

SHORT COMMUNICATION

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The effects of diet induced obesity on breast cancer associated pathways in mice deficient in SFRP1

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Abstract

Background: Secreted frizzled-related proteins (SFRPs) are a family of proteins that block the Wnt signaling pathway and loss of *Sfrp1* expression is observed in breast cancer. The molecular mechanisms by which obesity contributes to breast tumorigenesis are not well defined, but involve increased inflammation. Mice deficient in *Sfrp1* show enhanced mammary gland inflammation in response to diet induced obesity (DIO). Furthermore, mammary glands from *Sfrp1*^{-/-} mice exhibit increased Wnt signaling, decreased cell death responses, and excessive hyper branching. The work described here was initiated to investigate whether obesity exacerbates the aforementioned pathways, as they each play a key roles in the development of breast cancer.

Findings: Wnt signaling is significantly affected by DIO and *Sfrp1*^{-/-} loss as revealed by analysis of *Myc* mRNA expression and active β -catenin protein expression. Furthermore, *Sfrp1*^{-/-} mice fed a high fat diet (HFD) exhibit an increase in mammary cell proliferation. The death response is also impaired in the mammary gland of *Sfrp1*^{-/-} mice fed a normal diet (ND) as well as a HFD. In response to γ -irradiation, mammary glands from *Sfrp1*^{-/-} mice express significantly less *Bax* and *Bbc3* mRNA, caspase-3 positive cells, and p53 protein. The expression of *Wnt4* and *Tnfs11* are critical for normal progesterone mediated mammary gland development and in response to obesity, *Sfrp1*^{-/-} mice express significantly more *Wnt4* and *Tnfs11* mRNA expression. Evaluation of progesterone receptor (PR) expression showed that DIO increases the number of PR positive cells.

Conclusions: Our data indicate that the expression of *Sfrp1* is a critical factor required for maintaining appropriate cellular homeostasis in response to the onset of obesity.

Keywords: *Sfrp1*, Obesity, Breast cancer, Wnt signaling, Apoptosis, p53, RANKL

Findings

Obesity has increased with an alarming rate in the United States. It is estimated that by 2015, 75% of the population will be either overweight or obese. This fact has elicited a serious public health concern since obesity increases the incidence, progression, and mortality from breast cancer [1]. Cancer results from cellular mutations that enhance proliferation and decrease programmed cell death [2]. Our earlier published studies focused on the role a tumor

suppressor gene, secreted frizzled related protein 1 (*Sfrp1*), plays in mammary gland development and cell death. We revealed that loss of *Sfrp1* alters the growth and behavior of mammary epithelial in such a manner that they exhibit characteristics of breast cancer cells [3,4]. Moreover, *Sfrp1* plays a critical role in mediating the mammary epithelial cellular apoptotic response to DNA damage *in vivo* [5]. Recently, we found that mice deficient in *Sfrp1* (*Sfrp1*^{-/-}) fed a high fat diet (HFD) exhibit a significant increase in body mass, body fat percentage, as well as adipocyte size and have elevated fasting glucose levels and impaired glucose clearance abilities [6]. Additionally, the inflammatory state of mammary glands from *Sfrp1*^{-/-} mice fed a HFD is elevated as revealed by increased macrophage infiltration

