transfection. Cells were transfected for 5 h with 15 μ g of BAC clone DNA and 0.1 μ g of P1b-Bla which carries the blasticidin resistance gene using Lipofectamine Plus reagent (Invitrogen). Blasticidin (10 μ g/ml) was added into the culture medium 48h later. The individual clones were picked up 7 days later and expanded. The mRNA expression of Fat4 in these clones was examined by RT-PCR.

In vivo tumorigenicity assay

Two clones expressing Fat4 and two clones not expressing Fat4 were used for the tumorigenicity assays in vivo. The selected clones were injected subcutaneously into 8-week-old BALB/c athymic nude mice (1×10^6 cells per injection, 4 injections for each clone). The maximal tumor diameter was determined by caliber measurements once a week up to sixth week.

Suppression of Fat4 expression by shRNA

A vector expressing shRNA targeting Fat4 (V2MM_94721) was obtained from Openbiosystem. Phoenix-Eco packaging cells were transfected with the calcium phosphate precipitation method. The viral supernatant was added to 5×10^5 NOG8 cells with 8 μ g/ml of polybrene 36h after the transfection. One day later, the cells were selected with 1 μ g/ml of puromycin. One week later, 1×10^6 cells expressing shRNA targeting Fat4 or control shRNA were injected subcutaneously into BALB/c athymic nude mice for tumor formation.

Assay for YAP transcriptional activity

PM-YAP was constructed by inserting YAP full-length cDNA into EcoRI/SalI sites of PM (Clontech) vector. For transient transfection, 3×10^5 of NOG8, Fat4-deficient tumor cells, NOG8 cells expressing Fat4-shRNA or control shRNA were seeded in 6-well plates 24 h before transfection. Cells were transfected for 5 h with 1.25 μ g of luciferase reporter DNA, 0.2 μ g of PM or PM-YAP plasmid, 0.1 μ g of β -galactosidase expression vector pCMV β (CLONTECH) using Lipofectamine Plus reagent (Invitrogen). Cell extracts were prepared 24 h after transfection and assayed for luciferase and β -galactosidase activities (Tropix, Bedford, MA). Three independent transfections were performed for each assay.

RESULTS

A ~3Mbp deletion in mouse chromosome 3 associated with tumorigenesis

NOG8 cell line was derived from NMuMG mouse mammary epithelial cell line.²⁷ NOG8 shows a normal cuboidal epithelial morphology and a non-tumorigenic phenotype. NOG8 cells are almost diploid, carrying 41 chromosomes with trisomy 8. To generate random chromosomal deletion, a total of 20 plates of NOG8 cells were sequentially infected with LacZ/Neo-5'Pur-LoxP-TK virus, Hyg-3'Pur-loxP virus and Cre adenovirus. Cells from each plate (1×10^6) after selection with puromycin were injected subcutaneously into nude mice. Two months later, 9 tumors developed and were isolated from the mice. We have characterized 4 of the 9 tumors so far. We identified 3 deletions and 1 translocation. In addition to the deletion described in detail below, the other two deletions are ~20.0 Mbp deletion on chromosome 11, which contains tumor suppressor gene BRCA1, and ~12.6 Mbp deletion on chromosome 14, which includes tumor suppressor gene Lats2.

For one tumor, inverse-PCR and sequencing revealed that the 5'LTR of the retrovirus was located at 37,747kb on chromosome 3 while the 3'LTR was found to be at 40,623 kb on the same chromosome. Therefore, a ~3Mbp fragment of chromosome 3 from 37,747 kb to 40,623 kb was deleted in this tumor, which was further confirmed by PCR using a pair of primers located ~ 3Mbp away from each other (Fig. 1a). The PCR generated a 8.3 kb band corresponding to the size of the recombinant retroviral vector as a result of deletion. Histology revealed that the tumor was a poorly differentiated carcinoma with occasional gland differentiation (Fig. 1b).

