# Adhesion signaling in mammary gland development, tumorgenesis and progression

Miltenburg, M.H.A.M.

# Chapter 4

## FAK is required for p53 R270H-induced mammary tumorigenesis

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# FAK is required for p53 R270H-induced mammary tumorigenesis

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**Abstract**

A majority of breast cancers show alterations in the p53 tumor suppressor gene. Li-Fraumeni syndrome patients carry a germ line mutation in p53 gene and as a result develop early onset tumors, including breast cancer. In addition to mutations in the p53 gene, elevated levels of focal adhesion kinase (FAK) are frequently found in breast cancer. Wild type p53 binds to the promoter region of FAK thereby suppressing its transcription. Since p53 point mutations often result in dominant-negative inhibition of wild type p53 function, we hypothesized that due to the lack of p53 wild type function, the FAK promoter is no longer suppressed by p53. Likewise enhanced levels of FAK may support tumorigenesis in a p53 mutant background. To study the role of FAK in p53R270H-induced mammary tumorigenesis we used conditional expression of p53 point mutation R270H, the mouse equivalent to human R273H, in combination with conditional deletion of FAK, We show that deletion of FAK reduces the incidence of p53R270H-induced mammary gland tumors. Since the majority of mammary glands with reduced expression of FAK showed mammary gland hyperplasia, FAK seems important for the progression of p53R270H-induced hyperplasia into carcinomas.

## Introduction

The tumor suppressor gene p53 has been implicated in diverse biological processes such as apoptosis, cell-cycle arrest, DNA repair, cellular differentiation and senescence; and p53 status is altered in over 50% of spontaneous tumors in humans (1-3). In addition to spontaneous tumors, germ-line mutations in p53 are observed in Li-Fraumeni patients which predisposes them to a wide spectrum of early-onset cancers (4-6). Normally, p53 is expressed in a latent form and is maintained at low levels through targeted degradation. However, in response to DNA damage or other forms of cellular stress, p53 is activated to function as a transcription factor, resulting in a cascade of events that eventually prevents tumor development (1). P53 is the most frequently mutated gene in spontaneous breast tumors. Approximately 30% of sporadic breast tumors acquire p53 mutations, which are related to breast cancer progression and resistance to doxorubicin therapy in breast cancer patients (6, 7). Hot-spot mutations are frequently found in sporadic breast cancer and often accompanied by loss of heterozygosity (LOH). Higher frequency of *p53* mutations are found in patients with advanced disease and the incidence of *p53* mutations is higher in recurrent tumors than in the primary ones (8), indicating that acquiring *p53* mutations in breast cancer predisposes to increased tumor malignancy.

Focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase, is located in the cell-matrix adhesions where it integrates signals from integrins and growth factor receptors to coordinate and control processes such as actin cytoskeleton dynamics and migration. FAK expression is elevated in numerous cancers including colon-, cervix- and breast cancer (9-12) often associated with gene amplification (13, 14). In several recent publications a role has been shown for FAK in breast cancer development and progression mostly using the MMTV-PyMT/PyVT model to induce mammary tumors (15-18). FAK deletion in these models reduced the incidence of MMTV-PyMT/PyVT mammary tumors, suggesting an important role for FAK in breast tumor development. Though these studies have implicated an important role for FAK in breast cancer progression, the PyMT mouse model for breast cancer does not mimic the natural course of mammary tumorigenesis. Development of cancer is a multistep process; characterized by genomic instability and mutations, which can finally give rise to cancer. In the time frame in which the PyMT model induces mammary tumors, approximately within 10 weeks, these naturally occurring transformations can not take place. We used a conditional mouse model mammary gland specific expression of mutant p53 which induces mammary tumorigenesis after approximately 40 weeks, making this a more clinically relevant model.

In human breast cancer specimens complete loss of p53 is rarely found but hot-spot p53 mutations occur frequently in human breast cancers. P53 has been shown to affect FAK levels by binding to the N-terminal region and the

promoter region of FAK to inhibit the transcription of FAK (19). Vice versa, FAK facilitates p53 turnover via enhanced mdm2-dependent p53 ubiquitination, and subsequent proteosomal degradation leading to inactivation of p53 (20). Increased FAK expression is correlated with p53 mutations in human breast cancer (21). However, the role of FAK in p53-mutant induced mammary tumorigenesis has not been defined.

Targeted point mutations of p53 lead to dominant-negative inhibition of wild-type p53 function. Here, we made use of p53 R270H point mutation; the mouse equivalent of human R273H mutation, to study the role of FAK in p53 R270H-induced mammary tumorigenesis. Expression of mammary gland–specific p53.R270H mutation has been shown to mimic human breast cancer development (22). We crossed $p53^{R270H/wt}$ mice with $FAK^{lox/-}$, and $FAK^{lox/Wt}$, in a WapCre background. Upon pregnancy and weaning the WapCre promoter is activated leading to the deletion of the stop codon in front of the hot spot p53 mutation 270, resulting in expression of the R270H mutant and to the deletion of the FAK allele that is flanked by loxP sites. Here we show that loss of FAK does not affect tumor initiation, whereas FAK is essential for the progression of mammary epithelial hyperplasia to breast cancer.

