Mammary gland-specific ablation of focal adhesion kinase reduces the incidence of p53-mediated mammary tumour formation

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Background: Elevated expression of focal adhesion kinase (FAK) occurs in numerous human cancers including colon-, cervix- and breast cancer. Although several studies have implicated FAK in mammary tumour formation induced by ectopic oncogene expression, evidence supporting a role for FAK in spontaneous mammary tumour development caused by loss of tumour suppressor genes such as p53 is lacking. Alterations in the tumour suppressor gene p53 have been implicated in over 50% of human breast cancers. Given that elevated FAK expression highly correlates with p53 mutation status in human breast cancer, we set out to investigate the importance of FAK in p53-mediated spontaneous mammary tumour development.

Methods: To directly assess the role of FAK, we generated mice with conditional inactivation of FAK and p53. We generated female p53$^{lox/lox}$/FAK$^{+/+}$/WapCre, p53$^{lox/lox}$/FAK$^{flox/+}$/WapCre and p53$^{lox/lox}$/FAK$^{flox/-}$/WapCre mice, and mice with WapCre-mediated conditional expression of p53^{R270H}, the mouse equivalent of human p53^{R273H} hot spot mutation, together with conditional deletion of FAK, P53$^{R270H/+}$/FAK$^{lox/+}$/WapCre and p53$^{R270H/+}$/FAK$^{flox/-}$/WapCre mice. All mice were subjected to one pregnancy to induce WapCre-mediated deletion of *p53* or expression of p53 R270H, and *Fak* genes flanked by two loxP sites, and subsequently followed the development of mammary tumours.

Results: Using this approach, we show that FAK is important for p53-induced mammary tumour development. In addition, mice with the mammary gland-specific conditional expression of p53 point mutation R270H, the mouse equivalent to human R273H, in combination with conditional deletion of *Fak* showed reduced incidence of p53^{R270H}-induced mammary tumours. In both models these effects of FAK were related to reduced proliferation in preneoplastic lesions in the mammary gland ductal structures.

Conclusions: Mammary gland-specific ablation of FAK hampers p53-regulated spontaneous mammary tumour formation. Focal adhesion kinase deletion reduced proliferative capacity of p53 null and p53^{R270H} mammary epithelial cells but did not lead to increased apoptosis *in vivo*. Our data identify FAK as an important regulator in mammary epithelial cell proliferation in p53-mediated and p53^{R270H}-induced mammary tumour development.

The tumour suppressor p53 has been implicated in diverse biological processes such as apoptosis, cell-cycle arrest, DNA repair, cellular differentiation and senescence, and has been shown to be altered in over 50% of spontaneous tumours in humans (Cance et al, 2000; Wijnhoven et al, 2005; Dey et al, 2008). In addition to spontaneous tumours, germ-line mutations in p53 are observed in Li-Fraumeni patients, which predisposes them to a wide spectrum of early-onset cancers, including breast cancers (Malkin et al, 1990; Srivastava et al, 1990; Hainaut and Hollstein, 2000). Normally, p53 is expressed in a latent form and is maintained at low levels through targeted degradation. However, in response to DNA damage or stress, p53 is activated to function as a transcription factor, resulting in a cascade of events that eventually prevents tumour development. P53 is the most frequently mutated gene in spontaneous breast tumours. Approximately 30% of sporadic breast tumours acquire p53 mutations, which are related to breast cancer progression and resistance to doxorubicin therapy in breast cancer patients (Hainaut and Hollstein, 2000; Wijnhoven et al, 2005; Dey et al, 2008).

Focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase, specifically associates with 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. Focal adhesion kinase expression is elevated in numerous cancers including colon-, cervix- and breast cancer (Cance et al, 2000; Moon et al, 2003; Lightfoot et al, 2004; Madan et al, 2006; Pylayeva et al, 2009), and FAK overexpression and p53 mutations correlate with human breast cancer (Lightfoot et al, 2004). To study the role of FAK, FAK gene knockout mice were developed but led to an embryonic lethal phenotype at day E8.5 due to defects in the axial mesodermal tissues and cardiovascular system (Ilic et al, 1995, 2003). Using conditional mouse models, the importance of FAK in skin tumour progression was shown (McLean et al, 2001), and several studies were performed to determine the role of FAK in mammary gland tumour development and progression (Lahlou et al, 2007, 2012; Provenzano et al, 2008; Luo et al, 2009; Pylayeva et al, 2009). Focal adhesion kinase deletion in MMTV-PyMT and activated ErbB2 (NDL2-5) models reduced the incidence of mammary tumours, suggesting an important role for FAK in mammary gland tumour development. PyMT is a strong proto-oncogene inducing the onset of mammary gland tumours within 10 weeks, and also ErbB2-induced mammary tumours developed early, around 20 weeks (Lahlou et al, 2012). This precludes the analysis of the role of FAK in spontaneous mammary gland tumour development as a consequence of loss of heterozygosity of tumour suppressors, which is more relevant to the human situation. Consequently, these studies were unable to address the role of FAK in the natural course of mammary tumorigenesis (Lahlou et al, 2007; Provenzano et al, 2008; Luo et al, 2009; Pylayeva et al, 2009). p53 has been shown to affect FAK levels by binding to the N-terminal region of FAK as well as to the promoter region of FAK to inhibit the transcription of FAK (Golubovskaya et al, 2008). Vice versa, FAK facilitates p53 turnover via enhanced MDM2-dependent p53 ubiquitination, and subsequent proteosomal degradation leading to inactivation of p53 (Lim et al, 2008). This mutual interaction suggests interplay in cancer development, which so far has not been investigated.

To study the role of FAK in spontaneous mammary gland tumour development, here, we used two independent spontaneous mammary tumorigenesis models that are dependent on the tumour suppressor protein p53. The first model was based on conditional deletion of the p53, resulting in induction of mammary tumours after 45 weeks. In the second model, we explored the contribution of FAK to mammary gland tumour development induced by a p53 point mutation p53^{R270H}, the mouse equivalent of human p53^{R273H} mutation. Such p53 point mutation leads to dominant-negative inhibition of wild-type p53 function, and expression of mammary gland-specific p53^{R270H} mutation has been shown to mimic human breast cancer development (De Vries et al, 2002). Both mouse models were crossed with conditional FAK$^{flox/-}$ mice and WapCre mice. Expression of the WapCre transgene is restricted to the mammary glands of late pregnant and lactating mice, and is not expressed in virgin mice (Wagner et al, 1997). Mice were subjected to one pregnancy to allow mammary-specific inactivation of both p53 and FAK. Using these two independent spontaneous mammary tumorigenesis models, we show that FAK is essential for the progression of mammary epithelial hyperplasia to mammary gland tumours caused by either loss of p53 or expression of the p53^{R270H} mutant through inhibition of cell proliferation in preneoplastic lesions.