Loss of Fat4 expression in the tumor

By looking into the mouse genome map, we identified 14 genes within the ~3M bp deleted region. As the first step to linking any of the genes to tumorigenesis, we examined the expression of these 14 genes in the tumor and wild type NOG8 cells by real time RT-PCR. We found that Fat4 mRNA level was substantially decreased in the tumor (Fig. 2a) while the mRNA of the other 13 genes remained unchanged (data not shown). As only partial Fat4 cDNA sequence and hypothetical Fat4 coding sequence derived from the mouse genomic sequence using gene prediction method were available in Genbank, we determined the exact mouse Fat4 cDNA through RT-PCR and sequencing (Genbank No: DQ286572). The Fat4 gene turned out to encode a protein with 4981 amino acids, which contains 5 epidermal growth factor (EGF) domains and 2 laminin-A-G domain repeats in addition to the 34 cadherin repeats in the extracellular domain.

Promoter methylation of Fat4 gene

A CpG island encompassing about 2.0 kb was found in the mouse Fat4 gene promoter region (Fig. 2b). We examined Fat4 promoter to see if it was methylated. We amplified and sequenced the promoter region of Fat4 using sodium bisulfite-treated genomic DNA prepared from tumor cells. Indeed, the vast majority of CpG sites (20/22) were methylated in the tumor cells while the CpG sites in the wild type NOG8 cells were not methylated (Fig. 2b). When tumor cells were treated with the “demethylating” agent 5-aza-2'-deoxycytidine for 48 h, the expression of Fat4 mRNA was dramatically increased (Fig. 2a), indicating that methylation of the Fat4 promoter silenced the expression of Fat4 from the second allele following the deletion of the first Fat4 allele.

Reintroduction of Fat4 inhibited the tumor growth

To confirm that the loss of Fat4 expression was indeed involved in the tumorigenesis of NOG8 cells, we expressed Fat4 gene in the tumor cells to see if Fat4 could suppress the tumor formation in nude mice. A BAC clone containing the mouse Fat4 gene was cotransfected along with a blasticidin resistance gene into cells derived from the tumor. The Fat4 expression in individual cell clones was examined with RT-PCR (Fig. 3a). Two clones expressing Fat4 gene, and two clones not expressing Fat4 gene were selected for further studies. Cells from each of the selected clones (1×10^6) were injected subcutaneously into nude mice. As shown in Fig. 3b, tumor cells expressing Fat4 grew much slower than those not expressing Fat4, confirming that Fat4 expression inhibited the growth of tumor cells in nude mice.

Inhibition of Fat4 expression led to tumorigenesis

To further confirm that Fat4 down regulation contributed to the tumorigenesis, we inhibited Fat4 expression by shRNA. The NOG8 cells were infected with a retroviral vector expressing shRNA targeting Fat4 or control shRNA. The cells were then injected into nude mice. Only NOG8 cells expressing Fat4-shRNA developed into tumors in nude mice (Fig. 4a). Real time RT-PCR revealed that the expression level of Fat4 in NOG8 cells expressing Fat4-shRNA was decreased to ~20% of that of NOG8 cells expressing control shRNA. The tumors developed from NOG8 cells expressing Fat4-shRNA, however, expressed less than 5% of Fat4 mRNA from the control cells, possibly because the extent of inhibition by Fat4-shRNA varied among individual cells and cells expressing lower level of Fat4 were more likely to grow in nude mice (Fig. 4b).

Loss of Fat4 expression increases the transcriptional activity of YAP

As YAP, the homologue of Yorkie in mammalian, is the direct effector of the Hippo kinase cascade, the transcriptional activity of YAP serves as the indicator of Hippo signaling

activity. Plasmid encoding fusion protein between YAP and DNA binding domain from Gal4 transcription factor was cotransfected with reporter gene into NOG8 cells, cells derived from tumors not expressing Fat4, and NOG8 cells expressing Fat4-shRNA or control shRNA. While NOG8 cells and NOG8 cells expressing control shRNA showed minimal YAP transcriptional activities (Fig. 5), this activity was dramatically increased in cells derived from tumors not expressing Fat4 and to a less extent in NOG8 cells expressing Fat4-shRNA (Fig. 5), indicating that loss of Fat4 expression disrupts Hippo signaling pathway and leads to the abnormal activation of YAP.

