Sfrp3 modulates stromal–epithelial crosstalk during mammary gland development by regulating Wnt levels

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Mammary stroma is essential for epithelial morphogenesis and development. Indeed, post-natal mammary gland (MG) development is controlled locally by the repetitive and bi-directional cross-talk between the epithelial and the stromal compartment. However, the signalling pathways involved in stromal-epithelial communication are not entirely understood. Here, we identify Sfrp3 as a mediator of the stromal-epithelial communication that is required for normal mouse MG development. Using Drosophila wing imaginal disc, we demonstrate that Sfrp3 functions as an extracellular transporter of Wnts that facilitates their diffusion, and thus, their levels in the boundaries of different compartments. Indeed, loss of Sfrp3 in mice leads to an increase of ductal invasion and branching mirroring an early pregnancy state. Finally, we observe that loss of Sfrp3 predisposes for invasive breast cancer. Altogether, our study shows that Sfrp3 controls MG morphogenesis by modulating the stromal-epithelial cross-talk during pubertal development.
The mammary gland (MG) is one of the few organs that undergoes most of its development postnatally. At puberty, the ducts of the rudimentary embryonic tree start to invade the mammary fat pad through a process of ductal elongation and bifurcation, driven by the terminal end buds (TEBs) structures located at the tips of the ducts, and through a process of secondary side branching. Once the ducts reach the edges of the mammary fat pad, the TEBs regresses and the arborized ductal tree remains quiescent until pregnancy, except for the formation of tertiary side branches at each oestrus cycle. During pregnancy a massive process of tertiary side branching results into lobuloalveolar structures that ultimately differentiate into alveoli, producing and secreting milk during lactation. At weaning, the MG reverts to the adult virgin-like MG state. During each phase of MG development profound changes in cell proliferation, apoptosis and differentiation occur, allowing a constant remodelling of the gland structure.

The MG is composed of two compartments, the epithelial ducts and the stroma in which ducts are embedded. Postnatal MG development is controlled globally by systemic hormones and locally by the repetitive and bi-directional cross-talk between the epithelial and the stromal compartment. Many studies have shown that mammary stroma instructs differentiation and branching of mammary epithelial cells through paracrine signals, and sustains cell fate maintenance during postnatal life. The paracrine signalling between epithelial and stromal cells is based on secreted factors like the Wnt ligands.

The Wnt signalling pathway control multiple steps of MG development and dysregulation of Wnt signalling has been associated with breast cancer (BC). While mutations in the core genes of the canonical Wnt signalling pathway, namely βcatenin, APC, or axin2, are rare, loss by epigenetic silencing of Wnt inhibitors like secreted frizzled-related protein (Sfrp) family members is more frequent, and it has been associated with human BC.

The Sfrps are a family of secreted glycoproteins composed of five members in mammals, containing an N-terminal domain homologous to the cysteine-rich domain (CRD) of the Frizzled Receptor and a C-terminal domain with some homology to the netrin domain (NTR). Sfrp3 (also known as FRZB) is considered as the founding member of the Sfrp family, and identified as an inhibitor of Wnt signalling. Sfrp3 has an essential role in bone development and cartilage homeostasis, dependent and independent of Wnt signalling. Sfrp3 is expressed in stromal cells compared to relatively low levels present in the epithelial compartment, which includes basal/myoepithelial and luminal cells. Furthermore, we detected Sfrp3 mRNA in the mammary cleared fat pad, showing a decline during puberty. To better dissect the source of Sfrp3, we isolated the most abundant cell populations of the stroma, and we identified that Sfrp3 is mostly expressed by fibroblasts.

In summary, we found that Sfrp3 is highly expressed at prepuberty before mammary ducts morphogenesis occurs, and it is more abundant in the stromal compartment. The developmentally regulated pattern of expression of Sfrp3 suggests a potential role of Sfrp3 on MG development.

**Sfrp3 prevents early ductal invasion and side branching.** To address the function of Sfrp3 during MG development, we used the Sfrp3 knockout mouse. Homozygous (Sfrp3−/−) mice are viable with no apparent morphological postnatal phenotype. We confirmed that Sfrp3 expression is absent in Sfrp3−/− mice in MGs. To detect defects in MG morphology, we examined wild-type and Sfrp3−/− virgin females with matching oestrous cycles at different developmental stages. Both wild-type and Sfrp3−/− mice present a regular mammary epithelial tree in pre-puberty. Differences start to be evident at mid-puberty, when epithelial ducts are markedly longer in Sfrp3−/− mice, almost reaching the edges of the fat pad. At the end of puberty, Sfrp3−/− ducts not only show an accelerated invasion into the fat pad, but also a four-fold increase increased side branching. Branches terminate with small protrusions, lobuloalveolar buds, which are typical of pregnancy. This precocious hyperbranched phenotype persists in adult Sfrp3−/− (Fig. 1d, e). These results suggest that Sfrp3 controls MG development by preventing unwarranted mammary epithelial invasion and branching during puberty.

**Sfrp3 prevents ducts defects and altered differentiation.** To determine whether structural defects in Sfrp3−/− epithelial ducts, we histologically analysed MG. Sfrp3−/− MG sections confirmed the hyperbranched phenotype and further revealed a lumen filling phenotype of the ducts (Fig. 2a). Whereas control ductal structures are characterised by a normal hollowed lumen, in Sfrp3−/− MGs the lumen of 30% ducts is partially or entirely occluded (Fig. 2a, b). Sfrp3−/− ducts display remarkable alterations in the

MMTV-Neu animals induces the precocious appearance of premalignant lesions, suggesting a role for Sfrp3 as a marker for BC susceptibility. Collectively, our results show that stromal Sfrp3 regulates the Wnt-mediated epithelial–stromal interactions in the MG development.

**Results**

**Stromal and epithelial cells of the MG express Sfrp3.** In order to investigate the role of Sfrp3 in MG morphogenesis, we examined the expression of Sfrp3 during MG development. Sfrp3 mRNA expression is high at the pre-pubertal stage (3 weeks old) and decreases throughout puberty (6–8 weeks old). During pregnancy, its expression is further reduced (Fig. 1a). We also examined the expression of all Sfrp family members during MG development. Similarly, Sfrp2, 4 and five mRNA expression are high at the pre-pubertal stage and decreases throughout puberty, whereas, the levels of sfrp1 increases (Supplementary Fig. 1a). To determine which cellular compartment within the MG expresses Sfrp3, we separated stromal and epithelial cells from adult MGs (12 weeks old), based on their expression of CD49f and EpCAM cell surface markers (Supplementary Fig. 1b). Sfrp3 is highly expressed in stromal cells compared to relatively low levels present in the epithelial compartment, which includes basal/myoepithelial and luminal cells (Fig. 1c). Furthermore, we detected Sfrp3 mRNA in the mammary cleared fat pad, showing a decline during puberty (Fig. 1b). To better dissect the source of Sfrp3, we isolated the most abundant cell populations of the stroma, and we identified that Sfrp3 is mostly expressed by fibroblasts (Supplementary Fig. 1c).