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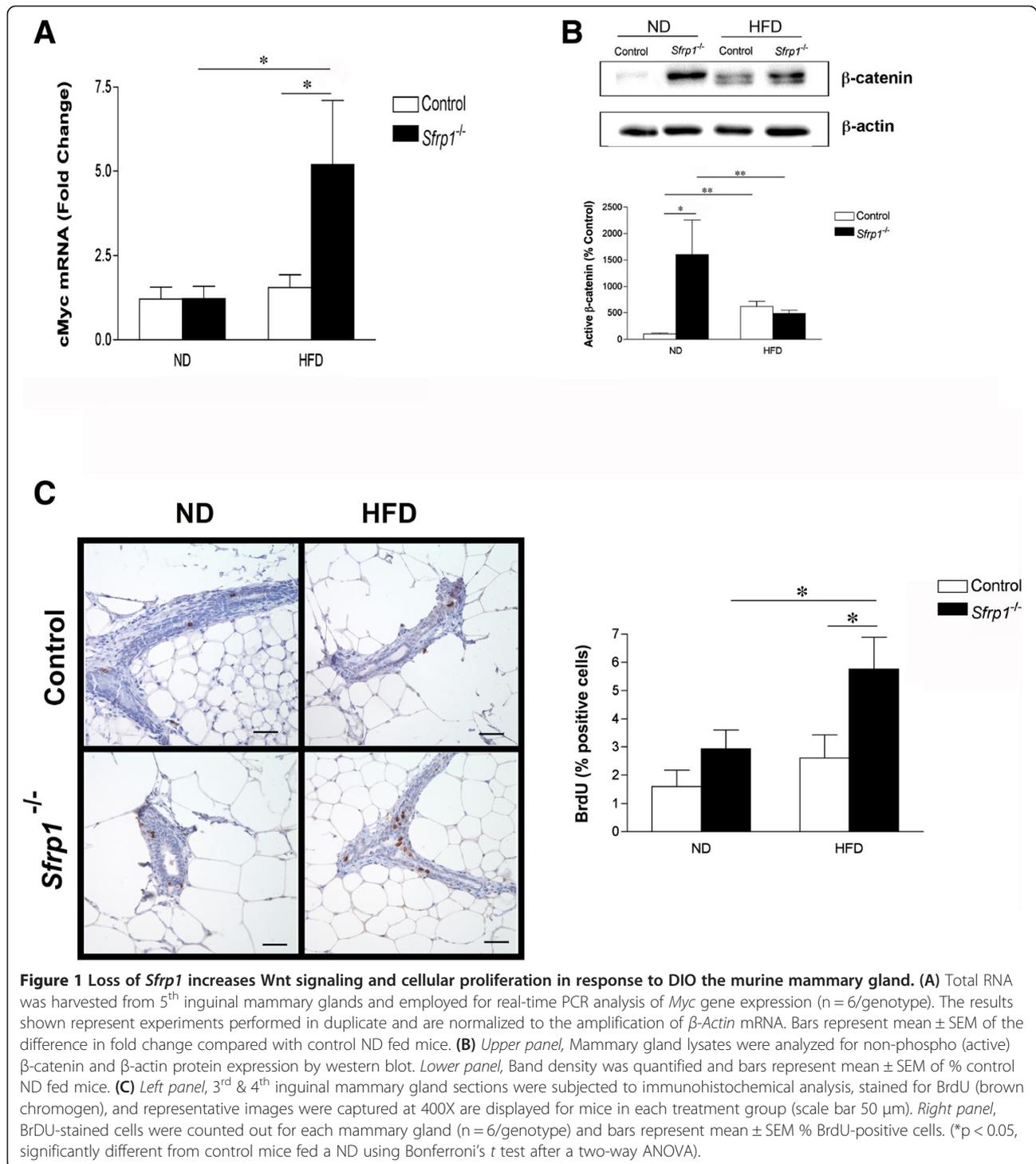
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and pro-inflammatory cytokine expression [6]. Considering the connection between obesity and inflammation, loss of *Sfrp1* may be a critical early event in obesity associated breast cancer initiation.

The Wnt family of secreted proteins is implicated in the regulation of cell fate during development, as well as in cell proliferation, morphology, and migration [7]. The best characterized Wnt pathway is the canonical Wnt/ β -catenin pathway whereby Wnt signaling leads to the stabilization of β -catenin and activation of β -catenin-responsive gene expression. *Sfrp1* antagonizes Wnt signaling by binding to Wnt ligands and preventing ligand-receptor interactions and signal transduction [8]. Indeed, loss of SFRP1 increases Wnt signaling in mammary epithelial cells [3], a deleterious effect considering that inappropriate activation of the Wnt/ β -catenin pathway contributes to the development of breast cancer [7]. To determine whether increased adiposity exacerbates the effect of *Sfrp1* loss on Wnt/ β -catenin signaling, we measured the mRNA expression of the β -catenin target gene, *Myc*, in control and *Sfrp1*^{-/-} mice [9] (Additional file 1: Figure S1) fed a normal diet (ND) and HFD. A two-way ANOVA revealed that *Myc* was significantly affected in response to *Sfrp1* loss on the HFD ($F_{1,17} = 5.17$; $P < 0.05$; $F_{1,17} = 5.23$; $P < 0.05$). In addition, there was a significant interaction between these two main effects ($F_{1,17} = 7.34$; $P < 0.05$) (Figure 1A). These findings are consistent with our recently published results demonstrating that *Axin2*, a hallmark Wnt target gene, is significantly elevated in the mammary gland of *Sfrp1*^{-/-} mice fed a HFD [6]. To investigate whether Wnt signaling is activated in the absence of *Sfrp1*, we employed western blot analysis with a non-phospho (active) β -catenin antibody (Figure 1B, upper panel). Densitometry measurements revealed that the active form of β -catenin was significantly upregulated in response to *Sfrp1* loss ($F_{1,10} = 8.50$; $P < 0.05$) as well as the HFD ($F_{1,10} = 5.94$; $P < 0.05$), but there was no interaction between these two main effects ($F_{1,10} = 1.15$; $P > 0.05$) (Figure 1B). We show that in response to DIO, β -catenin activity was significantly increased, but the absence of *Sfrp1* did not further enhance the expression of active β -catenin. These data may be partially explained by published findings and our previous results which demonstrate that adiposity increases the expression of other Wnt signaling antagonists, including *Sfrp5*, and thus may act to diminish the effect of *Sfrp1* loss on β -catenin activity [10,11]. Given the role Wnt/ β -catenin plays in cellular proliferation, mice were injected with BrdU to evaluate the effect of *Sfrp1* loss and diet induced obesity (DIO) on proliferation. We reveal that the percentage of BrdU positive epithelial cells was significantly increased in response to *Sfrp1* loss ($F_{1,18} = 7.02$; $P < 0.05$) as well as the HFD ($F_{1,18} = 5.10$; $P < 0.05$), but there was no interaction between these two main effects ($F_{1,18} = 1.13$; $P > 0.05$)

