Oncogene (2007) 26, 7423–7431

www.nature.com/onc

ORIGINAL ARTICLE

Deletion of Tip30 leads to rapid immortalization of murine mammary epithelial cells and ductal hyperplasia in the mammary gland

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Transformation of mammary epithelial cells (MECs) from the normal to the neoplastic stage requires the dysregulation of tumor suppressor genes and proto-oncogenes. Tip30 is a tumor suppressor that can inhibit estrogen receptor-mediated transcription in MECs, but its role in MEC proliferation remains unknown. Here, we show that deleting the *Tip30* gene leads to ductal hyperplasia in mouse mammary glands early in life and extensive mammary hyperplasia with age. *Tip30*$^{-/-}$ mammary glands transplanted into wild-type mammary fat pads also display mammary trees with extensive ductal hyperplasia. Strikingly, *Tip30* deletion promotes proliferation of primary MECs and results in rapid immortalization of MECs *in vitro* relative to wild-type cells. Gene array analysis identified significant increases in the expression of mammary epithelial growth factors Wisp2 and Igf-1 in *Tip30*$^{-/-}$ cells. Knockdown of either Wisp2 or Igf-1 using short interfering RNA dramatically inhibited proliferation of *Tip30*$^{-/-}$ cells. Together, these results suggest that Tip30 is an intrinsic and negative regulator of MEC proliferation partly through the inhibition of Wisp2 and Igf-1 expression, and its absence in the mammary gland may predispose MECs to neoplastic transformation.
Oncogene (2007) **26,** 7423–7431; doi:10.1038/sj.onc.1210548; published online 28 May 2007

Keywords: Tip30; mammary epithelial cell; hyperplasia; proliferation

Introduction

In the mammary gland, the progression of a cell from normal to neoplastic is a multi-step process. Experimental evidence suggests that mouse mammary epithelial cells (MECs) advance through a period of hyperplasia before becoming neoplastic (Medina and Kittrell, 1993; Medina, 2002). This process is facilitated by genetic and epigenetic alterations of proto-oncogenes and tumor suppressor genes that regulate DNA repair, cell growth and apoptosis. As predicted from this concept, several genetically engineered mouse models with activation of a proto-oncogene, or inactivation of a tumor suppressor gene, show MEC hyperplasia occurring early in life, and mammary tumors arising stochastically after a long latency. For instance, mammary glands of Brca1 conditional mutant mice display hyperplastic lesions by 2–10 months of age, but no tumors; after 10–13 months, spontaneous mammary tumors develop (Xu *et al*., 1999). However, other mouse models, such as the cdc25B transgenic and the P-cadherin and Caveolin-1 knockout models, exhibit mammary epithelial hyperplasia that does not progress to mammary tumor formation (Radice *et al*., 1997; Ma *et al*., 1999; Lee *et al*., 2002). The development of hyperplasia before the appearance of mammary cancer suggests that additional genotypic changes are required for cancer progression. Thus, the identification of genes altered in preneoplasia will not only help us understand the molecular basis of mammary gland tumorigenesis but may also present potential diagnostic and chemopreventive targets for the treatment of human breast cancer.

Tip30, also called CC3, is a tumor suppressor that promotes apoptosis and inhibits angiogenesis (Shtivelman, 1997; Xiao *et al*., 1998). Its expression is altered in human prostate, lung, colon and breast cancer (Hewitt *et al*., 2000; Varambally *et al*., 2002; Lee *et al*., 2004; Zhao *et al*., 2006). Recent analysis of Tip30 expression in 87 breast cancer specimens suggests that its expression is inversely associated with metastasis (Zhao *et al*., 2006). We previously reported that Tip30-deficient mice of C57BL6/J and 129SvJ mixed genetic background spontaneously develop tumors by 18–20 months of age (Ito *et al*., 2003). The majority of these tumors occur in female mice, and many of them arise in estrogen-targeted organs (Ito *et al*., 2003). However, the molecular mechanism by which Tip30 regulates tumorigenesis remains unidentified. A previous study suggested that Tip30 functions as an inhibitor of nuclear import (King and Shtivelman, 2004) in cells. Other studies implicated Tip30 as a transcription cofactor (Xiao *et al*., 1998; Jiang *et al*., 2004). A recent study revealed that Tip30 interacts with an estrogen

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Received 13 December 2006; revised 8 April 2007; accepted 19 April 2007; published online 28 May 2007

receptor (ER)-interacting coactivator, called CIA (*c*oactivator *i*ndependent of *A*F2), that is capable of both stimulation and repression of transcription (Jiang *et al*., 2004). Tip30 can dynamically assemble on the promoter of *c-Myc*, an ER-target gene. Tip30 acts as a repressor in ERα-mediated transcription in both estrogen-dependent and independent manners (Jiang *et al*., 2004).

Given the role of Tip30 in the regulation of ER-mediated transcription (Jiang *et al*., 2004), we investigated whether Tip30 played a role in the development and tumorigenesis of the mammary gland in Tip30-knockout mice. Our observations suggest that alteration of Tip30 expression may be an early event that contributes to the progression of MECs from the normal to the neoplastic state.