Loss of Fat4 expression in a fraction of human breast tumors due to promoter methylation

We examined Fat4 mRNA in 5 primary breast cancers and 6 established breast cancer cell lines. Loss of Fat4 mRNA expression was observed in 3 of 5 primary breast cancers (Fig. 6a). Of the 6 breast cancer cell lines examined (BT474, BT20, MDA-MB-157, MDA-MB-231, MCF-7, and ZR-75-1), 3 cell lines, namely BT474, BT20, and ZR-75-1, exhibited no expression of Fat4 mRNA (Fig. 6a).

Like mouse Fat4 promoter region, human Fat4 promoter region also contains a CpG island spanning about 2.5 kb. To understand the mechanism underlying the down-regulation of Fat4 mRNA, we examined the methylated status of CpG sites in the Fat4 promoter from two cell lines by bisulfite genomic sequencing. BT20 showed complete methylation in the ten CpG sites examined while MCF7 had no methylation at all (0/10) (Fig. 6b). Methylation-specific PCR was then performed for the 6 tumor cell lines and 5 primary tumors (Fig. 6c). Cell lines and primary tumors not expressing Fat4 carried methylated templates. We did not detect promoter methylation in the cell lines or primary tumors expressing Fat4. Therefore, methylation of Fat4 gene promoter is associated with the silenced expression of Fat4 in breast cancers.

DISCUSSION

In an effort to isolate novel tumor suppressor genes for breast cancers, we applied the random chromosome deletion method based on the Cre-LoxP system to an immortalized non-tumorigenic mammary epithelial cell line NOG8 cells. A ~3Mbp deletion on chromosome 4 was found to be associated with the tumorigenesis of NOG8 cells. We identified Fat4 in the deleted ~3Mbp region as a candidate for a breast tumor suppressor gene. There are four members of Fat family in mice and humans, which share high homology to the *Drosophila* Fat gene. Just as other membrane proteins, the cytoplasmic regions of Fats are expected to play an important role in the signaling transduction. However, we found that the cytoplasmic regions from Fat1, Fat2 and Fat3 exhibited no homology to that of the *Drosophila* Fat protein. This may explain the observation that unlike the *Drosophila* Fat, the null mutation of Fat1 in mice did not show overgrowth of any organs. Instead, several small regions from the cytoplasmic region of Fat4 are highly conserved in the *Drosophila* Fat gene (Fig. 7), suggesting that only Fat4 is the homologue of *Drosophila* Fat gene.

In addition to point mutations, epigenetic modifications such as gene methylation have been found to be a cause of inactivation of tumor suppressor genes in tumors.²⁸⁻³⁰ The mouse Fat4 gene promoter region contains a CpG island which was methylated in the tumor cells and Fat4 could re-express with the treatment of a demethylating agent. Therefore, the second allele of Fat4 in the NOG8 cells was inactivated through promoter methylation following the deletion of the first Fat4 allele. While how the gene methylation is regulated and how loss of an allele triggers the methylation of the other allele remains unknown, many other tumor suppressor genes could be inactivated in this way as loss of heterozygosity is very common in almost all types of tumors.

As tumorigenesis is an evolving process, cells losing Fat4 expression might undergo additional genetic changes involved in tumorigenesis. Re-expression of Fat4 would not completely inhibit the tumorigenesis when tumor cells carried these additional genetic changes. The critical role of Fat4 in tumorigenesis is further demonstrated by the finding that knockdown of Fat4 was sufficient for tumorigenesis.

We found that loss of Fat4 expression occurred to some of primary breast tumors and breast cell lines, implicating its important role in breast tumorigenesis. Methylation of Fat4 gene promoter was found to be associated with the silenced expression of Fat4 in breast cancers. In addition to promoter methylation, a mutation could potentially inactivate the Fat4 gene. We are currently examining a large number of specimens to identify the potential mutation. As the loss of heterozygosity in the chromosome 4q28, where the human Fat4 gene is located, was observed in several cancers including colon, prostate lung, and liver, 31–35 Fat4 could be involved in other types of tumors.