## Results

### Mammary gland-specific Fak deletion inhibits p53.R270H-induced breast tumor formation

FAK gene knockout in mice results in an embryonic lethal phenotype at day E8.5 due to defects in the axial mesodermal tissues and cardiovascular system (23, 24). To study the effect of mammary-specific loss of FAK in mammary tumorigenesis we generated mice with WapCre-mediated conditional expression of p53.R270H and conditional deletion of FAK (Fig. 1). We generated $FAK^{lox/wt}$ and $FAK^{lox/-}$, $FAK^{lox/wt}$ resulting in FAK heterozygous after recombination, and $FAK^{lox/-}$ resulting in complete FAK knockout in the mammary gland (Fig. 1).

To evaluate the role of FAK in p53R270H-induced mammary tumorigenesis we subjected female $p53^{R270H/wt}/FAK^{lox/wt}$/WapCre (referred to as $FAK^{lox/wt}$) and $p53^{R270H/wt}/FAK^{lox/-}$/WapCre (referred to as $FAK^{lox/-}$) to one pregnancy to mediate WapCre-mediated expression of p53.R270H point mutation and deletion of the FAK allele that is flanked by loxP sites, and subsequently followed the mice for the development of mammary tumors for a total time period of 70 weeks. The first onset of mammary tumors was at 38 weeks for $FAK^{lox/wt}$ and 39 weeks for $FAK^{lox/-}$ after induction of the p53.R270H point mutation and overall survival is similar (Fig. 2A). Though the onset of mammary tumors was comparable, only few mammary tumors developed in $FAK^{lox/-}$ mice, which all developed within 50 weeks. $FAK^{lox/wt}$ mice were more susceptible for mammary tumor development than $FAK^{lox/-}$ mice (Fig. 2B).

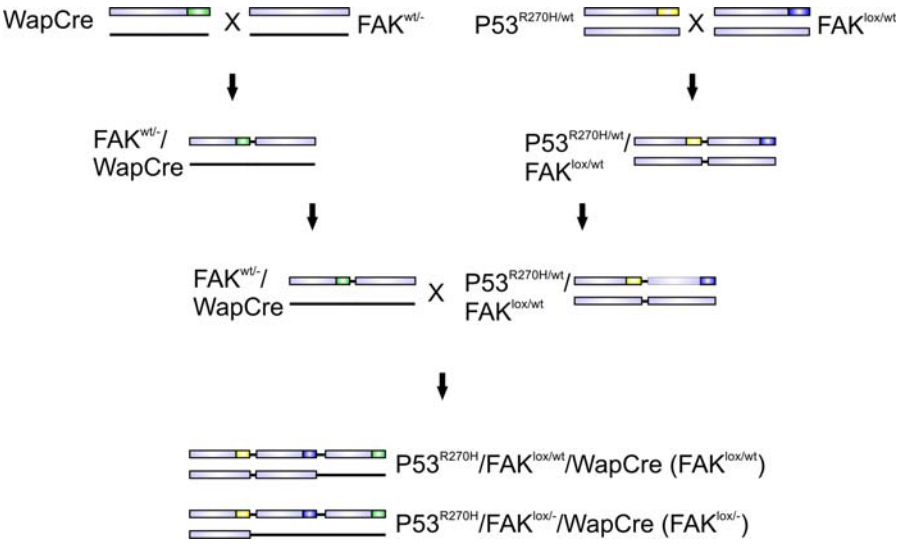


**Figure 1. Generation of mice**
Schematic representation of all the breeding steps necessary for the generation of $p53^{R270H}/FAK^{lox/wt}$/WapCre ($FAK^{lox/wt}$) and $p53^{R270H}/FAK^{lox/-}$/WapCre ($FAK^{lox/-}$) mice. P53R270H indicated by yellow bar, FAK lox indicated by blue bar, and WapCre is indicated by a green bar. Breeding overview of the generation of $p53^{lox/lox}/FAK^{lox/wt}$/WapCre mice can be found in chapter 3.

The number of mammary tumor-bearing mice was significantly higher in $FAK^{lox/wt}$ mice. Sixty-nine percent (9 of a total of 13 mice) of $FAK^{lox/wt}$ mice were tumor-bearing, all bearing one or more mammary gland tumors, with a mean latency of 59 $\pm$ 9 weeks (Fig. 2C and table 1). In contrast, only 21 percent (4 of a total of 19 mice) of $FAK^{lox/-}$ mice developed mammary tumors, with a mean latency of 81 $\pm$ 4 weeks, representing a significant difference in tumor formation ($p<0.05$, Fig. 2B). Most mammary gland tumors developed in the $4^{th}$ or $5^{th}$ mammary gland, 67% in $FAK^{lox/wt}$ and 50% in $FAK^{lox/-}$ mice.