Results

Loss of Tip30 results in ductal hyperplasia in virgin mice early in life

We analysed the phenotypic consequences of Tip30 deficiency on mammogenesis in genetically engineered mice by examining whole-mount stained mammary glands from 5- and 8-week-old virgin $Tip30^{+/+}$ and $Tip30^{-/-}$ mice. At 5 weeks, $Tip30^{-/-}$ glands appear to have more end terminal buds than their wild-type counterparts (Figures 1c and d). At 8 weeks, ducts of $Tip30^{-/-}$ glands penetrated the mammary fat pad similar to ducts in wild-type glands, indicating that Tip30 is not essential for ductal elongation (Figures 1a and b). Although $Tip30^{-/-}$ glands had a normal composition of epithelial cells with an extended lumen, ducts in

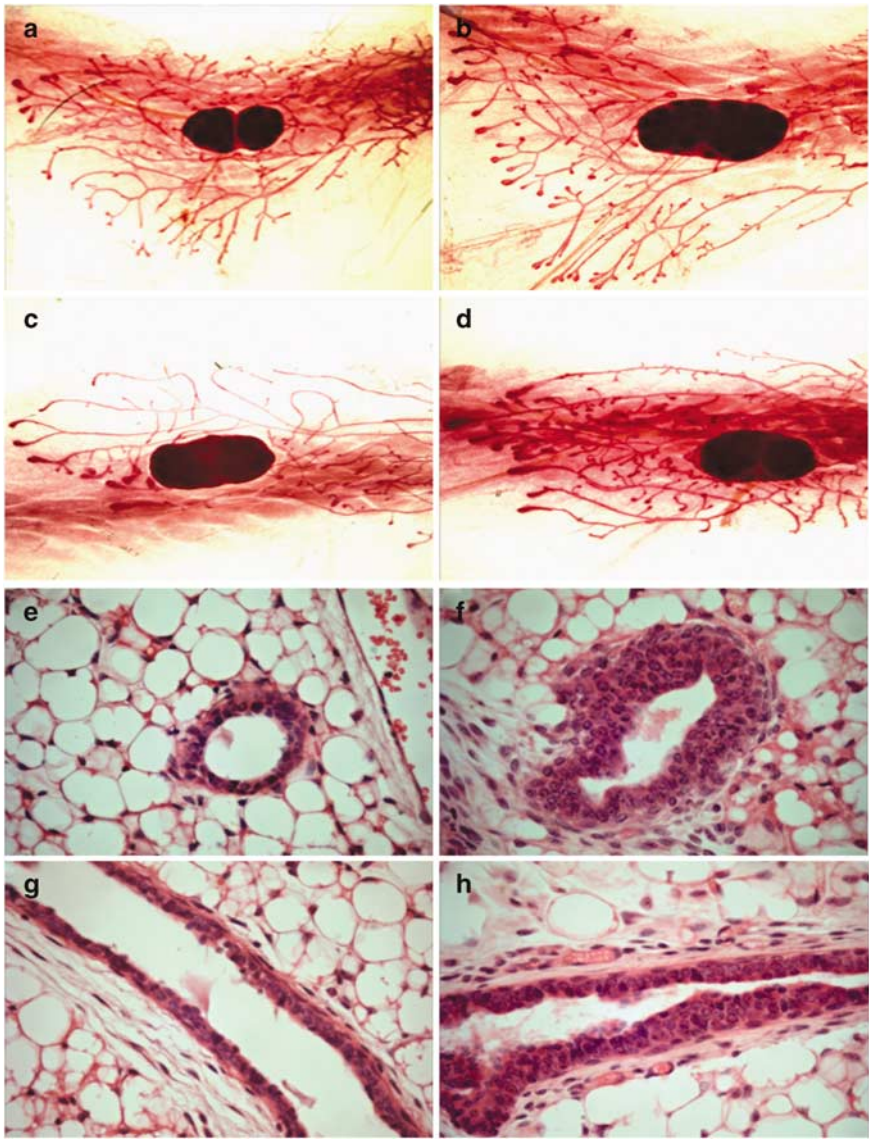

Figure 1 Loss of Tip30 results in enlarged ductal lumens and ductal hyperplasia. Whole-mount analysis of mammary glands from 8-week-old $Tip30^{+/+}$ (**a**) and $Tip30^{-/-}$ (**b**) or 5-week-old $Tip30^{+/+}$ (**c**) and $Tip30^{-/-}$ (**d**) female mice. Hematoxylin and eosin staining of ducts from $Tip30^{+/+}$ (**e** and **g**) and $Tip30^{-/-}$ (**f** and **h**) 8-week-old mammary glands. Original magnification in (**a**–**d**): $\times 40$ and in (**e**–**h**): $\times 400$.

approximately 80% of *Tip30*$^{-/-}$ glands appeared to have a mild hyperplasia characterized by an increased layer of crowded ductal cells and cells focally forming micropapillary fronds (Figures 1f and h). In addition, there is clear evidence of increased mitotic activity and individual necrotic epithelial cells characterized by nuclear karyorrhexis, cytoplasmic eosinophilic change and fragmentation, and association with occasional neutrophils (data not shown). *Tip30*$^{+/-}$ glands did not exhibit ductal hyperplasia observed in *Tip30*$^{-/-}$ glands (data not shown). These results suggest that Tip30 loss results in ductal hyperplasia in mammary glands early in life.