The evidence supporting Fat4 as a tumor suppressor gene is the loss of Fat4 in nontumorigenic mammary epithelial cells transformed the cells into tumorigenic cells and restoring Fat4 in these tumor cells markedly inhibited the tumorigenicity. As the studies were done with a mammary epithelial cell line which had been immortalized, it is important to understand the role of Fat4 in mammary epithelial tumorigenesis under physiological conditions. As a tumor suppressor gene, Fat4 could be an initiating factor during mammary tumorigenesis or a factor involved in tumor progression. A definitive conclusion can be reached through the establishment of an animal model with Fat4 deficiency in mammary glands.

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REFERENCES

1. Mahoney PA, Weber U, Onofrechuk P, Biessmann H, Bryant PJ, Goodman CS. The fat tumor suppressor gene in *Drosophila* encodes a novel member of the cadherin gene superfamily. *Cell*. 1991; 67(5):853–868. [PubMed: 1959133]
2. Garoia F, Guerra D, Pezzoli MC, Lopez-Varea A, Cavicchi S, Garcia-Bellido A. Cell behaviour of *Drosophila* fat cadherin mutations in wing development. *Mech Dev*. 2000; 94(1–2):95–109. [PubMed: 10842062]
3. Steinberg MS, McNutt PM. Cadherins and their connections: adhesion junctions have broader functions. *Curr Opin Cell Biol*. 1999; 11(5):554–560. [PubMed: 10508659]
4. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science*. 1991; 251(5000):1451–1455. [PubMed: 2006419]
5. Yagi T, Takeichi M. Cadherin superfamily genes: functions, genomic organization, and neurologic diversity. *Genes Dev*. 2000; 14(10):1169–1180. [PubMed: 10817752]
6. Shapiro L, Fannon AM, Kwong PD, Thompson A, Lehmann MS, Grubel G, Legrand JF, Als-Nielsen J, Colman DR, Hendrickson WA. Structural basis of cell-cell adhesion by cadherins. *Nature*. 1995; 374(6520):327–337. [PubMed: 7885471]
7. Harvey K, Tapon N. The Salvador-Warts-Hippo pathway - an emerging tumour-suppressor network. *Nat Rev Cancer*. 2007; 7(3):182–191. [PubMed: 17318211]
8. Pan D. Hippo signaling in organ size control. *Genes Dev*. 2007; 21(8):886–897. [PubMed: 17437995]