To determine whether the induction of WapCre leads to mammary specific removal of the translational stop cassette and subsequent expression of the p53.R270H protein and deletion of FAK, we performed PCR analysis on mammary gland tumors that were isolated from $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice (Fig. 3A and 3B, respectively). Immunohistochemical staining of $FAK^{lox/wt}$ and $FAK^{lox/-}$ mammary glands and mammary tumors with the CM5 clone revealed CM5-positive cells, suggesting accumulation of p53 in these cells, indicating that the p53.R270H point mutant is detectable at the protein level in mammary glands and tumors of both $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice (Fig. 3C).

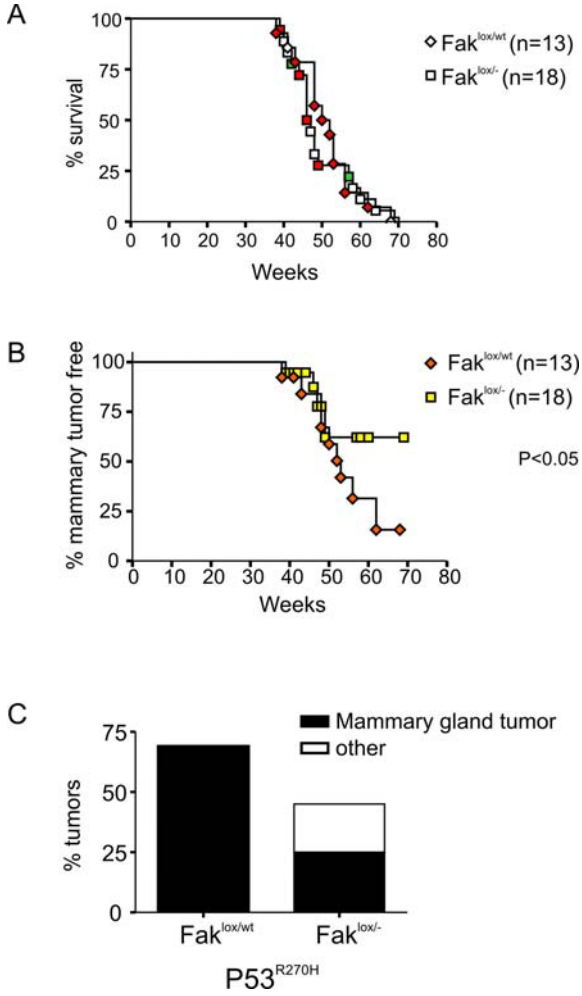


**Figure 2. Focal adhesion kinase is involved in progression of p53$^{R270H}$-induced breast tumors**
Female p53$^{R270H}$/FAK$^{lox/wt}$/WapCre (FAK$^{lox/wt}$) and p53$^{R270H}$/FAK$^{lox/-}$/WapCre (FAK$^{lox/-}$) mice were subjected to one pregnancy to activate WapCre resulting in expression of p53$^{R270H}$ point mutation and deletion of the floxed *Fak* allele. Mice were followed for the development of mammary tumors for a total time period of 70 weeks. (A) Overall survival of FAK$^{lox/wt}$ (n=13) and FAK$^{lox/-}$ (n=19) mice. Red indicates mammary tumors, green indicates other tumors, and white indicates non-tumor related deaths. (B) Mammary tumor-free survival curves of FAK$^{lox/wt}$ and (n=13) FAK$^{lox/-}$ (n=19) mice. Time is depicted in weeks after p53.R270H expression, set at time of birth of litters. Statistical analysis was performed using Student's t-test (*<0.05). (C) Schematic representation of FAK$^{lox/wt}$ and FAK$^{lox/-}$ tumor bearing mice. Black bars indicate mammary gland tumors. White bars indicate other tumor types.