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structure of the luminal compartment, while the basal compartment is normal (Fig. 2c). The inner luminal epithelium of Sfrp3−/− ducts is abnormally organised into multiple layers, filling in the luminal space of ducts and branches, compared to the single inner layer of luminal cells of the controls (Fig. 2c, d). Next, we analysed the distribution of polarity markers. In wild-type ducts, E-cadherin and ZO1 are localised at adherens and tight junctions, respectively, of luminal cells (Fig. 2e). In Sfrp3−/− ducts only the most apically localised luminal cells express E-cadherin and ZO1, which have both a normal distribution, comparable to controls. The expression of ZO1 is absent in the most internal cells, whereas E-cadherin distributes uniformly and widely along the entire cell membrane of Sfrp3−/− ducts (Fig. 2e).

Next, we determined whether the multistratified arrangement of Sfrp3−/− luminal cells is due to either an increase in proliferation rate or a decrease in apoptosis rate. In control MG only a few cells are proliferating, and almost no apoptotic cells are
detected (Fig. 3a, b), which is consistent with the entering of post-pubertal virgin females into a quiescent stage until pregnancy. There is a dramatic increase in the level of proliferation in Sfrp3−/− mature luminal cells, whereas the number of apoptotic cells is similar to control MGs (Fig. 3a–c). Accordingly, gene expression profiling of mammary epithelial cells from adult Sfrp3−/− mice showed an mRNA increase of classical cell cycle markers such as Ccnd2, Klf5, Hhf2, Id4 (Supplementary Data 1). Luminal cells are also classified by their expression of hormone receptors, particularly oestrogen receptor (ER). While ducts contain both ER− and ER+ luminal cells, alveolar luminal cells are mainly ER−20,21. We identified a substantial decrease in the ER+ population in the Sfrp3−/− MGs (Fig. 3d, e), that suggest a precocious alveolar development of Sfrp3−/− MGs.

The phenotype of Sfrp3−/− MGs resembles the state of early pregnancy in wild-type mice (P8.5) when epithelial cells undergo a process of proliferation and differentiation to form acini-like structures that ultimately mature into alveoli—the milk secretory structures—during pregnancy and lactation. This similarity, together with the critical increase in the number of ER− cells, suggested an altered differentiation of Sfrp3−/− MGs. Remarkably, MGs of adult virgin Sfrp3−/− virgin females expressed high levels of β casein compared to controls (Fig. 3f, g). β casein was detected inside the lumen and within luminal cells with a subapical localisation in Sfrp3−/− mammary ducts (Fig. 3f). Consistently, we detected higher levels of other two milk proteins, whey acidic protein (WAP) and alpha-lactalbumin (Lalba), in virgin Sfrp3−/− glands (Fig. 3g). Further, the expression of the lactating alveolar marker sodium–inorganic phosphate (NaPi)-type Ib is substantially increased (Fig. 3h). This premature differentiation does not impair the capability of Sfrp3−/− MGs to undergo the normal morphological changes associated with pregnancy (Supplementary Fig. 2a, b), suggesting that alveoli are functional and milk can flow through ducts, despite the occlusion of the luminal space in Sfrp3−/− MGs.

We further investigated whether Sfrp3−/− showed alterations in the stromal compartment. While we found some differences in the extracellular matrix organisation between wild-type and Sfrp3−/− MGs, they were not statistically relevant (Supplementary Fig. 2c–f).

Together, these observations suggest that loss of Sfrp3 leads to a disruption of the mammary ductal structures in virgin females, with overproliferating, and weakly polarised ER− luminal epithelial cells that arrange in multiple layers facing the ductal lumen and that are abnormally differentiated to a pregnancy state.

Sfrp3 is expressed by the stroma to control MG development. Since both stromal and epithelial cell compartments expressed Sfrp3 mRNA (Fig. 1c), we wondered whether the branching phenotype of Sfrp3−/− mice is due to loss of Sfrp3 either in the stroma or in the epithelium. To address this issue, we performed mammary epithelial transplantation experiments, consisting in the transplantation of mammary epithelial tissue from a donor adult mouse into the cleared fat pad of a recipient prepubertal mouse. Five weeks after transplantation we analysed the transplanted and the contralateral MGs (Supplementary Fig. 3a, b).

As expected, wild-type mammary tissue, when transplanted into wild-type cleared fat pad, grew and developed into an epithelial tree showing a regular branching pattern, while Sfrp3−/− transplanted epithelial cells into an Sfrp3−/− cleared fat pad formed hyperbranched epithelial ducts (Supplementary Fig. 3c). When Sfrp3−/− mammary epithelial cells were transplanted into wild-type fat pad, they grew and invaded the stroma, giving rise to a regular alveolar ductal tree similar to that observed in the contralateral MG (Fig. 4a, b). We obtained similar results with immunocompromised females as transplant recipients (Supplementary Fig. 3b, d). In contrast, wild-type tissue transplanted into Sfrp3−/− stroma generated almost four-fold increase hyperbranched epithelial network, comparable to that observed in the Sfrp3−/− MGs (Fig. 4a–c). We obtained a similar phenotype when wild-type tissue was transplanted into Sfrp3−/− cleared fat pad, and allocated in immunocompromised females, which discards a systemic effect of Sfrp3 (Supplementary Fig. 3f).

These results suggest that loss of Sfrp3 in the stroma is likely the cause of Sfrp3−/− phenotype in MGs. Indeed, wild-type outgrowths transplanted in Sfrp3−/− stroma show a phenotype similar to Sfrp3−/− MGs, with multilayering of luminal epithelial cells, polarity defects, and precocious alveolar differentiation (Fig. 4d, e), which suggests that loss of stromal Sfrp3 leads to loss of ductal architecture. In contrast, Sfrp3−/− outgrowths transplanted in wild-type fat pad form a network of ducts with a proper architecture (Fig. 4d, e, Supplementary Fig. 3e).