Tip30-*null mice have an increased incidence of alveolar bud formation in the mammary glands with age*

Given the hyperplasia we observed in 8-week-old virgin *Tip30*$^{-/-}$ mice, we next sought to determine the effects of loss of Tip30 on the mammary glands of 1-year-old virgin mice. Whole mounts (Figure 2a) and histological sections (Figure 2c) of glands of C57BL/6, 129SVJ or FVB/C57BL6 (25% FVB/75% C57BL/6) mixed background were scored for the presence of alveolar buds as defined by Cardiff *et al*. (2000). For glands from all genetic backgrounds, 60–67% of the *Tip30*$^{-/-}$ glands displayed a significantly increased number of alveolar buds as compared to wild-type glands (Figure 2b). These data suggest that the loss of Tip30 increases the incidence of alveolar bud formation in the mammary glands of older virgin mice. Histological sections from *Tip30*$^{-/-}$ glands showed an increased number of alveolar buds as compared to wild-type glands (Figure 2c) and the presence of ductal hyperplasia. These data show that in addition to the ductal hyperplasia observed in 8-week-old Tip30-knockout mice, older virgin female Tip30-knockout mice also have an increased incidence of alveolar budding in the mammary glands.

Transplantation experiments demonstrate that Tip30's effects on the mammary gland are intrinsic

To determine whether the observed effects of loss of Tip30 on the mammary glands were due to intrinsic genotypic and phenotypic changes in the mammary gland, pieces of mammary gland from 10-week-old *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mice were transplanted into contralateral cleared fat pads of 21-day-old *Tip30*$^{+/+}$ (Figure 3a) and *Tip30*$^{-/-}$ (Figure 3b) mice. Ten weeks after transplantation, transplanted *Tip30*$^{-/-}$ glands from wild-type and knockout recipients displayed ductal hyperplasia relative to transplanted *Tip30*$^{+/+}$ glands (Figures 3a and b). As with the short-term transplants, *Tip30*$^{-/-}$ glands from wild-type recipients, 1-year post-transplantation, displayed ductal hyperplasia relative to

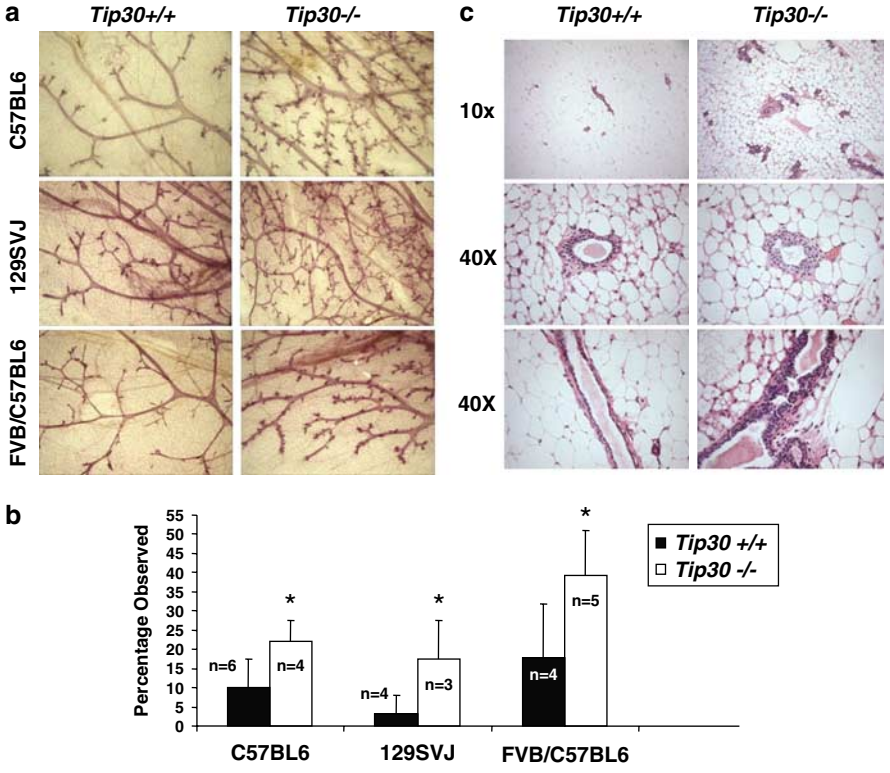

Figure 2 *Tip30*-null mice have an increased incidence of alveolar buds in the mammary glands. Mammary glands were prepared from 1-year-old virgin female mice in diestrus. One mammary gland no. 4 per mouse was used for mammary whole mount or histological analysis. (**a**) Whole-mount analysis of mammary glands from C57BL6, 129SV and FVB/C57BL6 *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ mice. (**b**) Percentage of alveolar budding in mammary glands. *Tip30*$^{-/-}$ mice with mammary hyperplasia were counted. $N =$ the number of mice. **P*-value of $\leqslant 0.05$ (Student's *t*-test). (**c**) Hematoxylin and eosin-stained sections of mammary glands from *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ female mice.