MATERIALS AND METHODS

Genotyping of mice. Genotyping of p53$^{lox/lox}$ mice was performed using the following primers. 5′-CAC AAA AAC AGG TTA AAC CCA G-3′ [p53-int1-fwd], 5′-AGC ACA TAG GAG GCA GAG AC-3′ [p53-int1-rev], 5′-AAG GGG TAT GAG GGA CAA GG-3′ [p53-int10-fwd], 5′GAA GAC AGA AAA GGG GAG GG-3′ [p53-int10-rev]. Deletion of p53 was confirmed with primers: 5′-CAC AAA AAC AGG TTA AAC CCA G-3′ [p53-int1-fwd] and 5′GAA GAC AGA AAA GGG GAG GG-3′ [p53-int10-rev]. Genotyping of p53$^{LSL-R270H}$ was performed using a PCR/digestion-based assay as described previously (Wijnhoven et al, 2005). Genotyping of fak was performed using specific primers for FAK: 5′-GAGAATCCAGCTTTGGCTGTT-3′; 5′-GGCTTCTTGA AGGAACTTCTC-3′; 5′-TGATATTGCTGAAGAGCTTGGCG G-3′. PCR products derived were FAK wt 88 and/or FAK mutant 170 bp; for FAK^{flox} mice the primers are fwd: 5′-GAGAATCCAGC TTTGGCTGTTG-3′, rev: 5′-GAATGCTACAGGAACC AAA TAAC-3′, PCR products were Wt 290 bp and lox 400 bp. For Cre-genotyping primers used were fwd: 5′-GTTCAGGGATCGC CAGGC G-3′ and rev: 5′-GCTGGCTGGTGGCAGATGG-3′. Focal adhesion kinase recombination was determined using the following primers fwd: 5′-GACCTTCAACTTCTCATTTCTCC-3′ and rev: 5′-GAATGCTACAGGAACCAAATAAC-3′; PCR products were Wt 1.4 kB, lox 1.6 kB and FAK-recombined allele 327 bp. All mice were on a mixed C57jBl6/Ola background.

Analysis of spontaneous tumour development. Spontaneous tumour development was determined in p53$^{lox/lox}$/FAK$^{+/+}$ (n = 25), p53$^{lox/lox}$/FAK$^{flox/-}$ (n = 25) and p53$^{lox/lox}$/FAK$^{flox/+}$ (n = 25), and in p53^{R270H}/FAK$^{flox/+}$/WapCre (n = 13) and p53^{R270H}/FAK$^{flox/-}$/WapCre mice (n = 20). At the age of 8 weeks, mice were placed in breeding; pregnancy and lactation stimulated the WapCre promoter. After 3 weeks of weaning, the pups were taken away from the mother. Weight development and mammary tumour formation were monitored once a week. Mammary tumour formation was followed on a weekly basis by palpation up to 70 weeks for p53^{R270H} mammary tumour development and up to 90 weeks for p53$^{lox/lox}$ mammary tumour development. Mice were killed before tumours reached the size of 1 cm^3 or when mice lost more than 10% of their total body weight.

All animal experiments were approved by the local ethical committee. Housing and experiments were performed according to the Dutch guidelines for the care and use of laboratory animals (UL-DEC-05099). Sterilised food and water were provided ad libitum.

Tissue processing and histology. Tumours and/or mammary glands isolated from the mice were fixed in 4% formaldehyde and subsequently processed and embedded in paraffin or snap-frozen in liquid nitrogen. Paraffin sections of 3 μm were de-paraffinised, and antigen retrieval was performed when needed. Sections were stained with the primary antibody overnight at 4°C and with the secondary

antibody for 1 h at room temperature. Slides were mounted with aqua polymount (Polysciences, Eppelheim, Germany).

Immunofluorescence microscopy and image analysis. Immunofluorescence staining was performed as described previously (De Graauw *et al*, 2005). Primary antibody incubation was performed overnight at 4 °C, and tumours were subsequently incubated with the Alexa-488 or Cy-3-conjugated secondary antibody (Molecular Probes, Bleiswijk, The Netherlands). Microscopic analysis was performed using a Bio-Rad Radiance 2100 confocal system with a × 60 or × 40 Plan Apo (NA 1.4; Nikon) objective lens. Image acquisition was controlled using the Laser Sharp software (Bio-Rad, Hercules, CA, USA). Images were processed, and quantitative image analysis was performed using Image-Pro Plus (Version 5.1; Media Cybernetics, Rockville, MD, USA).

Western blot analysis. A small biopsy from the tumours was lysed using lysis buffer (Tris-HCl (10 mM), sucrose (250 mM), EDTA (1 mM). Western blotting was performed as previously described (De Graauw *et al*, 2005). Primary antibody incubation was performed overnight at 4 °C. Thereafter, blots were incubated with horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Fairfield, CT, USA) or Cy5-conjugated secondary antibody in Tris-buffered saline-Tween 20 for 1 h at room temperature. Protein signals were detected with the 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 imager (GE Healthcare).

Antibodies. Anti-FAK clone 4.47, Upstate Biotechnology (Lake Placid, NY, USA) (#05-537); anti-E-cadherin, BD transduction (San Jose, CA, USA) (#610263); anti-vimentin, Sigma Aldrich (St Louis, MO, USA) (V-4630); anti-cytokeratin 5, Covance (Rotterdam, The Netherlands) (#PRB-160P); anti-cytokeratin 8, Fitzgerald (North Acton, MA, USA) (rdi-pro61038); anti-p53 CM5, Novocastra (Rijswijk, The Netherlands) (p53-CM5P); anti-p21, Santa Cruz (Dallas, TX, USA) (sc-397); anti-p53^{Ser15}, Cell Signaling Technology (Danvers, MA, USA) (9284); anti-Tubulin, Sigma Aldrich (T-9026); β-actin Santa Cruz (sc-47778), anti-FAK (IHC), Cell Signalling Technology (#3258).

Gene expression analysis. Mouse mammary tumour RNA was purified using an RNeasy minikit (Qiagen, Venlo, The Netherlands). The samples were hybridised to Affymetrix microarrays. We generated mu-sigma normalised log gene expression values and clustered the mice using Euclidean distance on average linkage using R version 2.15.2 and the package gplots version 2.11.0. We focused on selected basal-like genes, including Trp63, Krt17, Cryab, Ptn, Col17a1 and Slug (snai2). Genes were not reordered.