Consistently with the transplantation results, we did not detect any substantial difference between wild-type and Sfrp3−/− mammary epithelial organoids in terms of either organoid structure/size or lumen volume. Also, in the absence of growth factors neither wild-type nor Sfrp3−/− organoids form branches (Supplementary Fig. 4a). Neither, we did observe any substantial difference in the frequency of wild-type and Sfrp3−/− epithelial cells able to generate mammospheres, which is used to estimate the number of mammary stem cells (MaSC) in the epithelial tissue22 (Supplementary Fig. 4a). Together, these results indicate that the abnormal features of the ductal phenotype in Sfrp3−/− MG arise from loss of Sfrp3 in the stroma, rather than in the epithelium.

Sfrp3 functions in a hormonally stimulated environment. Ovarian oestrogen and progesterone are the central systemic regulators of MG development during puberty and pregnancy. Higher levels of progesterone23 accompany the formation of alveolar-like structures during the diestrus phase of the oestrous cycle and pregnancy23. In the absence of ovarian hormones, Sfrp3−/− ducts do not elongate and do not show any alteration in branching rate or structural organisation (Supplementary Fig. 4b). Additionally, we detected no increase in the serum levels of oestrogen and progesterone in Sfrp3−/− females.
Sfrp3 modulate of Wnt signalling in MG development. Taking into account that Wnt signalling pathway has been traditionally associated with MG development, and Sfrps are regulators of Wnt signalling, we hypothesise that Sfrp3 might control mammary development through modulating Wnt signalling. However, Sfrps present roles that could be either dependent or independent of Wnt.
To address the role of Sfrp3 we used the wing imaginal disc of *Drosophila*, a well-studied model to investigate morphogens during development. *Drosophila* represents a natural Sfrp null background since no *Sfrp* homologue has been identified in its genome. We observed that ectopic expression of Sfrp3 in the posterior compartment of the *Drosophila* wing imaginal disc (driven by HhGal4) does not interfere with the symmetrical gradient of Wingless (Wg) at the dorsoventral boundary of the wing imaginal disc, but reduced its peak of concentration there (Fig. 5a, b). Sfrp3 expression also dramatically abolishes the expression of Senseless, a canonical target of Wg, but not the expression of Cut, a target of Notch signalling (Fig. 5c, d). Consistently, the adult wings of *Drosophila* expressing NubGal4>UAS-Sfrp3 show a lack of sensory organs at the wing margins, which is a characteristic phenotype of the inhibition of Wg pathway (Fig. 5c). These results suggest that Sfrp3 acts as an inhibitor of Wg pathway in *Drosophila* wing-imaginal-disc. To validate this hypothesis, we also characterised the effect of Sfrp3 on Distalless (dll), a Wg target that response to low Wg concentrations. We observed that Sfrp3 expression does not inhibit dll, but instead broadens the dll territory in the P compartment (Fig. 5f). Thus, while the effect on Sen seems to support the conclusion that Sfrp3 overexpression inhibits Wg, the effect on dll suggests that Sfrp3 expression might induce the transport and diffusion of Wg, reducing its concentration at the D/V boundary. This change in Wg would inhibit Sens
(a high-concentration Wg-target) but expand dll territory (a low-concentration Wg-target). In order to prove this role of Sfrp3 in the transport and diffusion of Wg, we used a membrane-bound form of Wg (NRT-Wg), described before to allow persistent expression of relevant target genes. Importantly, the expression of Sfrp3 in the posterior compartment did not affect Sens (Fig. 5g), demonstrating that Sfrp3 is not a repressor or inhibitor of Wg, but more likely a transporter of Wg that allow the spreading of this morphogen.

Next, we investigated whether canonical Wnt signalling is abnormally activated in Sfrp3−/− MGs. Next-generation sequencing (NGS) analysis of sorted adult MG epithelial cells showed a
substantial up-regulation of LEF1 and cMYC in Sfrp3−/− epithelial cells (Supplementary Data 1). However, the expression levels of most canonical target genes like βcatenin, axin2, and cyclinD1 are unchanged in Sfrp3−/− adults (Supplementary Fig. 5, Supplementary Data 1). By contrast, we observed a substantial nuclear accumulation of βcatenin in pubertal Sfrp3−/− MGs (Fig. 6a, b), when the levels of expression of Sfrp3 are close to the maximum level (Fig. 1a). Together these results demonstrate that Sfrp3 acts as a specific modulator of canonical Wnt pathway.

Previous studies have shown that Wnt family members are differentially regulated in mouse MGs during puberty and pregnancy26. Since Sfrp3−/− MGs mimics an early pregnancy state, we investigated whether the loss of Sfrp3 associates with an alteration of Wnt ligands. We defined the cellular compartments that express these Wnt ligands by cell sorting and qPCR analyses. We determined that Wnt2 is more abundant in the stroma, while Wnt5a and Wnt7b are more expressed in the epithelial cells in adult virgin MGs (Fig. 6c). In line with this, NGS analysis detected shallow levels of Wnt2 and higher levels of Wnt5a and Wnt7b in wild-type epithelial cells (Supplementary Data 1). Furthermore, we found that Wnt2 protein accumulated at the stromal–epithelial boundary in Sfrp3−/− (Fig. 6d), while de-epithelialised cleared fat pads of adult animals presented reduced levels of expression of Wnt2 (Fig. 6e), which is consistent with the decrease we observed in pregnant mice for Wnt2 (Supplementary Fig. 6). This data suggests that Sfrp3 function might be related to control the accumulation of Wnt2 at the stromal–epithelial boundary since the loss of stromal Sfrp3 leads to an accumulation of Wnt2 in the boundary, which is consistent with the role found for Sfrp3 in Drosophila before. Altogether, our results suggest that stromal Sfrp3 mediates the crosstalk between stroma and epithelium through the modulation of stromal Wnt2 ligand.

To obtain a more precise idea of the molecular mechanism of Sfrp3, we studied the secretion of SFRP3 in vitro and its interaction with Wnt ligands. We found that myc-tagged SFRP3 requires the NTR domain to be secreted (Fig. 6f). Next, we analysed the interaction of SFRP3 with different Wnts present in the MGs. SFRP3 binds strongly to Wnt2 that is expressed by the stromal cells, and to a lesser extent with Wnt5a or Wnt7b expressed by the epithelial cells (Fig. 6g). Furthermore, we observed that the NRT domain of SFRP3 binds to all Wnts analysed, but this interaction is particularly strong with Wnt2 (Fig. 6h). The specific interaction of the NRT domain of Sfrp with Wnts/Wg was previously described for SFRP125,26, another member of the family. These results, combined with our previous data, suggest that SFRP3 interacts with Wnt2 to control the concentration levels of Wnt2 in the boundary of the stromal–epithelial mammary.