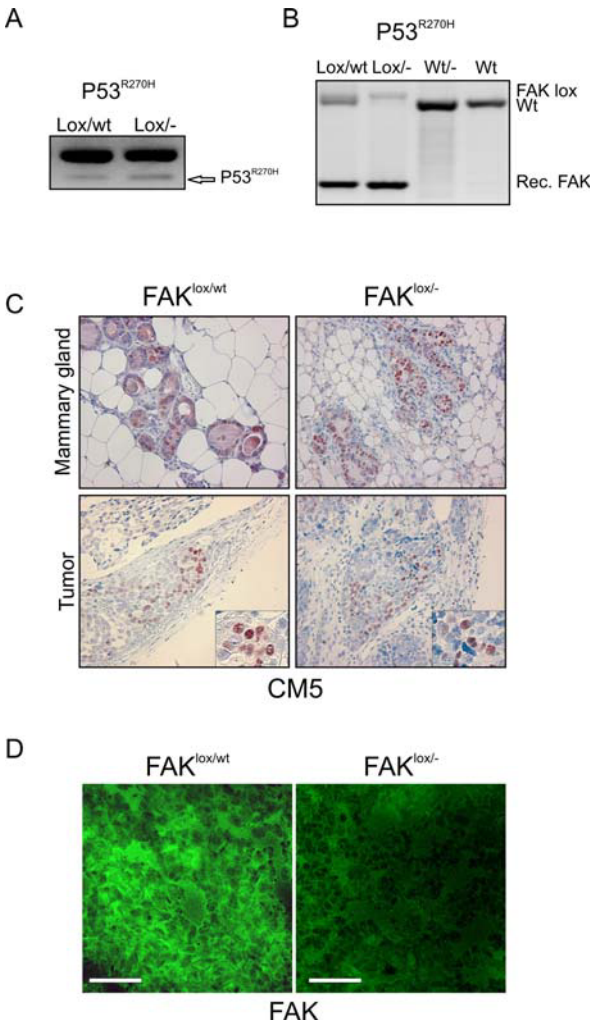


**Figure 3. Mammary gland-specific expression of p53.R270H and deletion of FAK**
RNA and DNA was isolated from mammary tumors and used for RT-PCR and PCR analysis to determine the expression of p53.R270H point mutation and recombination of the *Fak* gene, respectively. (A) Expression of the p53.R270H point mutation in mammary gland tissue of $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice. (B) *Fak* gene deletion in mammary gland tissue from $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice. $p53^{R270H}/FAK^{wt/wt}$/WapCre ($FAK^{wt/wt}$) and $p53^{R270H}/FAK^{wt/-}$/WapCre ($FAK^{wt/-}$) mice were used as negative control for recombination of the *Fak* gene. (C) p53 mutant expression was confirmed by CM5 staining on formalin-fixed paraffin embedded mammary gland (upper panel) and tumor tissue (bottom panel) of $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice. (D) Cryo-sections of tumor tissue isolated from $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice were stained for FAK.

In addition, FAK staining on cryosections of the isolated tumors showed a marked reduction in FAK protein levels in $FAK^{lox/-}$ tumors (Fig. 3D), indicating that in our model FAK is successfully deleted. Mammary tumors and (contra-lateral) 4th non-tumor bearing mammary glands were stained for hematoxylin/eosin and analyzed for mammary gland hyperplasia and tumor type, respectively (Fig. 4 and table 1). Solid carcinomas were isolated from both $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice. Two out of 9 tumors isolated from $FAK^{lox/wt}$ mice were adenocarcinomas (data not shown). None of the 4 mammary tumors that developed in $FAK^{lox/-}$ mice were adenocarcinomas.

| | $P53^{R270H}/FAK^{lox/Wt}$ | $P53^{R270H}/FAK^{lox/-}$ |
|---|---|---|
| Total number of mice analyzed | 13 | 19 |
| Tumor-bearing mice | 9 (69%) | 9 (45%) |
| Mammary gland tumor | 9 (69%) | 4 (20%) |
| - Carcinoma* | 9 | 4 |
| Mammary gland hyperplasia | 1 | 4 |
| Lymphoma | 0 | 1 |
| Other# | 0 | 5 |
| Mean mammary gland tumor latency (wks)§ | 51 ± 8.3 | 48 ± 8.3 |

**Tabel 1. Tumor spectrum in $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice**
* Carcinomas could be classified in adenocarcinoma and solid carcinomas.
# Other tumors were found in lung, bone, and colon.
§ Mean latency time is depicted as the number of weeks after expression of the $p53^{R270H}$ mutation.

**P53.R270H mutant induces mammary tumors with a basal-like character**

Expression of p53.R270H is initiated by activation of WapCre which is predominantly activated in luminal epithelial cells of the mammary gland. To evaluate if p53.R270H expression in luminal epithelial cells results in expansion of the luminal epithelial cell compartment and subsequent tumor formation, we stained mammary glands and tumors from $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice for luminal marker cytokeratin 8 (CK8) and myoepithelial marker cytokeratin 5 (CK5). Interestingly, though staining on contra lateral non-tumor bearing mammary glands indicated that the CK8-positive luminal epithelial cells expand upon expression of p53.R270H (Fig. 5A, white arrow); expanding luminal cells lose CK8 expression (Fig. 5A, blue arrow, cells indicated by white line) and tumors that ultimately develop are CK5 positive (Fig. 5A, bottom panel).