We employed principal components and hierarchical cluster analysis to determine gene expression differences in tumours derived from FAK$^{+/-}$ and FAK$^{-/-}$ mice in R version 3.0.1 (R Development Core Team).

We calculated the average expression values over all probes targeting the same gene, log-transformed and applied gene wise normalisation, resulting in a data set of 21 449 probes. We used the top 10 varying genes in these analyses for visualisation. To test sensitivity to this cut-off, we checked whether results were different if top5, top25, top50 or all genes were included in the analysis (data not shown). We used 1-Pearson correlation as distance metric and Ward's method for clustering. We used BootstrapClusterTest from the ClassDiscovery package for determining the robustness of the presence of two clusters by sampling 2000 genes 200 times (Coombes, 2009).

To determine significantly differing genes between FAK$^{+/-}$ and FAK$^{-/-}$ tumours, we employed a two-class unpaired SAM analysis (Tibshirani *et al*, 2011) on log10 gene expression values of the data set.

Statistics. Survival curves were generated using Kaplan–Meier statistics. Significance was estimated using unpaired two-tailed Student's *t*-test and Log-rank (Mantel-Cox) test.

RESULTS

Mammary-specific disruption of *Fak* inhibits spontaneous mammary gland tumour formation in p53$^{lox/lox}$ mice. Given the importance of FAK in mammary tumour formation as shown using two independent strong oncogenes (Lahlou *et al*, 2007, 2012; Provenzano *et al*, 2008; Luo *et al*, 2009; Pylayeva *et al*, 2009) and the potential interplay of FAK and p53 in cancer, we assessed whether FAK was important in p53-mediated spontaneous mammary tumour development. We generated female WapCre/ p53$^{lox/lox}$/FAK$^{+/+}$ (FAK$^{+/+}$), WapCre/p53$^{lox/lox}$/FAK$^{flox/+}$ (FAK$^{flox/+}$) and WapCre/p53$^{lox/lox}$/FAK$^{flox/-}$ (FAK$^{flox/-}$) mice. The mice were subjected to one pregnancy to induce WapCre-mediated deletion of *p53* and *Fak* genes flanked by two loxP sites, and subsequently followed the development of mammary tumours for a total time period of 90 weeks. The first onset of mammary tumours in FAK$^{flox/-}$ mice was comparable to that in FAK$^{flox/+}$ mice. However, mammary tumour formation reached a plateau in FAK$^{flox/-}$ mice after 60 weeks, but continued in FAK$^{+/+}$ and FAK$^{flox/+}$ animals (Figure 1A). This was reflected in the effect on the total number of mammary tumours that developed: 52% FAK$^{flox/+}$ mice developed mammary tumours (13 of a total of 25 mice), while only t36% FAK$^{flox/-}$ mice developed mammary tumours (9 of a total of 25 mice) (Table 1). FAK$^{flox/+}$ mice were similarly susceptible for mammary tumour development compared with FAK$^{+/+}$; however, FAK$^{flox/-}$ mice displayed a significant difference (Figure 1A, unpaired *t*-test P < 0.05). Sixty-four percent of FAK$^{+/+}$ mice were mammary tumour bearing (16 of a total of 25 mice), and mean latency time for spontaneous mammary tumour development was 62 weeks for FAK$^{+/+}$ mice. The mean latency time for spontaneous mammary tumour development was 67 weeks for FAK$^{flox/+}$ mice and 60 weeks for FAK$^{flox/-}$ mice. While contralateral mammary glands from tumour-bearing mice all displayed hyperplasia regardless of genotype, mammary glands from early and late dropouts in FAK$^{flox/-}$ mice (censored events) did not display hyperplasia, indicating that these mice would not have developed mammary tumours within our experimental setting. To determine FAK recombination efficiency, mammary tumours from FAK$^{flox/+}$ and FAK$^{flox/-}$ mice were analysed by PCR (Figure 1B). *Fak* gene recombination was confirmed in FAK$^{flox/+}$ and FAK$^{flox/-}$ mammary tumours (Figure 1B). Together, these data suggest that FAK is important for p53-mediated mammary tumour development.

On the basis of their histological and structural organisation, the vast majority of mammary tumours were classified as solid carcinomas and carcinosarcomas, the latter characterised by the presence of spindle-shaped cells (Figure 1C, Table 1). Confirmatively, carcinosarcomas stained positive for vimentin and negative for E-cadherin (Supplementary Figure 1). Histological analysis of mammary glands isolated from the contralateral side of the mammary tumour revealed neoplastic or hyperplastic lesions in all groups (Figure 1C, upper panel), indicating that oncogenic transformation occurred in both FAK-expressing and FAK-deleted mammary glands.

Focal adhesion kinase-proficient and -deficient mammary tumours are genetically comparable. To test the effect of FAK gene recombination on protein level, FAK$^{flox/+}$ and FAK$^{flox/-}$ and control mammary tumours (FAK$^{+/+}$) were analysed for FAK protein expression. Focal adhesion kinase protein expression determined by western blot analysis and FAK staining on paraffin sections were clearly reduced in FAK$^{flox/+}$ and FAK$^{flox/-}$
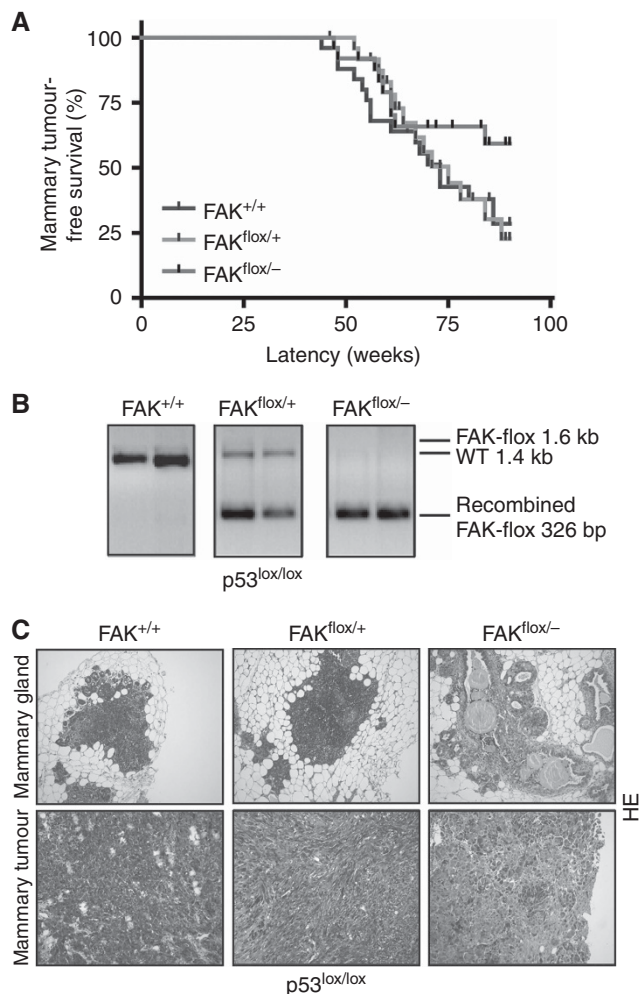
Figure 1. FAK is essential for p53$^{lox/lox}$-induced mammary tumorigenesis. Female FAK$^{+/+}$ ($n = 25$), FAK$^{flox/+}$ ($n = 25$) and FAK$^{flox/-}$ mice ($n = 25$) were subjected to one pregnancy to induce WapCre-mediated deletion of p53 and Fak, and subsequently followed for the development of mammary tumours for a total time period of 90 weeks. (A) Mammary tumour-free survival (%) (Log-rank $P < 0.05$, unpaired t-test $P < 0.05$) (B) FAK recombination PCR on mammary tumours. FAK wt is 1.4 kb, FAK lox is 1.6 kb and FAK recombined is 327 bp. (C) Paraffin sections of mammary tumours and non-tumour-bearing mammary glands stained for hematoxylin/eosin (HE).