Loss of Sfrp3 associates with cancer. Sfrp3−/− phenotype might resemble early stages of carcinogenesis27,28. However, we could not detect any defined tumour lesion like focal hyperplasia, disruption of the myoepithelial layer or change in collagen content in the Sfrp3−/− mice. Neither, did we observe any palpable tumour mass, or macroscopic lesion in nulliparous Sfrp3−/− females of 12/15 month of age. We also monitored Sfrp3−/− females mated and allowed to undergo several rounds of pregnancy, lactation, and involution. Again, Sfrp3−/− mice did not develop any mammary tumour lesion, indicating that aging and parity are not sufficient risk factors for tumour development in Sfrp3−/− mice. Although these results suggest that, in the absence of genetic changes that could provide a sensitised background, Sfrp3 does not act as tumour suppressor, we performed an NGS analysis of sorted mammary gland epithelial cells to identify transcriptomic changes pointing to tumorigenic susceptibility.

A total of 1408 differentially expressed genes (DEGs, q-value < 0.05) were identified, including 813 upregulated and 595 down-regulated genes in Sfrp3−/− compared to wild-type mammary epithelial cells (Supplementary Data 1). From the top 100 DEGs (50 upregulated and 50 downregulated) (Supplementary Data 2, Supplementary Data 3, Fig. 7a) we selected a group of 56 genes with a log2 fold change > 2 or < −2. To investigate their association with BC outcomes, we interrogated two independent breast tumour datasets deposited in the publicly available Oncomine database29, and we found data for 36 genes. Importantly, 15 upregulated genes in Sfrp3−/− epithelial cells are differentially expressed in BC versus normal samples. We observed that the expression of 8 genes (Scl1a2, Larp1b, Lef1, Edn2, Slc6a12, Fgfr2, Ct1qtnf5 and Fli3l) is appreciably elevated in invasive BC compared to normal breast samples using the Cancer Genome Atlas (TCGA) dataset (Fig. 7b). All eight genes are highly expressed in invasive ductal and/or lobular BC, except for Fli3l that is notably highly expressed only in invasive lobular BC, compared to controls. Furthermore, seven genes (Ecel1, Nr4a2, Plk3, Dpp10, Saa2, Csn2 and Nvxn3) are considerably highly expressed in the stroma of invasive BC (Fig. 7c).

We found similar evidence when we performed a tissue-specific analysis of NGS data. From the total 1408 DEGs, we selected 102 DEGs (74 upregulated and 28 downregulated genes) that have been already described in the literature as associated with mammalian gland for expression or functional data (Supplementary data 4) and we classified them into 20 KEGG pathways. Among the top four false discovery rate (FDR) ≤ 10%, we found “Proteoglycans in cancer” and “Pathways in cancer” signalling pathways (Fig. 7d). These data show that loss of Sfrp3 results in an epithelial gene expression profile that reflects oncogenic processes. Finally, when classified into the biological process (BP), the clusters with the top three enrichment scores include processes of regulation of cell proliferation, positive regulation of protein metabolic process and mammary gland alveolus development (Supplementary Table 1).
To further test the potential of sfrp3 as tumour suppressor in the presence of genetic changes that provide a sensitised background, we performed a crossed Sfrp3−/− mice with the MMTV-Neu (MMTV-Erbb2), which produced tumours classified as human luminal type carcinomas. We observed the formation of preneoplastic lesions in the sfrp3−/− MMTV-Neu mammary gland samples which are phenotypically similar to the MMTV-Neu premalignant lesions described before (Fig. 7e). Furthermore, we identified that lack of Sfrp3 in MMTV-Neu animals induces a significant reduction in the time of appearance of mammary tumours (from an estimated median of 405 days in Srrp3+/− animals to 344 days in Sfrp3−/− mice; p = 0.0148) (Fig. 7f), suggesting a role for Sfrp3 as relevant marker for BC susceptibility. The analysis of the Sfrp3−/−; MMTV-Neu mammary gland tumours by confocal microscopy showed that the tissues present the characteristic features of the Sfrp3−/− mammary ducts with β-casein accumulated in the lumen of ducts and hyperproliferative luminal cells (Fig. 7g).

Collectively, these results support the alterations in MG morphogenesis we observed in Sfrp3−/− virgin females, and reveal Sfrp3 as a potential marker for BC susceptibility.
**Discussion**

In this study, we have identified a role for Sfrp3 in regulating mammary epithelial development during puberty. We found that the absence of Sfrp3 leads to alterations of MG development, with ducts showing accelerated ductal invasion throughout the fat pad and precocious tertiary branching. Loss of Sfrp3 leads to luminal cell defects, bringing to alterations of the ductal architecture and integrity. Sfrp3−/− luminal epithelial cells show increased proliferation, with no change in the apoptosis level, loss of polarity features and arrangement into multiple layers protruding and occluding the duct lumen.

Furthermore, Sfrp3−/− luminal epithelial cells produce milk proteins, consistently with the observed increased side branching that is typical of pregnancy. Maturation of alveolar precursor cells and differential induction of milk protein genes are mainly controlled by prolactin signalling, which is mediated by JAK2 and STAT5A/B31,32. Our bioinformatic analysis of NGS data suggests prolactin signalling as a potential pathway acting in Sfrp3−/− epithelial cells (Fig. 7d). Future work will be needed to better characterise the role of Sfrp3 in the regulation of prolactin signalling.

Mammary stroma regulates the development and differentiation of epithelial cells, defining as well the side branching pattern33,34. This is partially due to a stromal–epithelial communication by secreted factors acting on either an autocrine or paracrine manner. A hallmark of MG development is the bidirectional activity of these factors, which complicates the identification of the source and target cells. Here we observed that Sfrp3 is expressed in both compartments, but with eightfold more expression in the stroma. Furthermore, we showed that loss of stromal Sfrp3 is responsible for the ductal phenotype observed in Sfrp3−/− MG. It is tempting to speculate that the signals from Sfrp3−/− stroma are instructive since they can target transplanted wild-type epithelial cells and participate in their differentiation into multi-layered cells abnormally proliferating and producing milk proteins. Recent studies have unravelled new molecules responsible for the stromal–epithelial communication in mammary epithelial morphogenesis35,36. We propose SFRP3 as a mediator of the mammary stromal–epithelial boundary (Fig. 8). Indeed, secreted SFRP3 binds to Wnt2 through its NRT domain, and we detected a substantial increase in Wnt2 accumulation at this boundary and the activation of the Wnt-β-catenin pathway in pubertal Sfrp3−/− whole MGs. Wnt-2, Wnt-5a and Wnt-7b are strongly expressed during ductal development, in the TEB microenvironment, and they seem to be regulators of mammary branching38,39. Indeed, the ectopic expression of Wnt2 results in a hypermorph branching phenotype40.