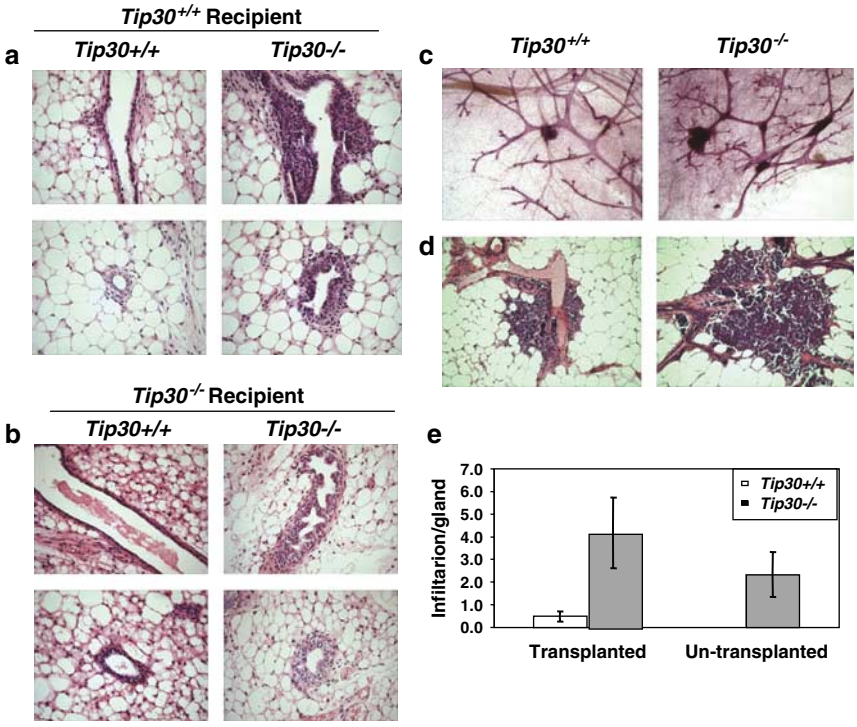

Figure 3 Effects of Tip30 on the mammary gland are intrinsic. Histological analysis of $Tip30^{+/+}$ and $Tip30^{-/-}$ mammary glands 10 weeks after transplantation into $Tip30^{+/+}$ (**a**) and $Tip30^{-/-}$ (**b**) recipients. (**c**) Whole-mount image of infiltrating lymphocytes observed in mammary glands 1 year post-transplantation into wild-type recipients. (**d**) Hematoxylin and eosin-stained sections of infiltrating lymphocytes observed in mammary glands 1 year post-transplantation into wild-type recipients. (**e**) Loss of Tip30 results in an increased incidence of lymphocyte infiltrates in the mammary glands. The number of lymphocyte infiltrations was counted in each mammary gland from $Tip30^{+/+}$ and $Tip30^{-/-}$ mice ($n=6$). First two columns: mammary glands harvested 1-year post-transplant from wild-type recipients. The third column: mammary glands from aged-matched $Tip30^{-/-}$ untransplanted mice. $Tip30^{+/+}$ mammary glands from aged-matched mice do not exhibit the nodule of lymphocyte infiltration. *P value of <0.05 as determined by Student's t-test.

transplanted $Tip30^{+/+}$ glands (data not shown). There was also increased alveolar budding in the transplanted $Tip30^{-/-}$ glands as compared to $Tip30^{+/+}$ transplants (data not shown). In addition, the nodules of lymphocyte infiltration were observed near some blood vessels and ducts in both the wild-type and knockout transplanted glands (Figures 3c and d), whereas they appeared larger in size and occurred much more frequently in the transplanted knockout glands (Figures 3d and e). This infiltration of lymphocytes around the ducts and blood vessels was also observed in the glands of untransplanted 1-year-old $Tip30^{-/-}$ mice, but not in the glands of their wild-type littermates (Figure 3e). These results suggested that the effects of Tip30 deficiency on mammary gland alveolar morphology and ductal hyperplasia are intrinsic to the gland itself.

Loss of Tip30 increases MEC proliferation

To assess Tip30's role in MEC proliferation, we used *in vivo* 5-bromodeoxyuridine (BrdU) labeling in 8-week-old virgin $Tip30^{+/+}$ and $Tip30^{-/-}$ mice (Figure 4a). An increase (12%) in labeled MECs was observed in the $Tip30^{-/-}$ glands, whereas few (1%) BrdU-labeled MECs were observed in wild-type glands. Additionally, apoptosis did not increase in Tip30-knockout glands as determined by the *in situ* TdT-mediated dNTP nick end labeling assay (data not shown). Thus, $Tip30^{-/-}$ MECs are more proliferative than $Tip30^{+/+}$ epithelial cells *in vivo*. To further verify this, we examined the effect of Tip30 loss on the growth of primary MECs *in vitro*. MECs from virgin 8-week-old $Tip30^{-/-}$ and $Tip30^{+/+}$ mice from the same litters were isolated and confirmed by *in situ* immunostaining of E-cadherin (data not shown), and their growth rates were compared. We found that $Tip30^{-/-}$ cells grew much faster than $Tip30^{+/+}$ cells as determined by both viable cell counting and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Figure 4b). This result suggests that loss of Tip30 promotes proliferation of MECs.