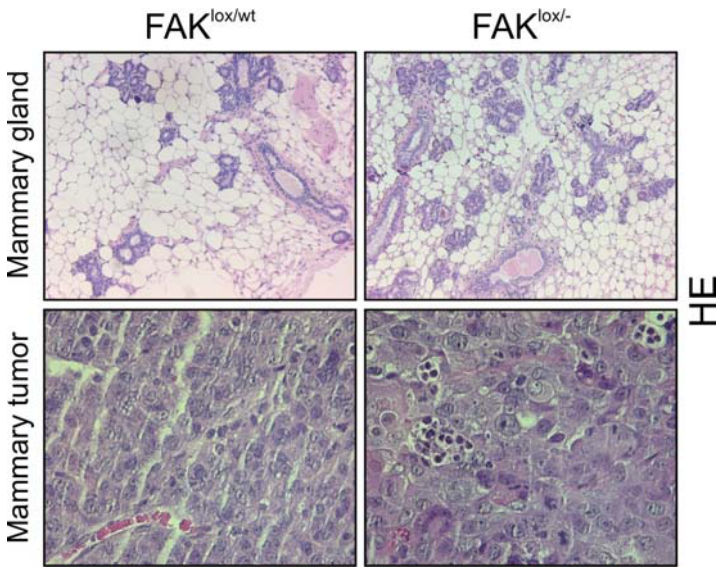


**Figure 4. Histological analysis of p53.R270H mammary tumors**
Sections of formalin-fixed paraffin embedded mammary gland tissue and mammary tumors of $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice were stained for HE.

These data suggest that p53.R270H induces tumors with a basal-like character, possibly switching cancer (stem) cell progenitors from a luminal phenotype to a basal-like phenotype.
Induction of p53.R270H was shown to cause a switch in cytokeratin expression in tumors that developed in $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice. In our previous study (described in chapter 3) we observed a decreased protein expression of E-cadherin in mammary tumor that lost expression of FAK. To determine if loss of FAK in our p53.R270H model was accompanied by down regulation of E-cadherin, we stained tumors for E-cadherin. Most tumors of both $FAK^{lox/wt}$ and $FAK^{lox/-}$ origin expressed E-cadherin (Fig. 5B and 6B), indicating that tumors that develop in $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice differ in their nature when comparing them to tumors that developed in the $p53^{lox/lox}$ model. Western blotting showed reduced FAK expression in $p53^{R270H}$/ $FAK^{lox/-}$/WapCre tumor cells (Fig. 6A), indicating that recombination and subsequent ablation of FAK was successful. We isolated tumor cell lines from two $FAK^{lox/wt}$ and two $FAK^{lox/-}$ mammary tumors. In line with our previous findings (25), *Fak* deletion in $p53^{R270H}$ tumor cells resulted in a disturbance of cell-matrix adhesion turnover, resulting in increased cell-matrix adhesions and displayed dense actin stress fibers (Fig. 6B).

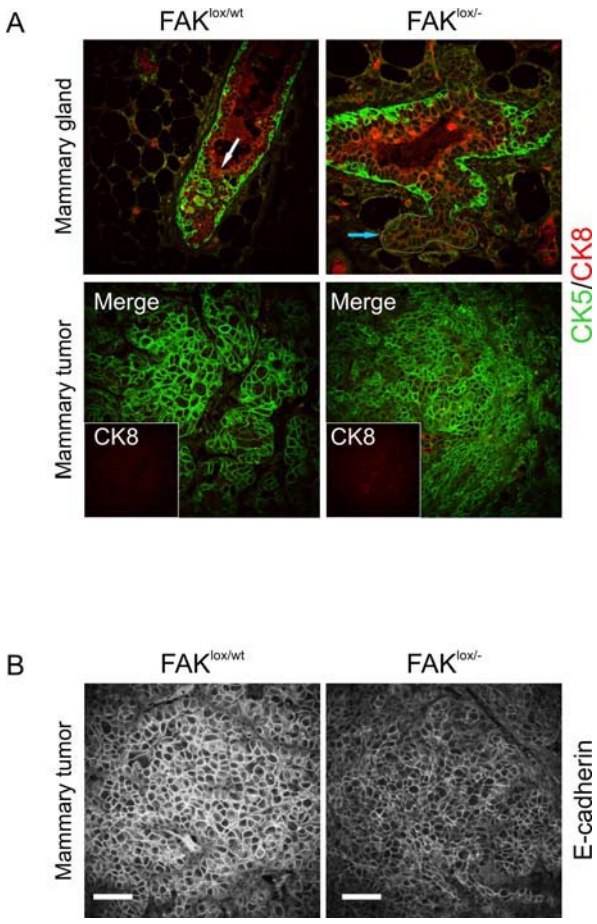


**Figure 5. p53.R270H mammary tumors are CK5 positive**
Paraffin sections of mammary glands and tumors isolated from $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice were stained for CK5, CK8 (A) and E-cadherin (B) and subsequently analyzed by confocal microscopy.