Table 1. Tumour spectrum in FAK$^{+/+}$, FAK$^{flox/+}$ and FAK$^{flox/-}$ p53$^{lox/lox}$ mice

	FAK$^{+/+}$	FAK$^{flox/+}$	FAK$^{flox/-}$
Total number of mice analysed	25	25	25
Tumour-bearing mice	18	14	11
Mammary gland tumour[a]	16 (64%)	13 (52%)	9 (36%)
Lymphoma	0	0	1
Other[b]	2	1	1
Mean mammary gland tumour latency (weeks)[c]	61.9 ± 12.3	67.2 ± 11.0	59.7 ± 10.7

[a]The majority of mammary gland tumours that developed were characterised as solid carcinomas and carcinosarcomas.
[b]Other tumours were found in the lung, bone and colon.
[c]Mean latency time is depicted as the number of weeks after ablation of p53$^{lox/lox}$.
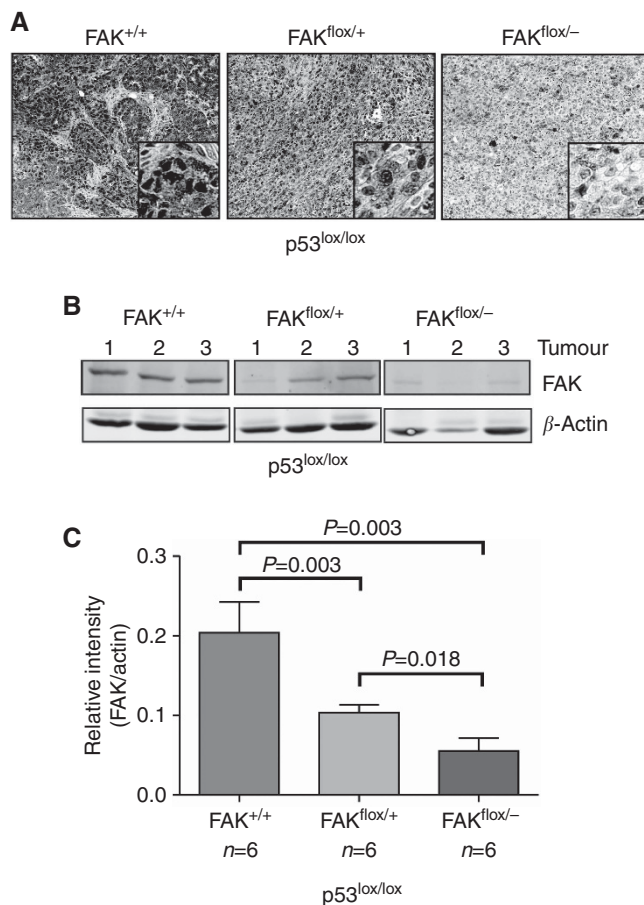
Figure 2. Fak gene recombination was successful on both gene and protein levels. (A) FAK staining on paraffin sections of mammary tumours. (B) Western blot analysis was performed on lysates of mammary tumours. Membranes were incubated with antibodies against FAK. Actin was used as a loading control. (C) Quantification of the FAK protein level. Results represent the mean of six individual tumours per genotype (± s.d.), from which a representative western blot is shown in B.

mammary tumours compared with control mammary tumours (FAK$^{+/+}$), indicating that ablation of FAK was successful on both gene and protein level (Figure 2A–C). An interesting observation is that the few mammary tumours that do arise in FAK$^{flox/-}$ mice share similar mammary tumour latency as compared with mammary tumour development in FAK$^{flox/+}$ mice. As FAK status was significantly reduced in FAK$^{flox/-}$ mammary tumours, we wondered whether FAK$^{flox/-}$ tumours use alternative strategies to facilitate tumour development. To unravel potential alternative pathways, we performed gene expression analysis on FAK$^{flox/-}$ and FAK$^{flox/+}$ mammary tumours. Unsupervised hierarchical clustering and principle component analysis on these tumours revealed that the FAK$^{flox/-}$ and FAK$^{flox/+}$ groups do not show clear separation based on gene expression, suggesting that the mammary tumours are genetically comparable (Supplementary Figure 2). This was supported by SAM analysis to determine significant changes in gene expression between FAK$^{+/-}$ and FAK$^{-/-}$ tumours, which revealed only 28 genes that showed significant higher expression in FAK$^{flox/+}$ compared with that in FAK$^{flox/-}$ (Supplementary Table 1). However, we did observe that hierarchical clustering and principal components analysis identified mainly separate histological subtype, that is, solid carcinoma vs carcinosarcoma, but this was not related to FAK status (Odd ratio = 3.6, $P = 0.34$). One outlier sample turned out to be a lymphoma and was excluded from SAM analysis. When we

sampled 200 times a clustering with 2000 genes (similar to visualisation, Supplementary Figure 2), we observed that the clusters were robust, with some samples from FAK$^{flox/+}$ clustering with FAK$^{flox/-}$ samples, representing similar tumour types.

Given that FAK-null mammary tumours genetically resemble FAK-proficient tumours and the sparse mammary tumours that arise show the same latency, a plausible explanation for this phenomenon is that, at the time of mammary tumour initiation and early mammary tumour development, FAK has not been deleted efficiently, which is supported by some residual cells that still showed clear FAK expression (Figure 2B).