(Figure 1C). Although both DIO and *Sfrp1* loss exhibited effects on their own that could participate in an increased risk for cancer, the expression of *Myc* was enhanced by the two main effects together suggesting that a HFD and *Sfrp1* loss, through methylation or mutation, could drive the expression of *Myc* to very high levels and thus work together to promote cancer risk. Thus, in the context of obesity, *Sfrp1* expression is especially important in preventing aberrant Wnt signaling.

Sfrp1 downregulation leads to a resistance to anoikis (apoptosis triggered by loss of attachment) [3]. Resistance to death triggers, due to mutations or loss of attachment, is an important capability for metastasis to occur by allowing cellular survival until colonization in a distant location. *Sfrp1* has been shown to induce apoptosis in numerous tissues [3,12-15] and loss of *Sfrp1* significantly impacts apoptotic related gene expression as well as activity [5] suggesting a causative role for reduced *Sfrp1* in premalignant breast changes leading to tumor progression. Given that loss of *Sfrp1*^{-/-} mice are more resistant to γ -irradiation induced cell death [5], we exposed control and *Sfrp1*^{-/-} mice fed a ND and a HFD to 5Gy whole body irradiation to assess whether loss of *Sfrp1* in our DIO model inhibits death responses. We first measured the expression of *Bax*, a major mediator of pro-apoptotic activity in mammary epithelial cells. Real-time PCR analysis demonstrated that the expression of *Bax* mRNA was significantly affected by *Sfrp1* loss ($F_{1,7} = 9.03$; $P < 0.05$) and the HFD ($F_{1,7} = 6.76$; $P < 0.05$) and there was also an interaction between these two main effects ($F_{1,7} = 4.83$; $P < 0.05$) (Figure 2A). Additionally, we assessed the expression of *Bbc3* (aka PUMA), a key p53 transcriptional target [16]. Our data show that *Bbc3* is significantly repressed in response to *Sfrp1* loss ($F_{1,7} = 6.1$; $P < 0.05$) as well as the HFD ($F_{1,7} = 5.57$; $P < 0.05$), but there was no interaction between these two main effects ($F_{1,7} = 1.41$; $P > 0.05$) (Figure 2A). Caspase-3 is a key intracellular effector of apoptosis by cleaving critical protein substrates required for apoptotic cell death [17]. Immunohistochemical analysis of the cleaved (activated) form of caspase-3 revealed that the immune cells within the lymph node of both genotypes underwent apoptosis serving as an excellent internal positive control for our assay (Figure 2B, left panel). The total number of cleaved-caspase-3 positive luminal epithelial cells were quantified and our data reveal that there was a significant reduction in caspase-3 positive cells of in response to *Sfrp1* loss ($F_{1,7} = 6.37$; $P < 0.05$) as well as the HFD ($F_{1,7} = 5.81$; $P < 0.05$), but there was no interaction between these two main effects ($F_{1,7} = 2.99$; $P > 0.05$) (Figure 2B, right panel). Finally, we wished to look at the effect DIO in *Sfrp1*^{-/-} mice on p53 expression. Consistent with our earlier findings, there are less intensely stained nuclei in *Sfrp1*^{-/-} mice compared to control mice fed a ND. Additionally, p53 expression is diminished in animals



fed a HFD independent of genotype (Figure 2C). Although work confirms previous studies which demonstrate that obesity inhibits cell death responses [18,19], these novel findings are the first to demonstrate that the DIO diminishes mammary epithelial cell death and that the expression of p53 is repressed by DIO in the mammary gland. These data may be partially explained by the elevated insulin

observed levels in these animals [6] as insulin has been shown to reduce apoptosis in mammary epithelial cells *in vitro* [20]. Taken together, our results suggest a possible mechanism by which obesity promotes mammary tumorigenesis.