Deletion of the Tip30 *gene, a single step, results in a rapid immortalization of mouse MECs*

Mouse primary MECs in culture undergo crisis after 3–5 passages, during which time they expand very slowly. After crisis, which can last several weeks, immortalized cells, which proliferate readily, begin to appear (Medina and Kittrell, 2000). While working with wild-type and *Tip30*-null MECs in culture, we observed that $Tip30^{-/-}$ cells continued to proliferate even after the wild-type cells had entered the crisis stage (Figure 5a). Thus, we constructed population doubling curves for $Tip30^{+/+}$ and $Tip30^{-/-}$ MECs. Proliferation of wild-type MECs

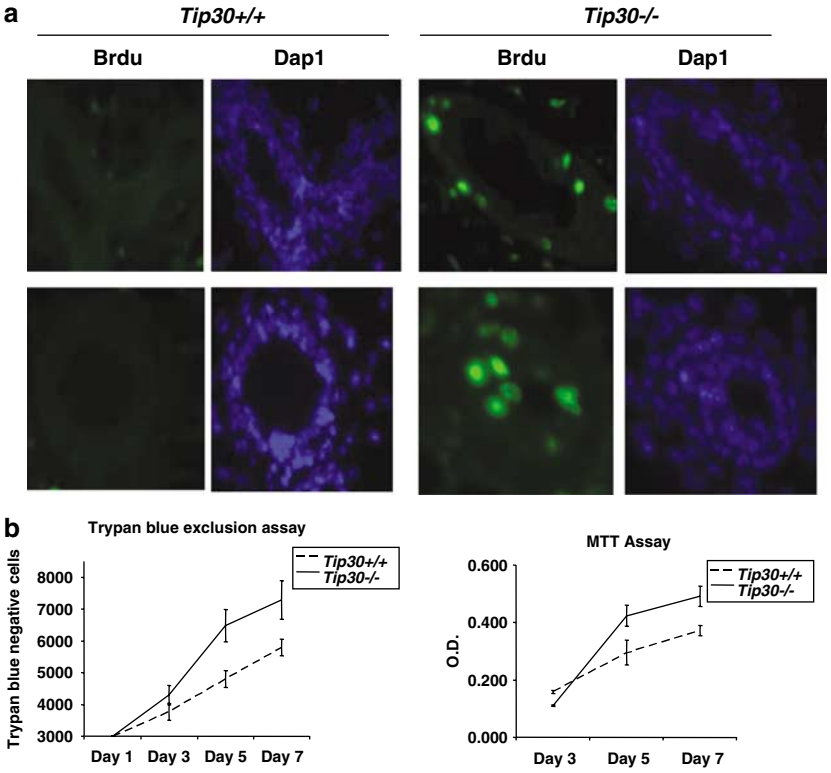

Figure 4 Loss of Tip30 increases proliferation of MECs. (**a**) BrdU labeling and DAPI (4′,6-diamidino-2-phenylindole) staining of wild-type and *Tip30*-null mammary glands from 8-week-old virgin female mice. (**b**) Cell counts of MECs using trypan blue dye (left panel) and MTT assay of MECs (right panel). All data points represent three independent samples. BrdU, 5-bromodeoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; MEC, mammary epithelial cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

slowed dramatically after 9 days in culture (that is, seven population doublings, Figure 5b), and after 16 days, ceased proliferating altogether for 4 weeks. After 7 weeks, immortalized clones began to arise; the growth rate of these post-crisis wild-type MECs was slower than the pre-crisis MECs until 2 additional weeks had passed. In contrast, $Tip30^{-/-}$ MECs, while showing a decrease in population doublings during week 3 (that is, population doublings 9–10), did not stop proliferating for any length of time. At 2 weeks post-crisis, the $Tip30^{-/-}$ MECs were proliferating at more than twice the rate of pre-crisis $Tip30^{-/-}$ MECs. Significantly, $Tip30^{-/-}$ MECs have doubled over 340 times *in vitro*, whereas $Tip30^{+/+}$ MECs have undergone only 120 population doublings. These data suggest that loss of Tip30 promotes rapid immortalization of MECs in culture, and that $Tip30^{-/-}$ MECs maintain their growth advantage over wild-type MECs even after long periods in culture. Although it is likely that those Tip30-null MECs were immortalized *in vitro*, we are not sure that those were immortalized *in vivo*. To further confirm, this would require *in vivo* serial transplantation studies.

Expression of two growth factors, Wisp2 and Igf-1, is increased in Tip30$^{-/-}$ *MECs and knockdown of their expression inhibits proliferation*

We used Affymetrix GeneChip assays to compare expression profiles in $Tip30^{+/+}$ and $Tip30^{-/-}$ MECs.