Mammary gland-specific Fak deletion inhibits p53^{R270H}-induced breast tumour formation. Using the p53$^{lox/lox}$ mouse model, we identified a contribution of FAK in loss of tumour suppressor gene-mediated spontaneous mammary tumour formation. However, FAK overexpression in breast cancers has been frequently correlated with p53 mutations, a feature that is not represented by our p53$^{lox/lox}$ mouse model (Golubovskaya et al, 2009). Therefore, in addition to our p53$^{lox/lox}$ mouse model, we developed mice with WapCre-mediated conditional expression of p53^{R270H}, the mouse equivalent of human p53^{R273H} hot spot mutation (Wijnhoven et al, 2005), together with conditional deletion of FAK. The conditional-floxed FAK strain was crossed with mice conditionally expressing p53^{R270H} and WapCre to generate WapCre/p53$^{R270H/+}$/FAK$^{lox/+}$ (referred to as FAK$^{flox/+}$) and WapCre/p53$^{R270H/+}$/FAK$^{flox/-}$ mice (referred to as FAK$^{lox/-}$). To evaluate the role of FAK in p53^{R270H}-induced mammary tumorigenesis, female FAK$^{lox/+}$ and FAK$^{flox/-}$ were subjected to one pregnancy to mediate the WapCre-mediated expression of p53^{R270H} point mutation and ablation FAK. Subsequently, the mice were monitored for mammary tumour development by physical palpation for a total time period of 70 weeks. The first onset of mammary tumours was already at 38 weeks for FAK$^{flox/+}$ and 39 weeks for FAK$^{flox/-}$ after p53^{R270H} induction (Figure 3A and Table 2) and overall survival was similar (data not shown). Although the onset of mammary tumours was comparable, only few mammary tumours developed in FAK$^{flox/-}$ mice, all of which were observed within 50 weeks. The number of mammary tumour-bearing mice was significantly higher in FAK$^{flox/+}$ mice, indicating that mice that still express FAK are more susceptible to mammary tumour development than FAK$^{lox/-}$ mice even later in time. Sixty-nine percent (9 out of 13 mice) of FAK$^{flox/+}$ mice were tumour bearing, all bearing one or more mammary gland tumours, with a mean latency of 51 ± 8.3 weeks (Figure 3A and Table 2). In contrast, only 20 percent (4 of a total of 20 mice) of FAK$^{flox/-}$ mice developed mammary tumours, with a mean latency of 48 ± 8.3 weeks, representing a clear difference in tumour formation (unpaired t-test $P < 0.05$, Figure 3A). Most mammary gland tumours developed in the 4th or 5th mammary gland, 67% in FAK$^{flox/+}$ and 50% in FAK$^{flox/-}$ mice. Focal adhesion kinase recombination efficiency was determined by PCR and confirmed that FAK ablation was evident in FAK$^{lox/+}$ and FAK$^{lox/-}$ mammary tumours (Figure 3B).

On the basis of their histological analysis, mammary tumours that developed in FAK$^{lox/+}$ and FAK$^{lox/-}$ animals were mostly adenocarcinomas and solid carcinomas (Figure 3C and Table 2). Strikingly, none of the p53^{R270H}-driven FAK$^{lox/+}$ and FAK$^{lox/-}$ mammary tumours were classified as carcinosarcomas. P53^{R270H} expression in FAK$^{lox/+}$ and FAK$^{lox/-}$ mammary tumours was assessed by immunohistochemical staining with CM5 and showed accumulation of p53 in nuclei of mammary tumour cells (Figure 4A). Accumulation of p53 was also observed in neoplastic and hyperplastic lesions of contralateral non-tumour-bearing glands (Figure 4A). We confirmed FAK protein level was reduced in FAK$^{flox/-}$ mammary tumours by performing immunohistochemistry and western blot analysis (Figure 4B–D). Focal adhesion
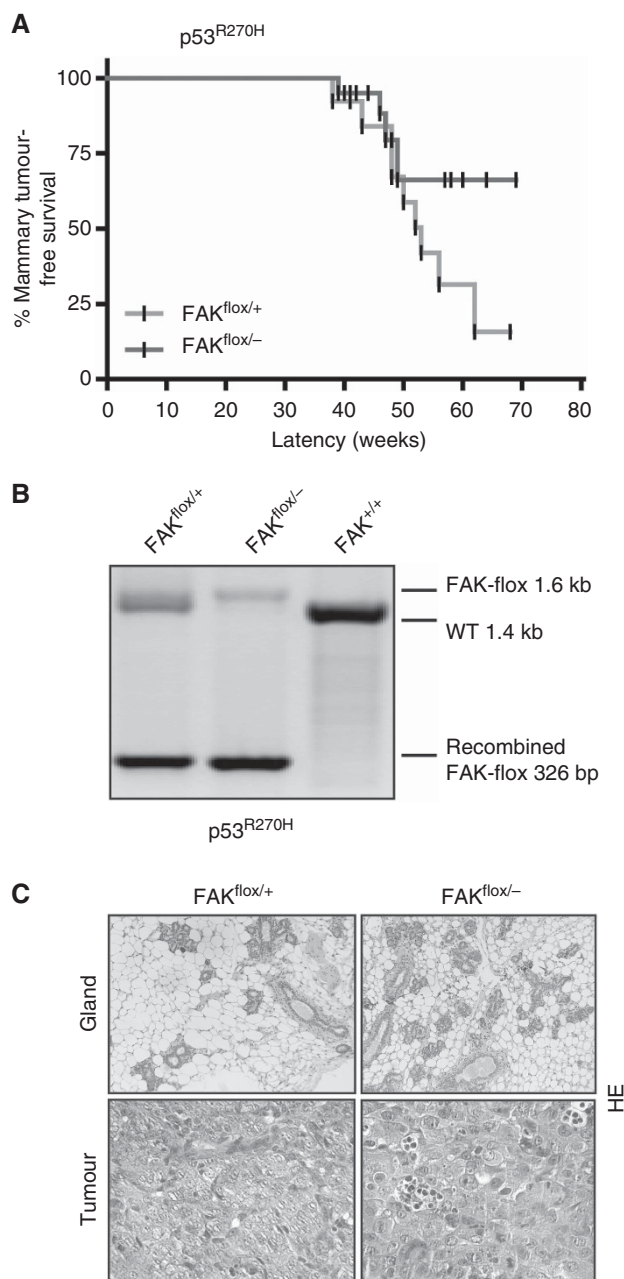
Figure 3. FAK expression is crucial for p53^{R270H}-induced mammary tumour progression. Female p53^{R270H}/FAK$^{flox/-}$ and p53^{R270H}/FAK$^{flox/+}$ mice were subjected to one pregnancy to activate WapCre resulting in the expression of p53^{R270H} point mutation and deletion of the floxed Fak allele. Mice were followed for the development of mammary tumours for a total time period of 70 weeks. (**A**) Mammary tumour-free survival curves of p53^{R270H}/FAK$^{flox/-}$/WapCre ($n = 20$) and p53^{R270H}/FAK$^{flox/+}$/WapCre mice ($n = 13$). Time is depicted in weeks after p53^{R270H} expression, set at time of birth of litters (Log-rank ns, unpaired t-test $P < 0.05$). (**B**) FAK recombination PCR was used to determine recombination of the FAK allele flanked by loxP sites. FAK wt is 1.4 kb, FAK lox is 1.6 kb and FAK recombined is 327 bp. (**C**) Mammary tumours were stained with haematoxylin/eosin (HE) to determine tumour type.