We previously showed that *Sfrp1*^{-/-} mice exhibit a higher density of ducts with distinct alveoli present

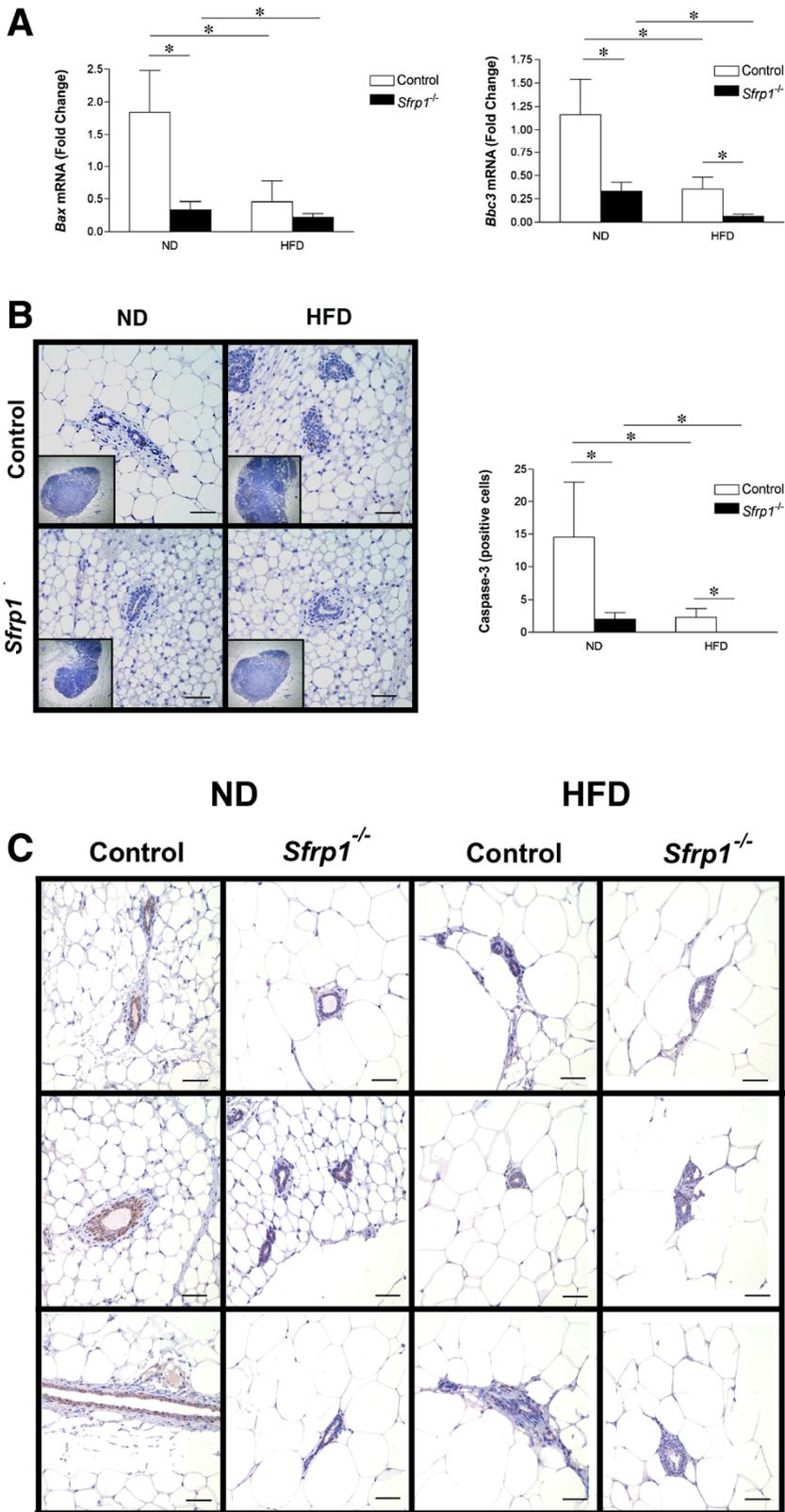


Figure 2 (See legend on next page.)