Furthermore, we found that Wnt2 is expressed by stromal fibroblast in the MG, and previous studies showed that Wnt2 expression accumulates preferentially in the stromal–epithelial boundary38. Thus, our current data suggest that SFRP3 might act in the MG as an extracellular transporter of Wnt ligands enhancing their diffusion across cellular compartments. Sfrp3 and Crescent have been shown to promote the diffusion of Wnt8 and Wnt11 in Xenopus embryo41. In line with this, in the presence of SFRP3, Wg displays a more diffuse distribution at the dorsoventral boundary of the Drosophila wing imaginal disc. We propose that SFRP3 binds to stromal Wnt2 in order to diffuse this ligand function to epithelial cells. In the absence of SFRP3, Wnt2 would accumulate in the stroma, resulting in an altered communication with the epithelium, and a defective mammary epithelial morphogenesis (Fig. 8). Future studies will be required to dissect the complex scenario of stromal–epithelial communications better.

Similarly to Sfrp3, loss of Sfrp1 also leads to excessive side branching with clear lobuloalveolar development in the mouse mammary gland42. It is to note that, although loss of either Sfrp1 or Sfrp3 results in a similar mammary phenotype, distinct molecular routes are activated in their absence at signalling level in order to prompt mammary modifications. For example, in contrast to Sfrp3−/− mice, Wnt4 and its downstream effector RANKL mRNA levels are increased in Sfrp1−/− mammary gland in comparison to wild-type glands42. Additionally, Sfrp1 expression is found mostly in basal/myoepithelial cells that lack Sfrp3 expression43,44. Lastly, previous work demonstrated that Sfrp1 and Sfrp2 control retinal neurogenesis in a Wnt-independent manner45. These results altogether suggest that Sfrp1 and Sfrp3, participate in alternative signalling cascades during MG development. It will be interesting to address the function of other members of the Sfrp family in light of the recent evidence pointing to a stromal expression of Sfrp2 and Sfrp4 in both normal and tumour samples16,46–48.

How is MG development regulated downstream of Wnt signalling? Previous studies reported that mouse models with the activated expression of β-catenin display a precocious...
Fig. 6 Sfrp3 modulates Wnt signalling in the mammary gland. a Representative images of immunofluorescence staining for β-catenin (red), Troma (green) and nuclei (blue), on 5-weeks-old MG. Arrow denote localisation of β-catenin at cellular junctions in controls (Sfrp3+/+). Scale bar: 50 μm. b Quantification of the number of β-catenin positive cells in mammary ducts of 5 weeks old Sfrp3+/+ (n = 2, 5 mice) and Sfrp3−/− (n = 2, 8 mice) females. Data represent mean ± SD (****p < 0.0001). c Relative mRNA level of Wnt2 (left), Wnt5a and Wnt7b (middle) and Wnt36 (right) in whole MG from Sfrp3+/+ and Sfrp3−/− females at 10 weeks of age. n = 6. d Representative images of immunofluorescence staining for Wnt2 (red), K8 (green), on 5-weeks-old MG. Arrow denote localisation of Wnt2 at stromal-epithelial boundary. Images are representative of 3 MG sections analysed in 3 different experiments. Scale bar: 20 μm. e Relative mRNA level of Wnt2 (left) and Wnt5a (right) in de-epithelialised cleared fat pad from Sfrp3+/+ and Sfrp3−/− females at 8 weeks of age. n = 4. f Left, schemes of wild-type myc-tagged SFRP3 and truncated variants. Numbers indicate the aa position of each conserved domain according to UniProtKB database. SP, signal peptide; CRD, cysteine-rich domain; NTR, netrin-like domain. Right, western blot detection of SFRP3-myc in cell lysates and α-experiments. Source data are provided as a Source Data file.

The Wnt pathway has emerged as a key signalling cascade participating in mammary organogenesis and oncogenesis7. Our NGS data analysis shows that the gene expression profile of Sfrp3−/− epithelial cells is associated with BC. Indeed, the hyperproliferation and disruption of epithelial apical polarity phenotype that we observed in Sfrp3−/− MG could be reasonably considered as the first steps of a tumorigenic process. Despite this evidence, Sfrp3−/− mice did not develop any evident mammary tumour lesion or palpable mass. Also, we did not detect any aberrant collagen deposition and organisation, features that can
initiate a process of mammary tumour growth and invasion. We hypothesise that the development of BC requires other genetic and epigenetic changes. Indeed, mutations in canonical Wnt genes, leading to abnormal activation of Wnt signalling, are genetic and epigenetic changes. Indeed, mutations in canonical Wnt genes, leading to abnormal activation of Wnt signalling, are genetic and epigenetic changes. Indeed, mutations in canonical Wnt genes, leading to abnormal activation of Wnt signalling, are genetic and epigenetic changes.

Methods

Mouse lines. Generation of Sfrp3+/− mice in Swiss/CD1 background were a kind gift from Prof. F.P. Luyten (KU Leuven, Belgium) and were maintained for our studies on pure CD1 background, as well as Sfrp3−/− mice. Virgin female mice
were sacrificed at 3, 8, 10 and 12 weeks of age for mammary gland developmental studies. At the end of puberty (8 weeks of age) the oestrus cycle was monitored daily by vaginal smear twice per day (early morning and late afternoon). Only age-matched wild-type and Sfrp3−/− females with matching oestrus cycle were used for studies. For pregnancy stages, mice were mated and inspected for the presence of vaginal plug the following morning. The day of the vaginal plug was counted as day 0.5 of pregnancy. The first day after delivery was considered as lactation day 1 (L1).