9. Edgar BA. From cell structure to transcription: Hippo forges a new path. *Cell*. 2006; 124(2):267–273. [PubMed: 16439203]
10. Wu S, Huang J, Dong J, Pan D. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell*. 2003; 114(4):445–456. [PubMed: 12941273]
11. Huang J, Wu S, Barrera J, Matthews K, Pan D. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. *Cell*. 2005; 122(3):421–434. [PubMed: 16096061]
12. Tapon N, Harvey KF, Bell DW, Wahrer DC, Schiripo TA, Haber DA, Hariharan IK. salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell*. 2002; 110(4):467–478. [PubMed: 12202036]
13. Cho E, Feng Y, Rauskolb C, Maitra S, Fehon R, Irvine KD. Delineation of a Fat tumor suppressor pathway. *Nat Genet*. 2006; 38(10):1142–1150. [PubMed: 16980976]
14. Willecke M, Hamaratoglu F, Kango-Singh M, Udan R, Chen CL, Tao C, Zhang X, Halder G. The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. *Curr Biol*. 2006; 16(21):2090–2100. [PubMed: 16996265]
15. Yang CH, Axelrod JD, Simon MA. Regulation of Frizzled by fat-like cadherins during planar polarity signaling in the Drosophila compound eye. *Cell*. 2002; 108(5):675–688. [PubMed: 11893338]
16. Fanto M, Clayton L, Meredith J, Hardiman K, Charroux B, Kerridge S, McNeill H. The tumor-suppressor and cell adhesion molecule Fat controls planar polarity via physical interactions with Atrophin, a transcriptional co-repressor. *Development*. 2003; 130(4):763–774. [PubMed: 12506006]
17. Zhang S, Xu L, Lee J, Xu T. Drosophila atrophin homolog functions as a transcriptional corepressor in multiple developmental processes. *Cell*. 2002; 108(1):45–56. [PubMed: 11792320]
18. Dunne J, Hanby AM, Poulosom R, Jones TA, Sheer D, Chin WG, Da SM, Zhao Q, Beverley PC, Owen MJ. Molecular cloning and tissue expression of FAT, the human homologue of the Drosophila fat gene that is located on chromosome 4q34-q35 and encodes a putative adhesion molecule. *Genomics*. 1995; 30(2):207–223. [PubMed: 8586420]
19. Hong JC, Ivanov NV, Hodor P, Xia M, Wei N, Blevins R, Gerhold D, Borodovsky M, Liu Y. Identification of new human cadherin genes using a combination of protein motif search and gene finding methods. *J Mol Biol*. 2004; 337(2):307–317. [PubMed: 15003449]
20. Mitsui K, Nakajima D, Ohara O, Nakayama M. Mammalian fat3: a large protein that contains multiple cadherin and EGF-like motifs. *Biochem Biophys Res Commun*. 2002; 290(4):1260–1266. [PubMed: 11811999]
21. Nakayama M, Nakajima D, Yoshimura R, Endo Y, Ohara O. MEGF1/fat2 proteins containing extraordinarily large extracellular domains are localized to thin parallel fibers of cerebellar granule cells. *Mol Cell Neurosci*. 2002; 20(4):563–578. [PubMed: 12213440]
22. Ciani L, Patel A, Allen ND, French-Constant C. Mice lacking the giant protocadherin mFAT1 exhibit renal slit junction abnormalities and a partially penetrant cyclopia and anophthalmia phenotype. *Mol Cell Biol*. 2003; 23(10):3575–3582. [PubMed: 12724416]
23. Nathanson KL, Wooster R, Weber BL. Breast cancer genetics: what we know and what we need. *Nat Med*. 2001; 7(5):552–556. [PubMed: 11329055]
24. Devilee P, Cornelisse CJ. Somatic genetic changes in human breast cancer. *Biochim Biophys Acta*. 1994; 1198(2–3):113–130. [PubMed: 7819270]
25. Osborne RJ, Hamshire MG. A genome-wide map showing common regions of loss of heterozygosity/allelic imbalance in breast cancer. *Cancer Res*. 2000; 60(14):3706–3712. [PubMed: 10919637]
26. Zhu YT, Qi C, Hu L, Zhu YJ. Efficient generation of random chromosome deletions. *Biotechniques*. 2007; 42(5):572, 74, 76. [PubMed: 17515194]
27. Redmond SM, Reichmann E, Muller RG, Friis RR, Groner B, Hynes NE. The transformation of primary and established mouse mammary epithelial cells by p21-ras is concentration dependent. *Oncogene*. 1988; 2(3):259–265. [PubMed: 3127778]

28. Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev.* 2002; 16(1):6–21. [PubMed: 11782440]
29. Feinberg AP, Tycko C. The history of cancer epigenetics. *Nat Rev Cancer.* 2004; 4(2):143–153. [PubMed: 14732866]
30. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med.* 2003; 349(21):2042–2054. [PubMed: 14627790]
31. Cho ES, Chang J, Chung KY, Shin DH, Kim YS, Kim SK. Identification of tumor suppressor loci on the long arm of chromosome 4 in primary small cell lung cancers. *Yonsei Med J.* 2002; 43(2): 145–151. [PubMed: 11971207]
32. Hammond C, Jeffers L, Carr BI, Simon D. Multiple genetic alterations, 4q28, a new suppressor region, and potential gender differences in human hepatocellular carcinoma. *Hepatology.* 1999; 29(5):1479–1485. [PubMed: 10216132]
33. Knosel T, Petersen S, Schwabe H, Schluns K, Stein U, Schlag PM, Dietel M, Petersen I. Incidence of chromosomal imbalances in advanced colorectal carcinomas and their metastases. *Virchows Arch.* 2002; 440(2):187–194. [PubMed: 11964050]
34. Knosel T, Schluns K, Stein U, Schwabe H, Schlag PM, Dietel M, Petersen I. Chromosomal alterations during lymphatic and liver metastasis formation of colorectal cancer. *Neoplasia.* 2004; 6(1):23–28. [PubMed: 15068668]
35. Matsui S, LaDuca J, Rossi MR, Nowak NJ, Cowell JK. Molecular characterization of a consistent 4.5-megabase deletion at 4q28 in prostate cancer cells. *Cancer Genet Cytogenet.* 2005; 159(1):18–26. [PubMed: 15860352]