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Figure 2 Mammary glands from *Sfrp1*^{-/-} exhibit a decrease and cell death signals in response to γ -irradiation. (A) For real-time PCR analysis of *Bax* and *Bbc3* gene expression, total RNA was isolated from the mammary glands of control and *Sfrp1*^{-/-} female mice fed a ND and HFD 6 hours following 5 Gy whole body irradiation (n = 3/genotype). The results shown represent experiments performed in duplicate and are normalized to the amplification of β -Actin mRNA. Bars represent mean \pm SEM of the difference in fold change compared with control ND fed mice. (B) *Left panel*, 3rd & 4th inguinal mammary gland sections were subjected to immunohistochemical analysis, stained for cleaved caspase-3 (brown chromogen), and images were captured at 400X. *Inset*, In addition to capturing 400X photographs of mammary gland ducts, lymph nodes were imaged as a positive control (40X). *Right panel*, The total number of cleaved caspase-3 positive cells was counted for each mammary gland (n = 3/genotype) and bars represent mean \pm SEM cell number. (C) 3rd & 4th inguinal mammary gland sections were subjected to immunohistochemical analysis, stained for p53 (brown chromogen), and representative images were captured at 400X (scale bar 50 μ m). Pictures illustrate the staining results obtained from each γ -irradiated mouse in the study. (*p < 0.05, significantly different from control mice fed a ND using Bonferroni's t test after a two-way ANOVA).

throughout the mammary gland with focal ductal epithelial hyperplasia [4]. These data are fully consistent with previous studies showing that upregulation of the Wnt/ β -catenin pathway and activation of β -catenin in mice induces precocious lobulo-alveolar hyperplasia [21,22]. Constitutive expression of *Wnt4* in the virgin mammary gland also induces structures with a morphology similar to that seen in pregnancy [23] and *Wnt4* is significantly up-regulated in pubescent *Sfrp1*^{-/-} mice. We employed real-time PCR analysis to examine the effects of *Wnt4* in *Sfrp1*^{-/-} mice in response to DIO and a two-way ANOVA revealed that *Wnt4* is significantly increased in response *Sfrp1* loss ($F_{1,19} = 6.44$; $P < 0.05$) as well as the HFD ($F_{1,19} = 4.34$; $P < 0.05$), but there was no interaction between these two main effects ($F_{1,19} = 1.65$; $P > 0.05$) (Figure 3A). The receptor of activated NF- κ B ligand (*Tnfs11* aka RANKL) is a critical downstream target of *Wnt4* [24,25]. Transgenic overexpression of *Tnfs11* into the murine mammary gland elicits ductal side branching, alveologenes, and mammary hyperplasia [26,27]. Furthermore, SFRP1 has been shown to bind to and inhibit *Tnfs11* mediated action [28], and loss of *Sfrp1* increases the expression of *Tnfs11* during puberty. Here we show that *Tnfs11* was significantly increased in response to *Sfrp1* loss ($F_{1,18} = 10.7$; $P < 0.05$) as well as the HFD ($F_{1,18} = 13.7$; $P < 0.05$), but there was no interaction between these two main effects ($F_{1,19} = 1.65$; $P > 0.05$) (Figure 3A). Since *Wnt4* and *Tnfs11* are downstream effectors of progesterone signaling [29], we evaluated progesterone receptor (PR) expression in mammary ducts. Consistent with the literature, immunohistochemical analysis of PR expression illustrated that DIO increases the percentage of PR expressing cells (Figure 3B, *left panel*). The total number of PR positive luminal epithelial cells were quantified and a two-way ANOVA confirmed that there was no difference in the percentage PR expressing cells response to *Sfrp1* loss ($F_{1,19} = 0.913$; $P > 0.05$), but the HFD significantly increased PR expression ($F_{1,19} = 5.55$; $P < 0.05$), although there was no interaction between these two main effects ($F_{1,7} = 0.8253$; $P > 0.05$) (Figure 3B, *right panel*). Thus, the DIO-induced increase in PR expression may exacerbate the expression of *Wnt4* and *Tnfs11* in *Sfrp1*^{-/-}

mice. The expression of *Sfrp1* is critical for maintaining proper mammary gland development and considering that the deleterious effects of *Sfrp1* depletion are exacerbated in response to DIO, loss of *Sfrp1* in the context of obesity may be a critical event in cancer initiation. Additionally, the increased adiposity and decreased death response observed in *Sfrp1*^{-/-} mice may lead to increased breast cancer susceptibility. Future studies are aimed at elucidating the molecular mechanisms by which obesity and *Sfrp1* downregulation affect tumorigenesis.