For weight recording, litters were normalised at 7 pups at birth, and weight was measured at day 11 and day 21 (weaning). Male mice were sacrificed at 6 weeks and 6 months of age for mammary gland studies. For transplantation experiments we used CD1 mice and immunocompromised mice. NOD.Cg-PrkdcscidIl2rgtm1WjlNga (NSG) mice, Jackson Laboratories, Maine, USA) were bred and housed in the CIB animal facility. For analysis of tumorigenesis the Sfrp3+/− mice were crossed with FVB/Tg(MMTVneu)202Mul/J mice (MMTVneu+/−/+) mice (Jackson Laboratories, Maine, USA); the F1 (Sfrp3+/−/− MMTVneu+/−/+) were backcrossed to Sfrp3−/−/− mice. Genotypic analyses allowed to identify the Sfrp3−/−/− MMTVneu−/−/− and Sfrp3−/−/− MMTVneu−/−/− genotypes. Appearance of mammary tumours in these animals was analysed twice weekly, to determine the fraction of tumour-free animals over time.

All mice were housed and bred in a specific pathogen-free (SPF) barrier facility with a 12-h light cycle and food and water ad libitum. All experiments were approved by the CBMSO’s Institutional and Spanish Regional Committees on Ethics of animal research and were in adherence to the NIH “Guide for the Care and Use of Laboratory Animals”. Investigators were not blinded to genotype group allocations.

**Fly stocks.** The Gal4 drivers for ectopic expression experiments using the Gal4/UAS system were hh-Gal4 (FBti0017278) and nub-Gal4− (FBti0016825). The drivers for ectopic expression experiments using the Gal4/hh-Gal4 (FBti0017278) and nub-Gal4− (FBti0016825) were crossed with FVB/N-Tg(MMTVneu)202Mul/J mice (MMTVneu−/−/−) mice (Jackson Laboratories, Maine, USA); the F1 (Sfrp3+/−/− MMTVneu+/−/+) were backcrossed to Sfrp3−/−/− mice. Genotypic analyses allowed to identify the Sfrp3−/−/− MMTVneu−/−/− and Sfrp3−/−/− MMTVneu−/−/− genotypes. Appearance of mammary tumours in these animals was analysed twice weekly, to determine the fraction of tumour-free animals over time.

All mice were housed and bred in a specific pathogen-free (SPF) barrier facility with a 12-h light cycle and food and water ad libitum. All experiments were approved by the CBMSO’s Institutional and Spanish Regional Committees on Ethics of animal research and were in adherence to the NIH “Guide for the Care and Use of Laboratory Animals”. Investigators were not blinded to genotype group allocations.

**Antibodies and reagents.** Monoclonal anti-E-cadherin (#610181) antibody was obtained from BD Transduction Laboratories. Polyclonal anti-Keratin 14 (#PRB-155P) antibody was obtained from BioLegend. Monoclonal anti-Keratin 8 (TROMA-I), anti-ZO1 (R26.4C), anti-Wingless (4D4) and anti-Cut (2B10) antibodies were obtained from Developmental Studies Hybridoma Bank. The rabbit anti-NaPi-11b sera was a kind gift of Dr J. Biber (University of Zurich, Zurich, Switzerland) and was used 1:400. Polyclonal anti-β casein (M-14, sc-17971) antibody, and anti-ERα (F10. sc-8002; MC-20, sc-542) was obtained from Santa Cruz Biotechnology. Polyclonal anti-Cleaved Caspase 3 (#9661) antibody was obtained from Cell Signalling. Monoclonal anti-CD328 (EpCAM)-PE (#130-102-967), anti-CD45-FITC (#130-102-778) and anti-CD49F:APC (#130-101-147) antibodies were obtained from Miltenyi Biotec. Polyclonal anti-c-myc antibody for staining on Drosophila (#PA1-981) were obtained from Thermo Fisher Scientific. Mouse monoclonal anti-myc tag (clone 4A6, cat#05-724) antibody for IP and western blot, and anti-active b-catenin (8E7) was obtained from Merck-Millipore. Polyclonal anti-V5 tag (GTX171997) was purchased from GeneTex. Polyclonal anti-HA tag (#H6908) antibody was obtained from Sigma. Alexa Fluor 488/555/647-conjugated antibodies were obtained from Thermo Fisher. Matrigel Growth Factor Reduced (#356230) was obtained from BD Biosciences. Chambered coverglasses (#153583) were obtained from LabTek.

**Genotyping PCR.** Mice were genotyped by PCR analysis on genomic DNA extracted from tail tip. Two combinations of primers were used, one specific for the Sfrp3+/− allele (primers Sfrp3+/− allele forward and reverse), the other specific for the Sfrp3−/− allele (primers Sfrp3−/− allele forward and reverse). MMTVneu−/− transgenic mice were genotyped by PCR using the primers olMMR0386 TTTCTGCAACCGACCGCAGGCAC TACC and olMMR0387 CCGAACCCCCATCACTGACCGC to amplify the transgene, using as internal control the amplification with the primers olMMR8744 CAAATTGTGCTTGTCTGAGT and olMMR8745 GTCACCTGACGTGACAGATTTT, following the conditions described by the provider (The Jackson Laboratory).

**Whole mount analysis of mammary glands.** Abdominal (no. 4) glands were dissected from mice and spread on glass slides. After fixation in Carnoy’s fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for either 2–4 h at room temperature or overnight at 4 °C, they were hydrated and stained with Carmin alcian overnight and mounted with Permount mounting medium. Images were taken with a Nikon D750 digital camera and processed with ImageJ.

For each gland, the ductal length was measured as the distance between the lymph node and the distal end of the longest duct. Branching was quantified by manually counting the total number of branch nodes within three fields either adjacent to the sides of the lymph node for mammary gland or randomly distributed for transplanted mammary gland.

**Histological staining and analysis.** For morphological analysis, carmine-stained mammary glands were cleared in xylene, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin according to standard procedures. Images were collected on a Zeiss Axioskop2 plus microscope equipped with a colour charge-coupled device camera. Ducts with filled lumen were manually counted in 5–7 images per mammary section and expressed as a percentage of the total number of ducts per image.

For collagen staining, mammary glands were fixed with 4% PFA overnight, embedded in paraffin, sectioned at 5 μm and stained with PicroSirius red. Images of Sirius red-stained sections were taken with a Nikon Eclipse TE2000-U microscope under bright and polarised light and processed by using ImageJ software. The
number of the different coloured collagen fibres was measured as previously described\(^1\). Briefly, we subtracted the black-component image from each polarised image in order to eliminate interstitial space and non-collagen elements that are birefringent materials as collagen. Within the subtracted image we determined the hue (colour) of each pixel by using the following hue definitions: red 1–9; orange 10–19; yellow 20–38; green 39–51; cyan 52–123; blue 124–182; purple 183–235; red 236–245; orange 246–255; yellow 256–265; green 266–285; cyan 286–305; blue 306–345; purple 346–360. The number of pixels within each hue range image was calculated and expressed as a percentage of the total number of collagen fibres. The proportion of thick collagen fibres per image is given as the sum of red, orange and yellow pixels while the proportion of thin collagen fibres is due to green pixels percentage.