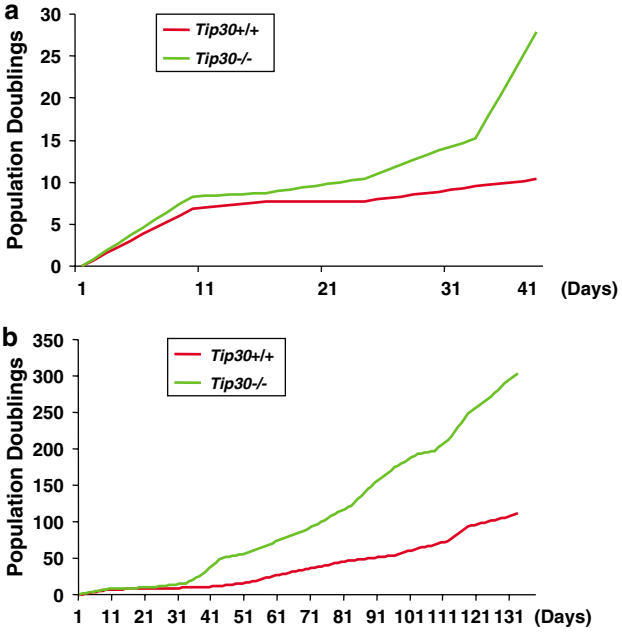

Figure 5 Tip30-null MECs rapidly immortalize *in vitro*. Population doubling curves for $Tip30^{+/+}$ and $Tip30^{-/-}$ MECs *in vitro*. Viable cells were counted every 3 days and then replated at lower density. The results represent the mean of three independent experiments. Similar results were obtained from three pairs of $Tip30^{+/+}$ and $Tip30^{-/-}$ mice. (**a**) Population doublings through day 41. (**b**) Population doublings through day 131.

Table 1 Summary of Affymetrix Genechip analysis

Fold change	*Name of protein/gene*
3.73	insulin-like growth factor 1
3.48	WNT1 inducible signaling pathway protein 2
2.83	early growth response 1
2.64	insulin-like growth factor binding protein 3
2.46	platelet-derived growth factor receptor, alpha polypeptide
2.3	Vascular endothelial growth factor-D
2.3	S100 calcium binding protein A4
2.14	adrenomedullin
2	matrix metalloproteinase 3
1.87	fibroblast growth factor 7
1.87	response gene to complement 32
1.74	fibulin 1
1.74	lysyl oxidase-like 4
1.74	KIT ligand
1.74	hepatocyte growth factor
1.74	interleukin 6 signal transducer
1.62	transferrin receptor
1.62	chemokine (C-X-C motif) ligand 12
1.62	fibroblast growth factor receptor 2
1.62	Kruppel-like factor 4 (gut)
−1.62	transforming growth factor, beta 2
−1.62	serine protease inhibitor, Kunitz type, 2
−1.62	inhibitor of DNA binding 2
−1.62	RAD51 homolog
−1.62	P53 apoptosis effector related to PMP-22
−1.74	retinoblastoma-like 1 (p107)
−1.74	roundabout homolog 1

Of the 20 genes that had mRNA levels increase by more than 1.6-fold (Table 1), the expression of two mammary gland growth factors, Wisp2 and Igf-1, was increased approximately 3.5- to 3.7-fold, respectively, in *Tip30*$^{-/-}$ MECs. Therefore, *Igf-1* and *Wisp2* mRNA levels were measured in *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ MECs by quantitative real-time (qRT)–PCR. As expected, *Tip30* mRNA was detected in the *Tip30*$^{+/+}$ MECs, but not in *Tip30*$^{-/-}$ MECs (Figure 6a). The levels of *Wisp2* and *Igf-1* mRNA were increased by 39.7- and 3.52-fold, respectively, in *Tip30*$^{-/-}$ MECs (Figure 6a, left and right panel). The result for *p107* was also in agreement with the microarray results, showing a 4.35-fold reduction in mRNA level in the *Tip30*$^{-/-}$ samples as compared to the *Tip30*$^{+/+}$ samples (Figure 6a, right panel). There was no significant change in ERα expression in *Tip30*$^{-/-}$ mammary glands by immunohistochemical analysis (Figure 1 of Supplementary Data).

To address whether increased Wisp2 or Igf-1 expression was responsible for the higher proliferative rate of *Tip30*$^{-/-}$ MECs, short interfering RNA (siRNA) knockdown experiments were performed to target either *Wisp2* or *Igf-1* mRNA. Targeted siRNA reduced *Wisp2* or *Igf-1* mRNA levels by approximately 90% relative to *Tip30*$^{-/-}$ cells treated with either transfection reagent only or with nonspecific siRNA, as determined by qRT–PCR (Figures 6b and c). Moreover, *Tip30*$^{-/-}$ MECs treated with either *Wisp2* or *Igf-1* siRNA had decreased cell numbers by 50–60% relative to control *Tip30*$^{-/-}$ cells following a 3-day proliferation assay (Figure 6d). These data suggest that both Igf-1 and Wisp2 play significant roles in the cell proliferative capacity of