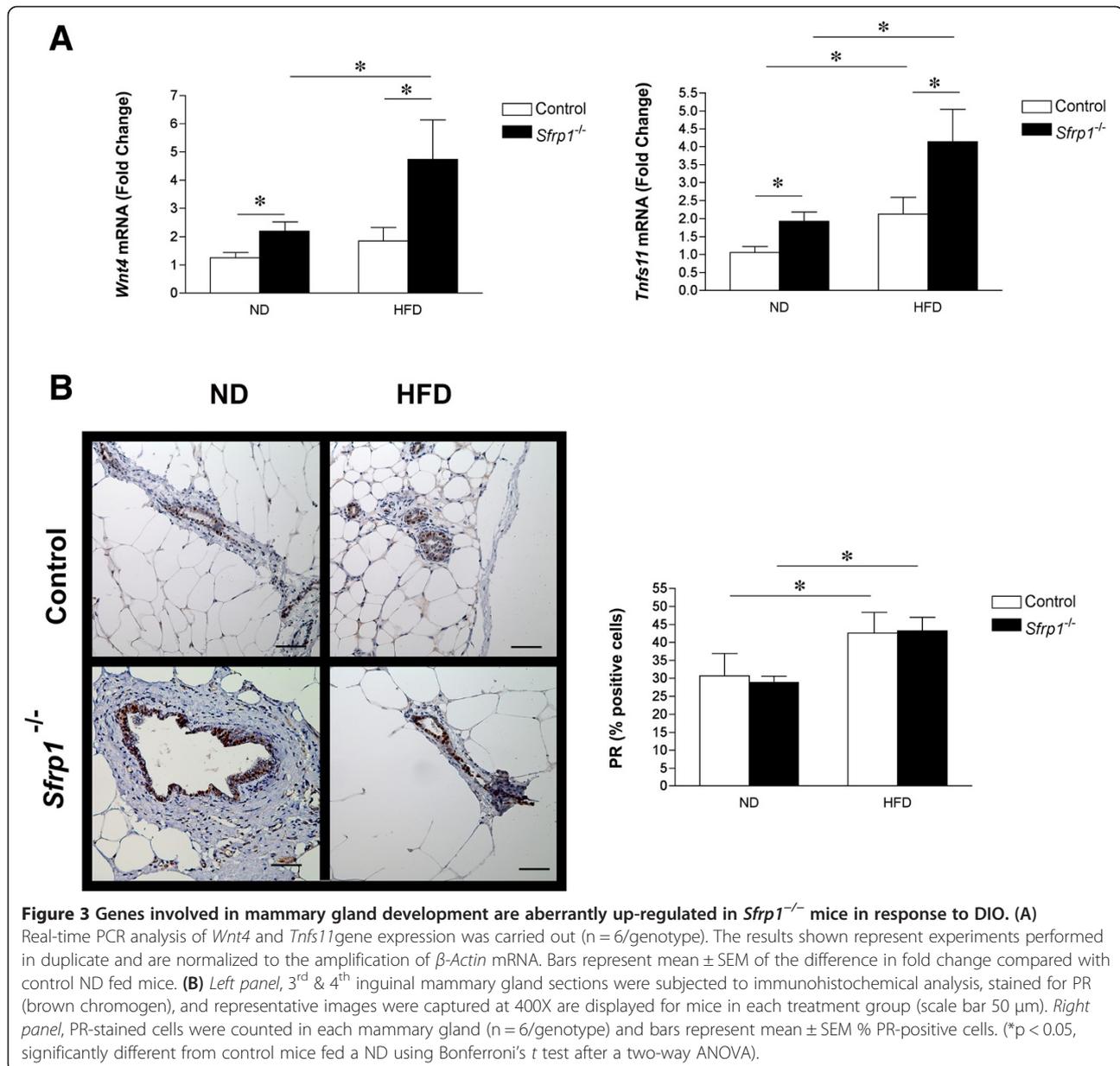
Materials and methods

Animals

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Baystate Medical Center Institutional Animal Care and Use Committee (Permit Number: 283237). Female 129/C57Blk6 control mice (n = 20) and 129/C57Blk6 *Sfrp1*^{-/-} mice (n = 20) were individually housed in plastic cages with food and water provided continuously, and maintained on a 12:12 light cycle. Mice (n = 10/genotype) were placed on either a normal diet [(ND) Harlan Teklad global 18% protein rodent diet (#2018) containing 2.8% fat, 18.6% protein] or placed on a high fat diet [(HFD) Bio-Serv (#F1850) containing 36.0% fat, 36.2% carbohydrate, and 20.5% protein] starting at 10 weeks of age for 12 weeks. Mice were injected 70 μ g/g body weight of 5-bromo-2-deoxyuridine (BrdU; Sigma, St Louis, MO) and the glands will be harvested 24 hours later. A select number of mice from each treatment group (n = 3) were subjected to 5 Gy of whole body γ -irradiation to induce DNA damage and mammary glands were harvested 6 hours later. Animals were euthanized by CO₂ followed by cervical dislocation and bled by cardiac puncture. The 3rd and 4th mammary glands were fixed in buffered formalin and 5th inguinal glands were flash frozen.

Genotyping

Tail DNA was obtained from control (*Sfrp1*^{+/+}), heterozygous (*Sfrp1*^{+/-}), and homozygous knockout (*Sfrp1*^{-/-}) mice as well as breeding pairs used to generate mice for