Eleven fields per mammary gland were imaged. For the quantification of collagen content the total number of collagen pixels (corresponding to hue range 1–255) was expressed as a percentage of the total number of pixels per image. Eleven images per mammary gland were analysed. The level of anisotropy of collagen fibres was measured by using the FractrUtool ImageJ plug as previously described\(^4\). For each image a ROI (region of interest) was selected including one single mammary duct and the surrounding area of collagen. A score of 0–4 was given for each ROI for no order (purely isotropic arrays) and 1 for perfectly ordered, i.e., parallel fibrils (purely anisotropic arrays). Between six and eleven images per mammary gland were analysed.

**3D culture of mammary organoids and staining.** We purified mammary organoids from CD1 mice following the protocol previously optimised and described for FVB mice\(^8\). Mammary glands pairs #3, #4 (without lymph node), and #5 were harvested from virgin 8-weeks-old females. They were minced and digested in collagenase buffer (DMEM/F12 buffer with 2 mg/ml type II collagenase, 5% FBS, 5 μg/ml insulin, and 50 μg/ml of gentamicin) for 30–40 min at 37 °C. Samples were centrifuged at 1500 rpm for 10 min. To recover additional epithelial tissue, the top fatty layer was resuspended in DMEM/F12 and centrifuged again. The epithelial pellets were resuspended in DMEM/F12 and treated with 40 μl DNase and 20 μl of NaPi buffer (1 mg/ml) for 3 min. They were then resuspended in DMEM/F12 and centrifuged at 1500 rpm for 10 min in order to obtain a compact red pellet. After four rounds of differential centrifugation (quick spin at 1500 rpm) the final white pellet consisted mostly of organoids. Organoids were embedded in Matrigel Growth Factor Reduced at 2 organoids/μl and plated as 75 μl suspensions in 4-well chambered coverglass over a 37 °C heating block. Gels were allowed to polymerize for 50 min at 37 °C, 5% CO₂, and then overlaid with organoid medium (DMEM/ F12 with 1% penicillin-streptomycin and 1% ITS) for 2 days and organoids were analysed for 6 days of culture. They were fixed with 2% PFA (15 min light shaking) and permeabilized with DPBS + 0.5% TritonX-100 for 1 h at room temperature. After blocking with DPBS + 2% BSA overnight at 4 °C antibody solution plus the secondary antibody rabbit 488 (1:500), mouse 568 (1:500), rat 555/647 (1:500), CC3 (1:500), anti-Ki67 (1:500), anti-ER (1:200), anti-K14 (1:200), anti-K8 (1:2), anti-E-cadherin (1:400), anti-ZO1 (1:500), anti-

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**Isolation of primary mammary cells by FACS.** Mammary glands pairs #2, #3, #4, and #5 were isolated from virgin 12-weeks-old females. They were harvested and incubated in the dissociation solution (DMEM/F12 medium with 10% gentle collagenase/hyaluronidase, 5% FBS and 50 μg/ml of gentamicin) overnight at 37 °C, 5% CO₂. After dissociation, cells were washed in HBBS + 2% FBS and centrifuged at 400 × g for 5 min. The pellet was incubated in HBBS + 2% FBS + NH₄Cl (mixture 1:4) for 10 minutes for lysis of red blood cells and centrifuged at 400 × g for 5 min. The resultant pellet contains epithelial cell organoids as well as stromal cells and large pieces of connective tissue. To obtain a single-cell suspension the pellet was gently pipetted for 3 min in 2 ml of trypsin-EDTA 0.25%, centrifuged, and then pipetted for 3 min in 2 ml Dispase II (5 mg/ml) and 200 μl DNAsel (1 mg/ml). After resuspension in HBBS + 2% FBS, cells were passed through a 40 μm cell strainer filter in order to dissociate cell clumps and centrifuged. The pellet was blocked in FACS buffer (PBS pH 7.4, 2 ml Dispase II, 10% FBS, 5 μg/ml of gentamicin) overnight at 37 °C, 5% CO₂. For each experiment, we subtracted the black-component image from each polarised cation image acquisition. We performed two separate lateral incisions in the lower back. Five weeks after MEF transplantation, mammary glands were harvested for whole mount carmine alum staining.

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**Mammary epithelial transplantation.** Transplantation of epithelial ducts was performed according to the standard procedure previously developed\(^3\). Three weeks old females were used as recipients and were anaesthetised. The region between the nipple and the lymph node, containing the endogenous epithelium, was separated from the fat pad (clearing) by performing a cut just after the lymph node. The tissue between the nipple and the lymph node was spread on a glass slide, fixed and stained with Carmine alum in order to confirm that the entire rudimentary tree was removed. The cleared fat pad was collected in RNA later (Invitrogen) and either stored at ~80 °C or immediately processed for RNA extraction.

For RNA extraction from 8-weeks-old cleared fat pad, mice of that age were sacrificed and the entire right abdominal mammary gland was excised. The region between the nipple and the lymph node, containing the endogenous epithelium, was separated from the fat pad (clearing) by performing a cut just after the lymph node. The tissue between the nipple and the lymph node was spread on a glass slide, fixed and stained with Carmine alum in order to confirm that the entire rudimentary tree was removed. The cleared fat pad was collected in RNA later (Invitrogen) and either stored at ~80 °C or immediately processed for RNA extraction.

**Ovariectomy.** Both ovaries were removed from anaesthetised 3-weeks-old females by performing two separate lateral incisions in the lower back. Five weeks after MEF transplantation, mammary glands were harvested for whole mount carmine alum staining.
lysed in 200 µl of Laemmli 2× sample buffer. Supernatants were centrifuged at 2000 rpm for 5 min to remove cell debris. The pellet was discarded and 4 volumes of cold 100% acetone were added to the supernatant. Samples were incubated at −80 °C for 10 min and centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant was discarded and the pellet was dried for 15 min at room temperature. Proteins were then resuspended in 400 µl of Laemmli 2× sample buffer (Sigma Aldrich). A total of 15 µl of cell lysate and 40 µl of medium were loaded on 10% SDS-PAGE. Transblotted nitrocellulose membranes were incubated with anti-myc antibody (1:500).