Fig. 1.
 (a). Confirmation of ~3Mbp deletion on mouse chromosome 3. The 5'LTR and 3'LTR of the recombinant retroviral vector was located at 37,747kb and 40,623 kb on chromosome 3, respectively. PCR using primers located in the two regions as indicated was performed to confirm the deletion, which generated a 8.3-kb band (lane 1). M: Lamda DNA /HindIII Marker. (b). The histology of the tumor with deletion on chromosome 3. The tumor was harvested from a nude mouse, sectioned and stained with H&E. The tumor was poorly differentiated with occasional gland formation.

Fig. 2.

(a). Loss of Fat4 expression in the tumor and increased expression of Fat4 gene in tumor cells with the treatment of 5-aza-2'-deoxycytidine(5-Aza-dC). Quantitative real-time RT-PCR was performed to determine the Fat4 mRNA levels, which were normalized to control β -actin mRNA levels. Tumor cells were treated with 5 mM 5-aza-2'-deoxycytidine(5-Aza-dC) for three days. (b). The methylation status of the CpG island in NOG8 cells and tumor cells. The ~2.0 kb CpG island of Fat4 promoter region is shown with the analyzed region (370bp) indicated by arrows. This region which contains 22 CpG sites was directly sequenced after being amplified from sodium bisulfite-treated genomic DNA. A closed circle indicates a methylated CpG and an open circle indicates an unmethylated CpG.

Fig. 3.

Expression of Fat4 gene inhibited the tumor growth in nude mice. (a). The mRNA expression of Fat4 gene as revealed by RT-PCR. Tumor Clone 3 and 4 expressed Fat4 while Clone 1 and 2 did not express Fat4. (b). The curve of tumor growth in nude mice. Two clones of tumor cells not expressing Fat4 or two clones of tumor cells expressing Fat4 (1×10^6 cells from each clone) were injected into nude mice, respectively. The experiment was performed in triplicate and the average tumor sizes were calculated. The tumors from clones expressing Fat4 were much smaller than that from tumor cell line not expressing Fat4.

Fig. 4.

Inhibition of Fat4 led to tumorigenesis. (a). Representative photo of tumor formation 60 days after injection of NOG8 cells (10^6) expressing Fat4-shRNA or control RNA into nude mice. Note the tumor on the right flank, corresponding to NOG8 expressing Fat4-shRNA, and no tumor formation on the left flank, corresponding to NOG8 expressing control shRNA. (b). Inhibition of Fat4 mRNA by shRNA. Quantitative RT-PCR was performed with total RNA prepared from NOG8 cells expressing control shRNA (Lane 1) or Fat4-

shRNA (Lane 2), and tumors developed from NOG8 cells expressing Fat4- shRNA (Lane 3). The Fat4 mRNA levels were normalized to control β -actin mRNA levels.

Fig. 5.

Increased YAP transcriptional activities in tumor cells not expressing Fat4 and NOG8 cells expressing Fat4-shRNA. Plasmid Gal4-YAP expressing fusion protein between YAP and Gal4 DNA binding domain or plasmid PM expressing Gal4 DNA binding domain was cotransfected with GAL-TKLUC vector into cells as indicated. The activity of luciferase from transfection with Gal4-YAP was divided by that from transfection with PM. Lane 1, NOG8; Lane 2, tumor cells not expressing Fat4; Lane 3, NOG8 expressing control shRNA; Lane 4, NOG8 expressing Fat4-shRNA.

Fig. 6.