## Accelerated mammary tumorigenesis in p53 R270H point mutation compared to p53 null mice

Point mutations in p53 often lead to a dominant-negative effect on wild type p53 (22). To determine if the time of induction and morphology of mammary tumors is similar in p53 mutant-induced and p53 null-induced mammary tumorigenesis, we compared mammary tumorigenesis in $FAK^{lox/wt}$ and $p53^{lox/lox}/FAK^{lox/wt}$/WapCre

mice. $FAK^{lox/wt}$ mice are clearly more susceptible in developing mammary tumors than $p53^{lox/lox}/FAK^{lox/wt}$/WapCre (Fig. 7A). Median survival of $p53^{R270H}/FAK^{lox/wt}$/WapCre mice is 53 weeks while median survival of $p53^{lox/lox}/FAK^{lox/wt}$/WapCre was 73 weeks. $P53^{R270H}/FAK^{lox/wt}$/WapCre tumors expressed the myoepithelial marker CK5 despite the restricted expression of p53.R270H to luminal epithelial cells (Fig. 7B). This suggests that the luminal cells switch CK8 expression for CK5 expression. In contrast, $p53^{lox/lox}/FAK^{lox/wt}$/WapCre tumors expressed CK8 (Fig. 7B).

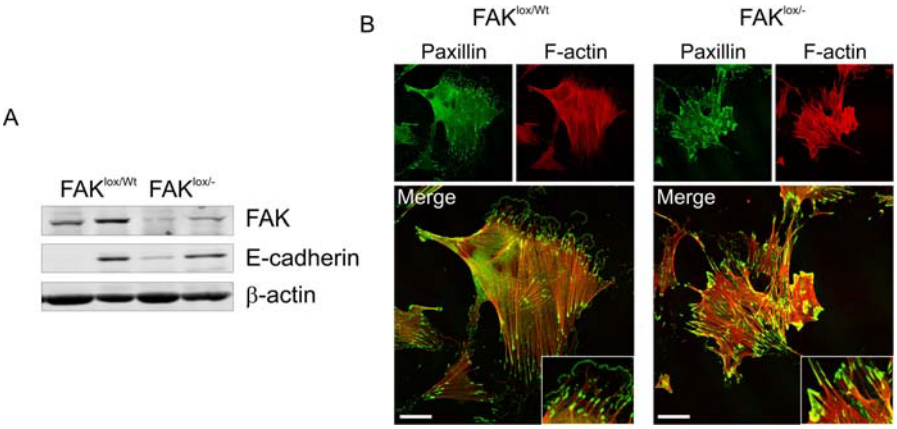


**Figure 6. Fak gene deletion affects cell-matrix adhesion turnover and actin cytoskeletal dynamics**
(A) FAK and E-cadherin protein levels of $FAK^{lox/wt}$ and $FAK^{lox/-}$ tumors were determined by Western blot analysis. Beta-actin was used as a loading control. (B) Tumor cells of $FAK^{lox/wt}$ and $FAK^{lox/-}$ mice were plated on collagen-coated coverslips, 24 hours thereafter formalin-fixed and stained for paxillin and the F-actin cytoskeleton. Images were taken using confocal laser scanning microscopy. Inserts: note the difference in paxillin-containing cell-matrix adhesions.

To determine if the levels of FAK are affected due to expression of the p53.R270H mutant or by the subsequent reduced p53 function, we determined FAK protein levels on tumor samples isolated from $p53^{R270H}/FAK^{lox/wt}$/WapCre and $p53^{lox/lox}/FAK^{lox/wt}$/WapCre tumors. Clearly, FAK protein levels were elevated in $p53^{R270H}/FAK^{lox/wt}$/WapCre tumors compared to $p53^{lox/lox}/FAK^{lox/wt}$/WapCre tumors (Fig. 7C and 7D), suggesting p53.R270H expression itself affects FAK protein levels, possibly leading to more aggressive tumors. The increased FAK expression was independent from CK5, suggesting that the higher levels of FAK may be a determinant of the CK8 to CK5 shift.