kinase staining on paraffin sections of the isolated tumours showed a manifest reduction in FAK protein levels in FAK$^{flox/-}$ tumours (Figure 4B), indicating that in our model FAK is successfully deleted. However, in line with the observations made in p53$^{lox/lox}$ mouse model, we obtained several mammary tumours that lack

FAK but show similar tumour latency compared with $FAK^{lox/+}$ mammary tumours. Unfortunately, the low number of tumours obtained from $FAK^{flox/-}$ mice in $p53^{R270H}$ model does not permit a further robust genetic comparison.

In conclusion, our data indicate that FAK is important for $p53^{lox/lox}$ and $P53^{R270H}$-induced spontaneous mammary tumour development.

Mammary-specific ablation of FAK results in a remarkable reduction in proliferation of $p53^{lox/lox}$ and $p53^{R270H}$ cells in neoplastic lesions. We have previously shown that FAK ablation affects mammary gland development and inhibits the lactational differentiation process (Van Miltenburg *et al*, 2009). Other studies indicate a role for FAK in tumour-cell proliferation (Lahlou *et al*, 2007; Provenzano *et al*, 2008). Deletion of FAK did not affect the

growth rate of tumours after they appeared in either $p53^{lox/lox}$ or $p53^{R270H}$ background: typically, after detection by palpation all tumours reached a volume of 1 cm^3 within a few days. Therefore, to determine the role of FAK in mammary tumour development in our models, we quantified the proliferative potential by staining for Ki67 in mammary glands that contained neoplastic and hyperplastic lesions, while no tumour was detected yet. In $p53^{lox/lox}$ and $p53^{R270H}$ mice neoplastic and hyperplastic lesions were evident already at 40 weeks, corresponding to early phases of oncogenic transformation. $P53^{lox/lox}/FAK^{flox/-}$ mammary glands showed a significant reduction in ki67-positive cells compared with $p53^{lox/lox}/FAK^{+/+}$ and $p53^{lox/lox}/FAK^{flox/+}$ mammary glands (Figure 5A and C), while number of apoptotic cells, determined by cleaved caspase-3, was comparable in all groups (Supplementary Figure 3). Confirmatively, reduced proliferation was also observed in $p53^{R270H/+}/FAK^{flox/-}$ mammary glands, albeit not significant, which is related to the limited number of $p53^{R270H}/FAK^{flox/-}$ tumours ($n = 4$), suggesting that also during mammary tumour development FAK is important for proliferation (Figure 5B and D). Taken together, these observations indicate that FAK is required at early stages of tumour initiation but becomes dispensable for expansion of established tumours.

Deletion of FAK does not influence tumour type. $P53^{R270H}$ mice develop mammary tumours that, based on their histomorphological characteristics, did not resemble the mammary tumours that arose in $p53^{lox/lox}$, and mammary tumour latency was significantly shorter in $P53^{R270H}/FAK^{lox/+}$ mice compared with $p53^{lox/lox}/FAK^{lox/+}$ (Supplementary Figure 4A). In human breast cancers, an inverse correlation has been observed between p53 activity and FAK expression. As $P53^{R270H}/FAK^{lox/+}$ tumours had a shorter latency than $p53^{lox/lox}/FAK^{lox/+}$ tumours, we determined whether this effect could be explained by differences in the FAK protein level. Indeed $P53^{R270H}/FAK^{lox/+}$ tumours show a significant higher FAK protein level (Supplementary Figure 4B and C),

Table 2. Tumour spectrum in $FAK^{flox/+}$ and $FAK^{flox/-}$ $p53^{R270H}$ mice		
	$FAK^{flox/+}$	$FAK^{flox/-}$
Total number of mice analysed	13	20
Tumour-bearing mice	9 (69%)	9 (45%)
Mammary gland tumour	9 (69%)	4 (20%)
Carcinoma[a]	9	4
Lymphoma	0	1
Other[b]	0	5
Mean mammary gland tumour latency (wks)[c]	51 ± 8.3	48 ± 8.3