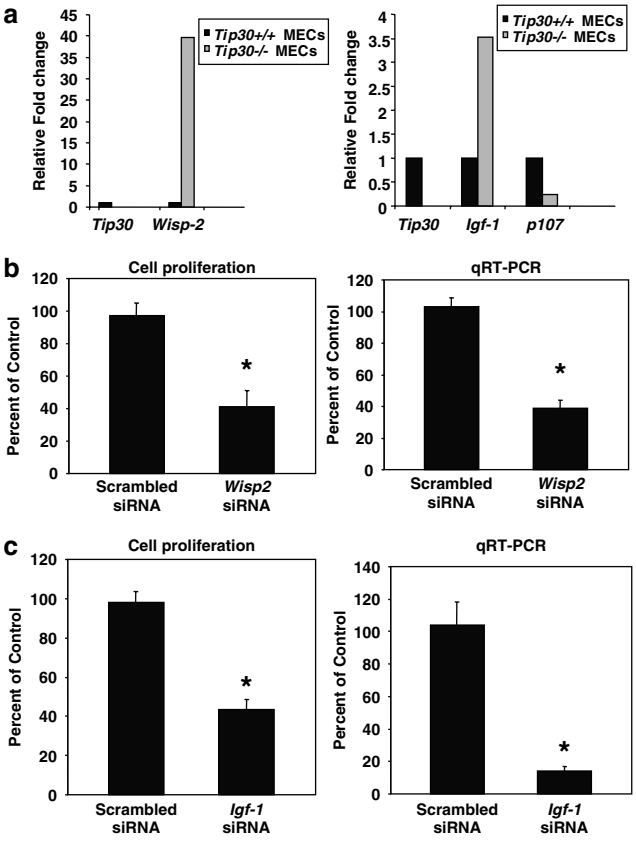

Figure 6 (**a**) Loss of Tip30 results in the dysregulation of multiple genes. Total cDNA was prepared from *Tip30*$^{+/+}$ and *Tip30*$^{-/-}$ MECs and subjected to qRT–PCR. Bars represent the relative amount of amplified target gene after normalization to β-actin. Left panel: *Tip30* and *Wisp2* expression. Right panel: *Tip30*, *Igf-1* and *p107* expression. Experiments were performed in triplicate. (**b** and **c**) Knockdown of *Wisp2* or *Igf-1* mRNA decreases cell proliferation *in vitro*. For qRT–PCR, bars represent the relative amount of amplified target gene after normalization to β-actin. For cell proliferation assays, bars represent the percentage of cells in treatment dishes relative to control cell numbers. Each data point is the mean of two experiments performed in triplicate. (**b**) Wisp2 knockdown assay. Left panel: *Wisp2* cell proliferation assay. Right panel: qRT–PCR analysis. (**c**) Igf-1 knockdown assay. Left panel: *Igf-1* cell proliferation assay. Right panel: qRT–PCR analysis. **P* value of <0.05 as determined by Student's *t*-test. qRT–PCR, reverse transcriptase–PCR.

Tip30$^{-/-}$ cells, and that Tip30, in part, regulates MEC cell proliferation by directly or indirectly modulating the expression of Igf-1 and Wisp2.

Discussion

Tip30 is a negative regulator of MEC proliferation

We demonstrate here that the loss of Tip30 in the mammary glands of virgin mice leads to rapid immortalization of MECs *in vitro* and ductal hyperplasia *in vivo*. We also show that *Tip30*$^{-/-}$ MECs proliferate faster than *Tip30*$^{+/+}$ MECs both *in vivo* and *ex vivo*, as demonstrated by BrdU labeling, viable cell counting and MTT assays. Moreover, loss of Tip30 results in an increased incidence of alveolar budding and lymphocyte

infiltration in the mammary glands of older virgin mice that becomes progressively worse as the mouse ages. We do not know why loss of Tip30 results in lymphocyte infiltration in the mammary glands. We speculate that infiltrating lymphocytes might be responding to preneoplastic phenotypic changes in Tip30-null MECs as a result of hyperplasia. The effects of Tip30 described here are not due to parenchymal or humoral factors, as shown in transplantation experiments and in *ex vivo* MEC proliferation assays. Tip30, therefore, plays an important role in the regulation of MEC proliferation and immortalization, and may contribute to the suppression of mammary tumorigenesis. We have also monitored tumor development in a cohort of *Tip30* wild-type, heterozygous and homozygous C57BL6 mice for 21 months. We found that only 1 of 31 $Tip30^{+/-}$ female mice developed mammary ductal adenocarcinoma and one developed mammary liposarcoma. Sixteen of 30 (52%) $Tip30^{-/-}$ female mice developed lymphoma and other diseases (unpublished data). The low incidence of mammary tumors in these mice is not surprising given that C57BL6 mice are less susceptible to mammary tumorigenesis (Ullrich *et al*., 1996). In addition, Tip30-knockout mice often die prematurely due to the development of lymphomas and other diseases, thereby possibly reducing the incidence of mammary tumors in Tip30-knockout mice.