our study as described previously [9]. PCR amplification was carried out using the Typeit Mutation Detect PCR Kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). Primer sequences used in the reaction were as follows: *SacII* forward, 5'-GATTGGTAACTGCGCGGCTG-3'; *SacII* reverse, 5'-GACTGGAAGCTCACGTAGTCG-3'; *LacZ* forward, 5'-TTCAGTGGCCGTCGTTTTACAACGTC-3'; *LacZ* reverse, 5'-TTCAGTGGCCGTCGTTTTACAACGTC-3'. *SacII* primers predicted to amplify 510 bp wild-type allele and *LacZ* primers predicted to amplify 364 LacZ target used in the generation of *Sfrp1* knockout mice. The conditions for the target DNA amplification were performed as follows: 1 cycle of 95°C for

15 min; 40 cycles each of 95°C for 30 s, 54.7°C for 1 min, and 72°C for 30 s; and 72°C for 10 min.

RNA isolation and real-time PCR analysis

Total RNA was extracted from the 5th inguinal mammary glands using an acid-phenol extraction procedure according to the manufacturer's instructions (Trizol, Invitrogen, Carlsbad, CA). Relative expression levels of mRNA was determined by quantitative real-time PCR using the Mx3005P™ real-time PCR system (Stratagene, La Jolla, CA) and all values were normalized to the amplification of β -Actin. PCR primers used for *Sfrp1* were as follows: *Sfrp1* forward, 5'-CACAACGTGGGCTACAAGAA-3'; *Sfrp1*

reverse, 5'- TCACCTCTGCCATGGTCT-3'. All other PCR primer sequences have been described previously [3-5]. The assays were performed using the 1-Step Brilliant® SYBRIII® Green QRT-PCR Master Mix Kit (Stratagene) containing 200 nM forward primer, 200 nM reverse primer, and 100 ng total RNA. The conditions for cDNA synthesis and target mRNA amplification were performed as follows: 1 cycle of 50°C for 30 min; 1 cycle of 95°C for 10 min; and 35 cycles each of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s.