[a]Carcinomas could be classified in adenocarcinoma and solid carcinomas.
[b]Other tumours were found in the lung, bone, and colon.
[c]Mean latency time is depicted as the number of weeks after expression of the $p53^{R270H}$ mutation.
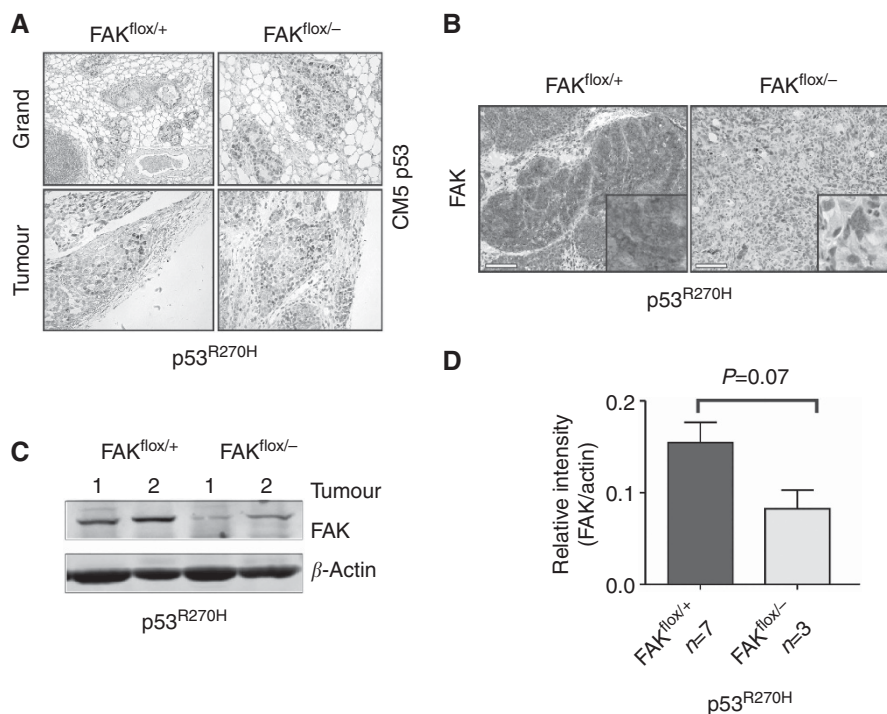
Figure 4. Successful p53 and Fak gene recombination in $p53^{R270H}$/WapCre mice. (**A**) Mammary glands and tumours were stained with p53 CM5 to validate p53 accumulation, which confirms $p53^{R270H}$ expression. (**B**) FAK staining on paraffin sections of mammary tumours. Sections were analysed by light microscopy. (**C**) Western blot analysis was performed on mammary tumour lysates to determine the FAK protein level. (**D**) Quantification of FAK protein level. Results represent the mean of seven and three individual tumours from $p53^{R270H}/FAK^{flox/+}$ and $p53^{R270H}/FAK^{flox/-}$, respectively (± SD), from which a representative western blot is shown in **C**.
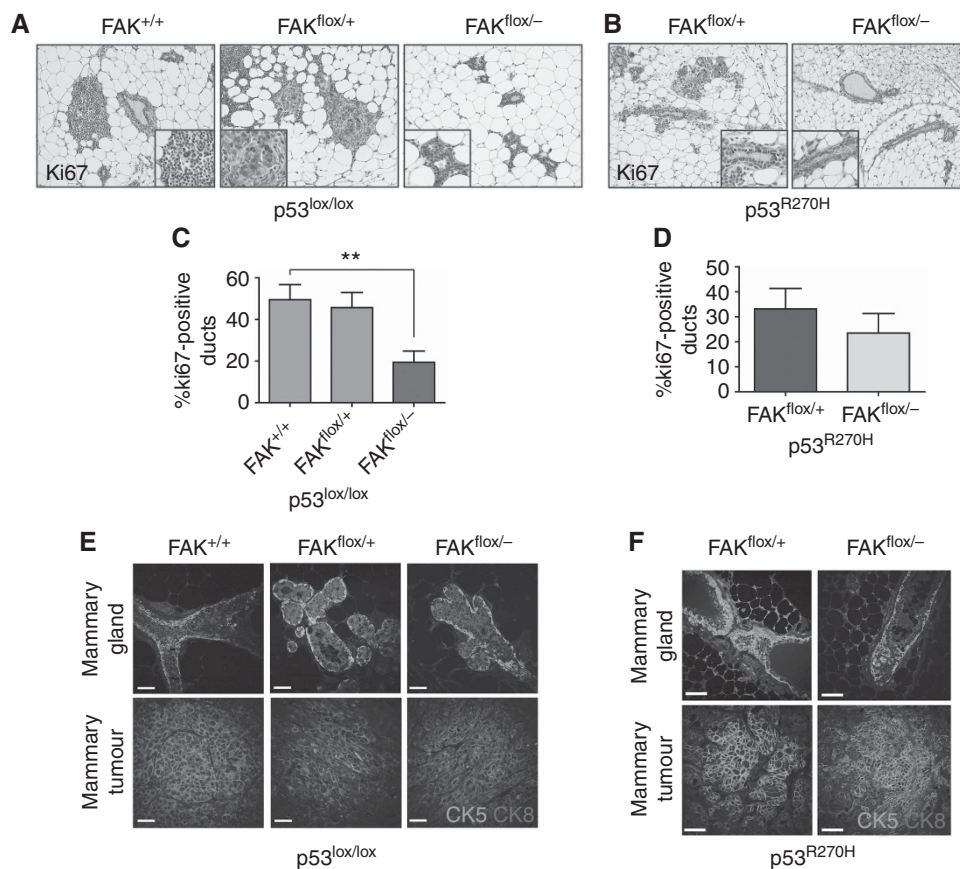
Figure 5. Mammary-specific ablation of FAK leads to reduced proliferation in preneoplastic ductal lesions. (A, B) Mammary glands from $p53^{lox/lox}$/$FAK^{+/+}$ ($n = 17$), $p53^{lox/lox}$/$FAK^{flox/+}$ ($n = 15$) and $p53^{lox/lox}$/$FAK^{flox/-}$ ($n = 11$) mice, and $p53^{R270H}$/$FAK^{flox/+}$ ($n = 6$) and $p53^{R270H}$/$FAK^{flox/-}$ ($n = 15$) mice, obtained over the course of the experiment, were stained for the proliferation marker Ki67. **(C, D)** Percentage of $P53^{lox/lox}$ ($P < 0.01$) and $p53^{R270H}$ ductal structures containing Ki67$^+$ mammary epithelial cells. Five $p53^{R270H}$/$FAK^{flox/-}$ mammary glands were excluded as they did contain too few ductal structures to quantify the ki67-positive cells. **(E, F)** Paraffin sections of $p53^{lox/lox}$ and $p53^{R270H}$ mammary tumours and non-tumour-bearing mammary glands were stained for luminal marker cytokeratin 8 (CK8) and myoepithelial marker cytokeratin 5 (CK5). Fluorescent staining was analysed by confocal laser scanning microscopy. Scale bar is 50 μm.

indicating that mammary tumour formation is dose dependent. This observation is in line with data showing a correlation between FAK protein level and p53 mutations (Golubovskaya *et al*, 2009).

Next, we wondered whether $P53^{R270H}$/$FAK^{lox/+}$ mice develop from the same mammary epithelial progenitors. WapCre is activated predominantly in the luminal compartment (Wagner *et al*, 1997). To determine whether the mammary tumours developed from the luminal-epithelial compartment, we stained all $P53^{R270H}$-driven and $p53^{lox/lox}$-induced mammary glands and tumours for cytokeratin 8 (CK8), a luminal marker, and cytokeratin 5 (CK5), a myoepithelial or basal marker. In contralateral non-tumour-bearing $p53^{lox/lox}$ mammary glands (Figure 5E), expansion of the CK8-positive luminal compartment was evident. Also, with exception of one $FAK^{+/+}$ mice, all tumours were CK8 positive (Figure 5E), indicating that the tumours developed from the luminal compartment. Interestingly, $p53^{R270H/+}$-expressing mammary glands show expansion of CK5- and CK8-positive epithelial cells upon expression of $p53^{R270H}$ (Figure 5F), and mammary tumours that ultimately develop in $p53^{R270H/+}$/$FAK^{lox/+}$ and $p53^{R270H/+}$/$FAK^{lox/-}$ animals were predominantly CK5 positive (Figure 5F). These observations were independent of FAK background. These data suggest that $p53^{R270H}$ induces tumours with basal-like characteristics, which are strikingly different from the mammary tumours developed in the $p53^{lox/lox}$ mouse model, as the $p53^{lox/lox}$ mouse model only gave rise to CK8-positive tumours. Cytokeratin 5 status was confirmed by western blot analysis, again clearly indicating that mammary