Preneoplastic MECs may advance through stages of immortality and hyperplasia before becoming neoplastic (Medina and Kittrell, 1993; Medina, 2002). The properties of immortality, hyperplasia and increased neoplastic potential have been demonstrated to be independent of one another. Characterization of four ductal mammary epithelial outgrowth lines by Medina and Kittrell (1993) showed these lines to be immortal, but morphologically normal and non-tumorigenic. Genes such as *c-Myc*, *ZNF217* and *Bmi-1*, when overexpressed *in vitro* can immortalize human MEC (Wang *et al*., 1998; Nonet *et al*., 2001; Dimri *et al*., 2002). Since loss of Tip30 results in increased expression of *c-Myc* in murine MECs (Jiang *et al*., 2004), we propose that increased c-Myc expression could contribute to both the hyperplastic and immortal phenotypes in the mammary gland. Our microarray analysis of $Tip30^{+/+}$ and $Tip30^{-/-}$ MECs did not reveal elevated c-Myc expression as previously demonstrated in $Tip30^{+/+}$ and $Tip30^{-/-}$ mammary glands (Jiang *et al*., 2004). This was likely due to the lack of estrogen in the culture medium used to grow wild-type and knockout MECs, thereby reducing the effects of Tip30 loss on estrogen-induced c-Myc expression (Jiang *et al*., 2004). Therefore, deletion of *Tip30* is able to contribute to both the immortality of MECs in *ex vivo* and hyperplasia of MECs *in vivo*. This could be due, in part, to the ability of Tip30 to repress *c-Myc* transcription as well as repress Wisp2 and Igf-1 expression. Consistent with this view, it was reported that knockdown of Wisp2 mRNA levels or the mRNA levels of components of the Igf-1 signaling pathway, such as 14-3-3 sigma protein, ERα, AIB1 (amplified in breast cancer 1), significantly reduces the proliferative capacity of MCF-7 breast cancer cells (Banerjee *et al*., 2003; Oh *et al*., 2004; Zhang *et al*., 2004, 2005). Moreover, *Wisp2* mRNA and protein expression is only detected in human breast cancer and not in normal human breast (Saxena *et al*., 2001). The data presented herein suggest that Tip30 could play a significant role in regulating Igf-1 and/or Wisp2 expression. Thus, Tip30 is able to contribute to the appearance of two preneoplastic phenotypes, immortality and hyperplasia. It therefore may play an important role in the progression of MECs from normal to preneoplastic, and possibly predispose them to tumor formation.

In conclusion, our data provide the first evidence that Tip30 is a new negative factor in the regulation of MEC proliferation and may represent a novel signal pathway in the suppression of breast cancer development.

Materials and methods

Mice and MECs
The genetic background of $Tip30^{+/+}$ and $Tip30^{-/-}$ mice was regarded to be identical since they were backcrossed 10 times with C57BL/6 mice. Primary MECs of each genotype were prepared from 8- to 10-week-old mice and maintained in culture, as described previously (Medina and Kittrell, 2000).

Cell culture
Cell growth by trypan blue exclusion A total of 3000 MECs of each genotype were seeded into 96-well plates and counted every other day in triplicate for 5 days using trypan blue dye.

Cell growth by MTT assay A total of 5000 MECs of each genotype were seeded into 24-well plates and cell viability measured every other day in triplicate for 5 days using MTT assay. Briefly, cells were incubated with 0.5 mg/ml MTT for 3 h at 37°C, and absorbance was measured at 584 nm using a FLUOstar OPTIMA spectrophotometer.

Population doublings Population doublings were determined by plating MECs (5×10^4 cells/well) in triplicate into six-well plates. When cells reached 80% confluency, they were counted and replated into new six-well plates (5×10^4 cells/well). Population doublings were calculated by considering the number of cells at splitting and the number of times they had been passaged.

Estrous stage determination
Estrous cycle stage was determined by cytological evaluation of vaginal smears using the Protocol Hema 3 stain set (122-911, Fisher Scientific, Pittsburgh, PA, USA), according to the manufacturer's instructions.

Mammary transplantation
Pieces of 10-week-old wild-type and $Tip30^{-/-}$ no. 4 mammary glands were transplanted individually into contralateral cleared fat pads of 21-day-old wild-type and $Tip30^{-/-}$ mice (Shillingford *et al*., 2002). Transplanted glands were harvested at 10 weeks or 1 year after transplantation.

Mammary whole mount, histology and BrdU labeling
Whole mounts and BrdU labeling of mammary gland no. 4 were performed in 8-week virgin mice according to Rosen, Baylor College of Medicine (Houston, TX, USA) (http://public.bcm.tmc.edu/rosenlab). Three to five mice of each

genotype were used and approximately 350 epithelial cells per gland were counted for BrdU staining.

qRT–PCR
Standard curves were constructed using serial dilutions of plasmids containing the target sequence. qRT–PCR was performed using the Cepheid SmartCycle with SYBR green fluorescence detection as described in the Supplementary Materials and methods.

siRNA knockdown assays
Target-specific siRNA for Igf-1 and Wisp2 and non-targeting siRNA (nonspecific control) were purchased from Dharmacon (Lafayette, CO, USA) and used at 100 nM concentrations according to the manufacturer's protocol. qRT–PCR and proliferation assays are described in the Supplementary Materials and methods.

Acknowledgements

We thank Drs C-Wu and Y Qin for their histopathological expertise, J Wahl for providing E-cadherin antibodies. We also thank C Eischen for her generous help and advice and S Haslam for critical reading of the paper. This work was supported by grants from National Institute of Health Grants DK066110-01 and American Cancer Society Grant RSG0216501GMC to HX. WT was supported by Department of Defense postdoctoral traineeship award, DAMD17-001-0361. JP was supported by an NIH graduate student fellowship.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).