Western blot analysis

The 5th inguinal mammary glands were homogenized in cold lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 100 mM NaF, 10 mM MgCl₂, 0.5% NP40, protease inhibitor cocktail, and phosphatase inhibitor I and II (Sigma)]. The lysates were passed 4 times through a 26 gauge syringe, kept on ice for 30 minutes, and then centrifuged for 20 minutes at 12,000 rpms at 4°C. The supernatant was transferred to a new tube and the protein was quantified utilizing the BCA™ Protein Assay Kit (Pierce, Rockford, IL). A total of 35 µg of protein was run on a 10% SDS-Page gel and transferred to a PVDF membrane. The membrane was blocked for 45 minutes with 5% milk in tris-buffered saline containing 0.05% Tween-20 (TBS-T). The primary antibodies used in this study were as follows: Rabbit non-phospho (Active) β-catenin (Ser33/37/Thr41) (D13A1) 1:1000 (Cell Signaling Technology, Danvers, MA); Rabbit β-actin 1:2000 (Abcam, Cambridge, MA). The primary antibodies were incubated over overnight at 4°C and the secondary antibody [goat anti-rabbit IgG-HRP 1:5000 (Santa Cruz Biotechnology, Dallas, TX) was incubated for 45 minutes at room temperature. The blot was washed and developed using a Western Blot Luminol Reagent (Santa Cruz Biotechnology) and imaged with a Synopsics 4.2 MP camera and G:Box Chemi-XT4 GENESys software (SYNGENE, Frederick, MD). Band density was quantified with Image J software).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on a Dako-Cytomation autostainer using the Envision HRP Detection system (Dako, Carpinteria, CA). Each mammary tissue block was sectioned at 4 µm on a graded slide, deparaffinized in xylene, rehydrated in graded ethanols, and rinsed in Tris-phosphate-buffered saline (TBS). Heat induced antigen retrieval was performed in a microwave at 98°C in 0.01 M citrate buffer. After cooling for 20 minutes, sections were rinsed in TBS and subjected to the following primary antibodies: Rat monoclonal anti-BrdU 1:100, (Abcam); Rabbit polyclonal anti-Cleaved Caspase-3 (Asp 175) 1:100 (Cell Signaling); Rabbit polyclonal anti-p53 antibody (CM5) 1:1000 (Leica, Wetzlar, Germany); Rabbit polyclonal anti-PR (C-19) 1:100, (Santa Cruz Technologies) for 45 minutes.

Immunoreactivity was visualized by incubation with chromogen diaminobenzidine (DAB) for 5 minutes. Tissue sections were counterstained with hematoxylin, dehydrated through graded ethanols and xylene, and cover-slipped. Images were captured with an Olympus BX41 light microscope using (SPOT™ Imaging Solutions, Detroit, MI).

Statistical analysis

Results were analyzed using a two-way ANOVA with *Sfrp1* loss and HFD treatment as the main effects unless otherwise stated. *Post hoc* tests, where appropriate, were performed by Bonferroni's *t* test. Bonferroni's *t* test uses the mean square error from the ANOVA table as a point estimate of the pooled variance (Graphpad Prism, San Diego, CA). Grubb's test was used on all data to identify statistical outliers (<http://www.graphpad.com/quickcalcs>). Statistical outliers were identified in some data sets, but the overall results were not altered by omission. A few samples were lost during processes; therefore, there are some unequal sample sizes.

Additional file

Additional file 1: Figure S1. Validation of *Sfrp1* mutation and *Sfrp1* mRNA loss in the mammary glands of *Sfrp1*^{-/-} mice. **(A)** PCR analysis of tail DNA from breeding pairs used to generate mice for the experiments described in the manuscript. Gel electrophoresis revealed that the *Saclf* and *Saclr* primer set yielded a 510-bp wild-type specific fragment by PCR in *Sfrp1*^{+/+} and *Sfrp1*^{+/-} mice and the *LacZf* and *LacZr* primer set yielded a 364-bp fragment in *Sfrp1*^{+/-} mice and *Sfrp1*^{-/-} mice as well as all breeders used for the study. **(B)** Total RNA was harvested from the 5th inguinal mammary glands of mice used in the described experiments and employed for real-time PCR analysis of *Sfrp1* gene expression (n = 6/genotype). The results shown represent experiments performed in duplicate and are normalized to the amplification of β-Actin mRNA. Bars represent mean ± SEM of the difference in fold change compared with control mice. (****p < 0.05, significantly different from control mice using student's t-test).

Abbreviations

Sfrp1: Secreted frizzled related protein 1; DIO: Diet induced obesity; ND: Normal diet; HFD: High fat diet; BrdU: 5-bromo-2-deoxyuridine; Bax: Bcl2 Associated X protein; PUMA: p53 upregulated modulator of apoptosis; Bbc3: Bcl2 binding component 3; Caspase: Cysteine aspartic acid specific protease; RANKL: Receptor of activated NF-κB ligand; Tnfs11: Tumor necrosis factor ligand superfamily member 11; PR: Progesterone receptor.

Competing interests

The authors do not have any financial or personal relationships with other people or organizations that could inappropriately influence the work described in this manuscript.

Authors' contributions

KG drafted the manuscript and performed all of the described experiments with the exception of the immunohistochemistry. AS provided our laboratory with the *Sfrp1*^{-/-} mice. LB and EH contributed to the mouse work. JW and JS processed the tissues and carried out the immunohistochemistry. SS participated in the study design, edited the manuscript, and gave final approval of the version to be published. All authors read and approved the final manuscript.

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