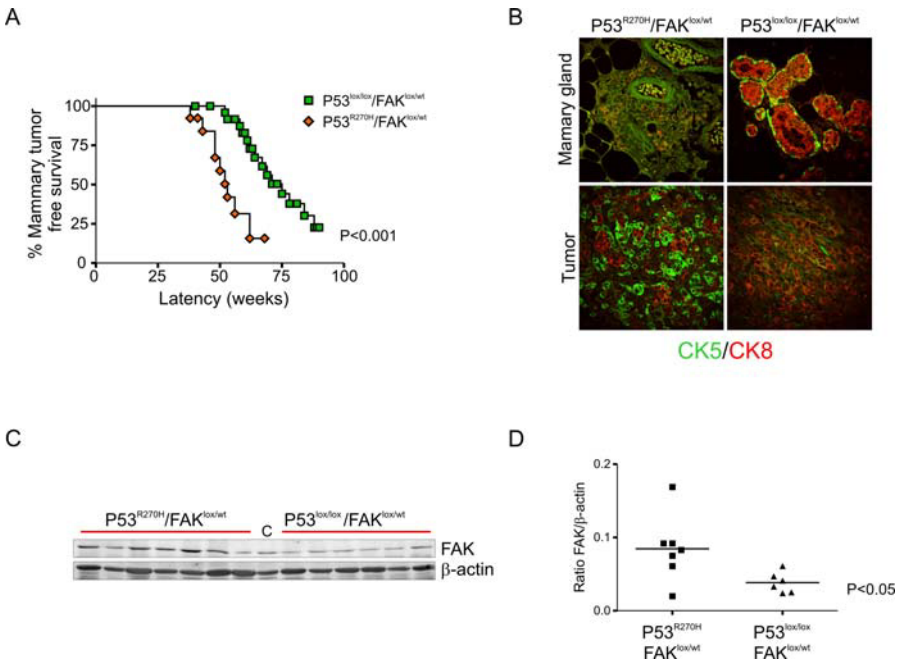


**Figure 7. FAK levels in p53.R270H mammary tumors are higher compared to $p53^{lox/lox}$ mammary tumors**

(A) Mammary tumor free survival of $p53^{R270H}/FAK^{lox/wt}$/WapCre (n=13) and $p53^{lox/lox}/FAK^{lox/wt}$/WapCre mice (n=24). *** P<0.001 (B) Mammary gland (upper panel) and tumors (bottom panel) were isolated from $p53^{R270H}/FAK^{lox/wt}$/WapCre and $p53^{lox/lox}/FAK^{lox/wt}$/WapCre mice and stained for CK5 and CK8. Images were taken using confocal laser scanning microscopy. (C) Total lysates were made from tumors isolated from $p53^{R270H}/FAK^{lox/wt}$/WapCre and $p53^{lox/lox}/FAK^{lox/wt}$/WapCre mice and Western blot analysis was performed. Blots were incubated with antibodies specific for FAK and CK5. Beta-actin was used as a loading control. (D) Quantification of FAK levels in tumors from $p53^{R270H}/FAK^{lox/wt}$/WapCre and $p53^{lox/lox}/FAK^{lox/wt}$/WapCre mice. FAK protein level was corrected for loading differences using β-actin. Graph represents ratio of FAK/β-actin. * P<0.05

## Discussion

Mutations in the tumor suppressor protein p53 and elevated FAK protein levels are frequently found in human cancers. In human breast cancer a high correlation was found for p53 mutations and FAK overexpression (21). In addition, FAK expression and activity in breast cancer are associated with poor prognosis (9, 26) and progression to metastases (18). P53 mutations affect p53 wild type function due to its dominant-negative effect on p53. Wild type p53 can bind to the FAK promoter thereby inhibiting its transcription. In this study we evaluated the role of

FAK in mouse mammary tumor formation. We show that mammary hyperplasia is evident in FAK deleted mammary glands, indicating that FAK does not directly influence this state of transformation. However, mammary tumor incidence was significantly reduced, indicating that FAK is essential for the progression of mammary hyperplasia towards mammary tumors. Interestingly, FAK levels were elevated in p53.R270H mammary tumors compared to $p53^{lox/lox}$ mammary tumors. In contrast to the p53.R270H model, $p53^{lox/lox}$ requires recombination of two p53 alleles. The delayed mammary tumorigenesis resulting from low FAK levels could be due to insufficient p53 gene recombination. If only one of the p53 alleles is present, p53 is still able to inhibit the FAK promoter thereby regulating FAK protein levels. Another possibility is that p53.R270H expression, apart from its dominant-negative effect on wild type p53, may elevate FAK expression. Though a gain-of-function for p53.R270H was not found by Wijnhoven et al (27), a recent paper describes that p53.R270H enhances the transition from intraepithelial neoplasia to invasive carcinoma (28). Combined data of the p53.R270H and the $p53^{lox/lox}$ mouse model indicate additional functions for p53.R270H.

The MMTV-PyMT mouse model for breast cancer has been extensively used to study the role of FAK in mammary tumorigenesis and implicated a role for FAK in breast cancer progression (15-18). In line with our findings mammary gland specific Fak deletion was shown to reduce the incidence of mammary tumors in the PyMT mice. The studies from Pylayeva et al revealed that the FAK deficient PyMT-transformed cells displayed diminished invasive and metastatic capacity. In addition, these cells displayed both growth arrest and apoptosis (18). However, another study suggested that the reduced mammary tumors incidence in FAK deficient mammary glands in their mouse model for mammary tumorigenesis was due to decreased proliferation, while no effect was observed in apoptosis (15). We have not yet determined if these processes are impaired in our mouse model for mammary tumorigenesis. FAK deficiency was shown to have a severe impact on mammary cancer stem/progenitor cells. FAK deletion reduced the pool of cancer stem/progenitors, decreased their self renewal and migration in vitro, and comprised their tumorigeneity and maintenance in vivo (16). The reduction in incidence of mammary tumors in FAK deficient-mammary glands in our model could be due to decrease number of mammary cancer stem/progenitor cells. To study this possibility we need to stain these tumors for specific stem cell markers such as CD24/CD29.