tumours that developed in the $p53^{R270H}$ mouse model were CK5 positive (Supplementary Figure 4B). In human patients, a significant association was found between elevated p53 levels, indicative of p53 mutations, and basal-like breast cancer, which is characterised by CK5 expression (Rakha *et al*, 2009). In addition to the expression of cytokeratin 5, gene expression analysis on $p53^{R270H}$ mammary tumours showed elevated levels of several basal markers including Trp63, Krt17, Cryab, Ptn, Col17a1 and Slug (snai2) (Perou *et al*, 2000; Liu *et al*, 2013) (Supplementary Figure 5). This finding is in line with human breast cancer patient data in which p53 missense and truncating mutations are most frequently found in the basal-like subgroup (Cancer Genome Atlas Network, 2012). Thus, p53 null and p53 mutants lead to distinct types of tumours, a feature that is not controlled by FAK.

DISCUSSION

Genetic alterations in the tumour suppressor protein p53 are frequently observed in breast cancer and are often correlated with FAK overexpression. In this study, we determined the role of FAK in $p53^{lox/lox}$- and $p53^{R270H}$-induced spontaneous mammary gland tumour development. We show that mammary neoplasia and hyperplasia are evident in FAK-deleted mammary glands, indicating that FAK does not fully prohibit the initiation of these lesions. However, these lesions contained reduced levels of proliferative

mammary epithelial cells, while no increase in apoptosis was observed. In conjunction, this led to a significant reduction in mammary tumour incidence in both p53-dependent mammary tumour development models, indicating that FAK is essential for the progression of mammary hyperplasia towards mammary tumours.

Comparison of the $p53^{R270H}$ and $p53^{lox/lox}$ mouse models revealed that the expression of $p53^{R270H}$ mutant resulted in accelerated mammary tumour development. These results indicate that the $p53^{R270H}$ point mutation, apart from having a dominant-negative affect on the wild-type p53 allele, may also acquire gain-of-function, which facilitates acceleration of mammary tumour development. Although a gain-of-function for $p53^{R270H}$ was not found by Wijnhoven et al (2007), a recent paper describes that $p53^{R270H}$ enhances the transition from intra-epithelial neoplasia to invasive carcinoma (Heinlein et al, 2008). Interestingly, FAK expression was elevated in $p53^{R270H}$ tumours compared with $p53^{lox/lox}$ tumours, suggesting that the increase in FAK protein contributes to the accelerated tumour development of $p53^{R270H}$-driven tumours.

Interestingly, based on morphology and cytokeratin markers $p53^{R270H}$ and $p53^{lox/lox}$ mammary tumours have a different appearance. Carcinosarcomas, as observed in $p53^{lox/lox}$ mouse model, are relatively uncommon in humans, but based on their appearance would likely be part of the metaplastic breast cancer subtype. Metaplastic breast carcinoma (MBC) is an umbrella term for a heterogenous group of cancers, which are characterised based on the presence of neoplastic cells with squamous or mesenchymal morphology, such as spindle cells. Metaplastic breast carcinomas frequently harbour mutations in the *Trp53* gene, indicating that the $p53^{lox/lox}$ mouse model potentially mimics a rare human breast cancer subtype (Weigelt et al, 2014). Although FAK depletion inhibited the development of mammary gland tumours in both models, FAK did not affect the differentiation status.

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 (Lahlou et al, 2007, 2012; Provenzano et al, 2008; Luo et al, 2009; Pylayeva et al, 2009). In line with our findings, mammary gland-specific *Fak* deletion was shown to reduce the incidence of mammary tumours in the PyMT mice. The studies from Pylayeva et al (2009) revealed that the FAK-deficient PyMT-transformed cells displayed diminished invasive and metastatic capacity. In addition, these cells displayed both growth arrest and apoptosis. After detection by palpation both $p53^{R270H}$ and the $p53^{lox/lox}$ tumours grew within a few days to a size that requires sacrifice of the animals. In these animals, we were unable to detect clear metastatic lesions at the macroscopic level, although we can not exclude that small microscopic lesions were present at distant sites. In addition, it was shown previously that FAK deficiency has a severe impact on mammary cancer stem/progenitor cells. Focal adhesion kinase deletion reduced the pool of cancer stem/progenitors, decreased their self renewal and migration *in vitro*, and comprised their tumorigenicity and maintenance *in vivo* (Luo et al, 2009). The reduction in incidence of mammary tumours we observed in our mouse models could be due to decreased numbers of mammary cancer stem/progenitor cells. This fits with the reduced proliferation capacity in preneoplastic lesions in FAK-null mammary glands. Yet, future studies should clarify a role for FAK in the regulation of the survival and/or proliferation of (tumour initiation) stem/progenitor cells. Regardless, the reduced proliferative capacity of preneoplastic cells in the FAK-deleted mammary glands would reduce the change for additional pro-oncogenic DNA lesions. The differential loss of such a population, which is most likely a dynamic event and progresses over time, may partly explain the bi-phasic effect of the consequences of FAK deletion on mammary gland tumour development. Remarkably, in dept analysis showed that the few tumours that did develop in

$FAK^{flox/-}$ mice were both genetically and phenotypically comparable to $FAK^{flox/+}$ tumours. Possibly, at the time of mammary tumour initiation and early mammary tumour development, FAK may not be efficiently deleted yet. Future next generation exon-sequencing studies of both preneoplastic and neoplastic material could unveil the underlying genetic events and possible escape mechanisms of the suppression of tumour progression in the absence of FAK.

Our data indicate a functional relationship between p53 and FAK in mammary tumour development. Since p53 regulates FAK expression (Golubovskaya et al, 2008), it would therefore be relevant to correlate the protein levels for FAK in human breast cancer patients expressing the $p53^{R273H}$ mutation (the human equivalent of mouse R270H). In addition, mRNA expression analysis on tumour samples from $p53^{R270H}$ and $p53^{lox/lox}$ tumours could provide more insight into pathways that are differentially regulated in these tumours. These data might shed light on additional functions of the $p53^{R270H}$ mutation. As most human breast cancers express mutant p53 and most tumours express elevated levels of FAK, these patients might respond well to FAK inhibitors, ultimately resulting in reduction of tumour size and/or aggressiveness.

In conclusion, we demonstrate that FAK is important in the spontaneous development of mammary tumours in $p53^{lox/lox}$ and $p53^{R270H}$ mouse models through inhibition of cell proliferation.

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