(a). Loss of Fat4 expression in a fraction of breast cancers. Total RNA was made from 6 breast tumor cell lines and 5 primary breast tumors. RT-PCR was performed. BT-20, ZR-75-1, BT474, and primary tumor 1 to 3 did not express Fat4. (b). Schematic depiction of the CpG island in the human Fat4 promoter region. The CpG island spanning ~2.7 kb was located in the promoter region and first exon of Fat4 gene. Location of bisulfite genomic sequencing PCR primers and methylation-specific PCR primers are indicated as white and gray arrows, respectively. Shown are results of bisulfite genomic sequencing of the fragment containing 10 CpG sites from BT-20 and MCF-7 cells, with an open circle representing an unmethylated CpG site and a close circle representing a methylated CpG site. (c).

Methylation of Fat4 promoter in breast cancers. Methylation-specific PCR was performed. BT20, ZR-75-1, and primary tumor 1 contained only methylated alleles. BT474, and primary tumor 2 and 3 contained both unmethylated and methylated alleles. MCF-7, MDA-MB-157, MDA-MB-231, and primary tumor 4 and 5 contained only unmethylated alleles. The water control did not show evidence of contamination.

mFat4	4573	RKQPEGNPKPDIIERENPYLIFDETDP----HNSETIPSAPLASPEQEIEHYDIDNASS	4628
		++Q + +PDIIERE+P LI ++ +P H ++ + + EHYD++NASS	
dFat	4696	QQQQRPQRPDIIERESP-LIREDHHLPIPLHPLPLEHASSVDMGSEYPEHYDLENASS	4754
mFat4	4629	IAPSDADIIQHYKQFRS----HTPKFSIQRHSPLGFARQSPMPLGASSLTYPSSYGQGL	4684
		IAPSD DI+ HYK +R K S+ P+ S Q +	
dFat	4755	IAPSDIDIVYHYKGYREAAGLRKYKASV--PPVSAYTHHKHQNSGSQQQQQHRHTAPF	4811
mFta4	4685	RTSSLSHSACPTPNPLSR-HSPAPFSKPSAFYRNSPARELHLPLRDGGTLEMHGDPQPG	4743
		T + P P SR H P ++ S S + L L D + G P Q	
dFat	4812	VTRNQGQPPPPPTSASRTHQSTPLARLSPSSELSSQQPRILTLHD-----ISGKPLQSA	4866
mFat4	4744	MFNYATRLG-----RRSKSP-QAMASHGSRPGSRLKQPIAQIPLESSPPVGLSI	4791
		+ + G R SP + S S SR K + Q + + +GL+	
dFat	4867	LLATTSSSGVGKDVHSNSERSLNSPVMSQLSGQSSASRQKPGVPQQQAQQT-SMGLTA	4925
mFat4	4792	EEVERLNTPRPRNPSICSADHGRSSSEEDCRRPLSRTRNPADG-IPAPES-SSDSDSHDS	4849
		EE+ERLN RPR S+ S SSS E R S G + A S S+D +DS	
dFat	4926	EEIERLN-GRPRTCSLISTLDAVSSSSEAPRVSSSALHMSLGGDVDAHSSTSTDESGNDS	4984
mFat4	4850	FTCSEMEYDRE-----KPVVYTSRMPKLSQVNESDADDEDNYG-----ARLKPRRY	4895
		FTCSE+EYD K S + S V+ + + E + + P Y	
dFat	4985	FTCSEIEYDNNLSGDKYSTSKSLLDGRSPVSRALSGGETSRNPPTTVVKTPPIPHAY	5044
mFat4	4896	HGRRAE-GGPVGTPAAASGAADSTLK-----LGQQAGNFNWDNLLNWGPFGFHYVD	4945
		G + G + T A+ + L A W+ LLNWGP + + +	
dFat	5045	DGFESSFRGSLSTLVASDDDIANHLSGIYRKANGAASPSATTLGWEYLLNWGPSYENLMG	5104
mFat4	4946	VFKDLASLPE 4955	
		VFKD+A LP+	
dFat	5105	VFKDIAELPD 5114	

Fig. 7.

Homology of the cytoplasmic region at the C-terminus between the mouse Fat4 (mFat4) and the *D. melanogaster* Fat (dFat). Pluses represent similar amino acids. Minuses represent space inserted for optimum alignment.