Immunoprecipitation. HEK293 cells were grown in MEM + 5% FBS. The day before transfection 2.2 × 106 cells were seeded in a P100 plate. Medium was changed 1–3 h before transfection by Calcium Phosphate performed as following. 6.25 µg of the construct expressing the wild-type variant of human myc-tagged SFRP3 (see Supplementary Information) and 750 ng of the construct expressing active V5/HA-tagged Wnt ligand were mixed in water plus 61 µl of 2 M CaCl2. The DNA mix was added to 500 µl of 2× HBS pH7, incubated for 30–60 s, and added dropwise to cells. The following day cells were lysed in 1 ml lysis buffer (150 mM NaCl, 50 mM Tris pH 8, 1% NP40, protease inhibitors). After clearing with unconjugated Sepharose beads, half of the lysate was incubated with anti-myc conjugated beads, and the remaining half was incubated with a negative control antibody conjugated beads for 2 h at 4 °C. After three washes with lysis buffer and one wash with PBS, the pellet was resuspended in 300 µl of Laemmli 2× sample buffer, and boiled. Fifteen microliter were loaded on 10% SDS-PAGE and analysed by western blot using anti-myc antibody (1:1000) for detection of SFRP3, anti-V5 antibody (1:1000) for detection of Wnt2 and Wnt5a, and anti-HA antibody (1:500) for detection of Wnt7b.

RNA extraction and quantitative PCR analysis. Mouse tissues were mechanically homogenised and total RNA was extracted by RNeasy Plus spin column kit. For RT–PCR on mouse tissues one µg RNA was reverse transcribed with the ThermoScript RT-PCR system using random hexamers. Sfpr3 (primers pair #1) and GAPDH (as normaliser gene, primers pair #1) were amplified from 2 µl of cDNA. For quantitative Real Time PCR one µg RNA for tissue sample or 600 ng RNA for FACs-sorted cells sample was reverse transcribed to cDNA with the High Capacity RNA-to-cDNA kit. Real Time PCR was performed on 10 ng of cDNA using the GoTaq qPCR Master Mix and 2.5 µM gene-specific primers in a final volume of 10 µl using the ABI 7900HT. The genes amplified from mouse tissue were: Sfpr3 (primers pair #2), Wnt2, Wnt5a, Wnt7b, Wnt16, β casein, Lalba, WAP, axin2, cyclinD1. The genes amplified from sorted cells were Sfpr3 (primers pair #2), Wnt2, Wnt5a, Wnt7b, CD24a, CD49f, Aldh1a1a, factin, GAPDH (primers pair #2) and Ppiα were used as internal controls. All primer sets were tested for primer efficiency using a six-fold dilution series containing six dilutions. Conditions of Real Time quantitative PCR were as follows: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Statistical analysis was performed using Prism software.

Low-input RNA sequencing. RNA sequencing libraries were prepared following the SMARTseq2 protocol with some modifications. Briefly, RNA was quantified using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific) and 10 ng of RNA was used for cDNA synthesis. Reverse transcription was performed using SuperScript II (Invitrogen) in the presence of oligo–dT30VN (1 µM), template-switching oligonucleotides (1 µM) and betaine (1 M). The cDNA was amplified using the KAPA HiFi Hotstart ReadyMix (Kapa Biosystems), 100 nM ISPCR primer and 8 cycles of amplification. Following purification with Agencourt AMPure XP beads (1:1 ratio; Beckmann Coulter), product size distribution and quantity were assessed on a Bioanalyzer using a High Sensitivity DNA Kit (Agilent). Two hundred nanogram of the amplified cDNA was fragmented for 10 min at 55 °C using Nextera® XT (Illumina) and amplified for 12 cycles with indexed Nextera® PCR primers. Products were purified twice with Agencourt AMPure XP beads (0.8:1 ratio) and quantified again on a Bioanalyzer using a High Sensitivity DNA Kit. The Nextera® libraries were sequenced on HiSeq2000 (Illumina, Inc.) in paired-end mode with a read length of 2 × 76 bp using TruSeq SBS Kit v4 and TruSeq Dual Index of 8 bp + 8 bp. We generated a mean of 42 million paired-end reads for each sample in a fraction of a sequencing v4 flow cell lane, following the manufacturer’s protocol. Image analysis, base calling and quality scoring of the run were processed using the manufacturer’s software Real Time Analysis (RTA 1.18.66.3) and followed by generation of FASTQ sequence files by CASAVA.

Bioinformatics analysis. More than 3 Gb of sequencing reads were obtained from each sample in fastq format. Quality analyses were performed over reads using FastQC software. Reads were trimmed with M. musculus reference genome (GRCh38) using tophat v2.0.14 with default parameters.

Differential expression analysis was performed using Cuffdiff v2.2.2 package, obtaining the expression values (RPKM) for each gene. A total of 1408 DEGs (RPKM > 1, q value < 0.05) were identified. Two datasets (TCGA and Finak) publicly available in Oncomine cancer microarray database were used to examine the expression of the top 100 DEGs in BC tissues. We selected p < 0.005 as a threshold. Also, a Functional Enrichment Analysis (FEA) on a subgroup of 102 DEGs genes of interest was performed with an in-house R script that uses the FGNET package.

Immunostaining on Drosophila wing imaginal discs. Imaginal discs were dissected in PBS, fixed in 4% PFA for 20 min at room temperature. They were blocked in PBS + 1% BSA + 0.3% TritonX-100 for 1 h at room temperature and then incubated with primary antibodies in PBST-BSA overnight at 4 °C, washed four times in blocking buffer and incubated with the appropriate fluorescent secondary antibody for 1 h at room temperature in the dark. They were then washed and mounted in Vectashield mounting medium. Primary antibodies used were: rabbit anti-c-myc (1:500), mouse anti-Wingless 4D4 (1:50), mouse anti-Cut 2B10 (1:100) and guinea pig anti-Sens (1:1000, a gift from Isabel Guerrero), and mouse anti Distalless (dil) (1:1000, a gift from Carlos Estella). Confocal images were captured using an A1R + Nikon confocal microscope.

Quantification and statistical analysis. Results are expressed as the mean ± standard deviation (SD). To calculate statistical significance, the two tailed Student’s t-test was used. Sample size n is reported for each experiment in figure legends. p value < 0.05 was considered significant. All calculations were performed using Prism software.

Data availability

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. The uncorrected and unprocessed scans of the most important blots, and the excel tables of all quantifications can be found in the Source Data file in the Supplementary Information.

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Additional information

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