The observation that FAK plays a critical role in p53.R270H-induced mammary tumor formation may have important therapeutic value for designing treatment of human p53.R273H-induced breast cancer. Elevated FAK expression is frequently found in human cancers, and elevated FAK protein levels correlates with p53 mutations (21). Given the frequently found elevated levels of FAK, inhibition of its activity by inhibitors in addition to cancer therapy might have a beneficial effect on the success of breast cancer treatment.

## Materials and Methods

*Generation of mice*
All breeding steps necessary to generate $p53^{R270H/wt}/FAK^{lox/wt}$ and $p53^{R270H/wt}/FAK^{lox/-}$ are depicted in Fig. 1.

*Mice and genotyping*
Genotyping of $p53^{LSL-R270H}$ was performed using a PCR/digestion-based assay as described previously (29). Genotyping of FAK was performed using specific primers for FAK: 5'-GAGAATCCAGCTTTGGCTGTT-3'; 5'-GGCTTCTTGAAGGAACTTCTC-3'; 5'-TGATATTGCTGAAGAGCTT-GGCGG-3'. PCR products are FAK wt 88 and/or FAK mutant 170bp, FAK lox fwd: 5'-GAGAATCCAGCTTTGGCTGTTG-3', rev: 5'-GAATGCTACAGGAACCAAATAAC-3', PCR products are Wt 290bp and lox 400bp, and Cre fwd: 5'-GTT CAG GGA TCG CCA GGC G-3', rev: 5'-GCT GGC TGG TGG CAG ATG G -3'. FAK recombination was determined using the following primers fwd: 5'-GACCTTCAACTTCTCATTTCTCC-3', rev: 5'-GAATGCTACAGGAACCAAATAA-C-3', PCR products are Wt 1.4 kB, lox 1.6kB, and FAK recombined allele 327bp.

*Analysis of spontaneous tumour development*
Spontaneous tumor development was determined in $p53^{R270H}/FAK^{lox/wt}$/WapCre (referred to as $FAK^{lox/wt}$) (n=13) and $p53^{R270H}/FAK^{lox/-}$/WapCre (referred to as $FAK^{lox/-}$) mice (n=18). At the age of 8 weeks mice were put in breeding; pregnancy and lactation stimulated the WapCre promoter. After three weeks of weaning the pups were removed from the mother. Weight development and mammary tumor formation was monitored once a week. Mammary tumor formation was followed on a weekly basis by palpation up to 70 weeks.

*Tissue processing and histology*
Tumors and/or mammary glands isolated from the mice were fixed in 4% formaldehyde and subsequently processed and embedded in paraffin. Sections of 3 µm were deparaffinized and antigen retrieval was performed when needed. Sections were stained with primary antibody overnight at 4°C and secondary antibody 1 hr at room temperature.

*Immunofluorescence microscopy and image analysis*
Immunofluorescence staining of coverslips was performed as described previously. Primary antibody incubation was performed overnight at 4 °C and cells were subsequently incubated with Alexa-488 or Cy-3 conjugated secondary antibody (Molecular Probes) in combination with rhodamin-phalloidin (Molecular Probes) to label the F-actin cytoskeletal network. Microscopic analysis was

performed using Bio-Rad Radiance 2100 confocal system with a 60 x or 40 x Plan Apo (NA 1.4; Nikon) objective lens. Image acquisition was controlled using the Laser Sharp software (Bio-Rad). Images were processed and quantitative image analysis was performed using Image-Pro® Plus (Version 5.1; Media Cybernetics) in combination with in house developed macros to analyze focal adhesion size.

*Western blot*

A small biopsy from the tumors was lysed in lysis buffer (Tris-HCL (10mM), sucrose (250 mM), EDTA (1 mM)). Twenty-five microgram of protein was loaded and run on SDS-PAGE and transferred onto nitrocellulose membrane. Primary antibody incubation was performed overnight at 4$^{o}$C. Thereafter blots were incubated with horseradish peroxidase conjugated secondary antibody (GE Healthcare) or Cy5-conjugated secondary antibody in TBS-T for 1h at room temperature. Protein signals were detected with ECL-plus method (GE Healthcare) followed by scanning of the blots or in case of Cy5-incubation, the blots were directly scanned with a Typhoon 9400 (GE Healthcare).

*Antibodies*

Anti-FAK clone 4.47 Upstate Biotechnology (#05-537), anti-p53 clone CM5 Novocastra (p53-CM5P), anti-paxillin Transduction laboratories (p-13520), anti-E-cadherin BD transduction (#610263), anti-cytokeratin 5 Covance (#PRB-160P), anti-cytokeratin 8 Fitzgerald (rdi-pro61038), anti-vimentin Sigma Aldrich (V-4630), anti-β-actin Santa Cruz (sc-47778).

*Statistics*

Student's t-test was used to determine if there was a significant difference between two means ($p<0.05$). Significant differences are marked in graphs.

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