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14. ABSTRACT

Technologies such as sequencing, epigenetic, proteomic or metabolomic profiling have resulted in large sets of data. Functional annotation of the genome is a key challenge for a fundamental understanding of disease. We combined genetic model organisms and repairable mutagenesis with *in vivo* mouse genetics and human cohort studies to functionally characterize candidate breast cancer genes. Using mouse genetics, we showed that RANKL and its receptor RANK are critical regulators of sex hormone and BRCA1 mutation-driven breast cancer providing a rationale for breast cancer prevention trials in BRCA1 carriers using RANKL blockade. We also showed that serum levels of RANKL are potentially indicative of predisposition and progression of breast cancer in humans. The ground work we have laid in this innovator award is now being translated into a clinical trial with the goal to prevent breast cancer, starting in November 2018. During the course of our work we realized that RANK links female sex hormones to lung cancer and gender bias in lung cancer. We also performed a near-genome wide screen in *Drosophila* for genes that control tumor progression, allowing us to identify multiple novel tumor suppressor genes. Finally, we have now generated more than 100000 murine haploid ES cell clones targeting ~ 16500 different genes. We also developed new methods to generate blood vessels from haploid ES cells which allows us to genetically explore novel factors for tumor angiogenesis. One of these factors, Apelin, indeed cooperates with VEGFR signaling and, in mutant mouse models, appears to be a key regulator of tumor neo-angiogenesis and survival in breast cancer. Thus, we have successfully initiated all proposed aims with the vision to identify and functionally annotate breast cancer genes. Importantly, the innovator award allowed us to lay the ground work on RANKL/RANK which is now directly being translated into a clinical trial with the goal to prevent breast cancer.

15. SUBJECT TERMS

Genome wide functional genetics, haploid stem cells, RANKL/RANK, breast cancer prevention, cancer angiogenesis, metastases, *Drosophila* cancer modeling, breast cancer prevention, BRCA1 carriers

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1. Introduction

The following background, objectives, and innovation/impacts were the basis of my DoD Era of Hope award – **“Novel approaches to breast cancer prevention and inhibition of metastases”**

Background: With the advent of modern genomics hundreds of candidate genes have been associated with breast cancer both in GWAS studies as well as by cancer genome sequencing approaches. The big challenge is now to determine which of these genes encode fundamental molecular pathways that initiate breast cancer and control metastases. It is paramount to rapidly translate such findings into strategies for the prevention, future treatment, and possible eradication of breast cancer. My group generated the first RANKL mutant mice, providing definitive proof that RANKL is the master regulator of bone loss, opening a rationale avenue for novel therapies against bone loss in osteoporosis, leukemia, arthritis, or cancer metastases. We found another essential function for RANKL and its receptor RANK: formation of a lactating mammary gland in pregnancy. Moreover, we showed that RANKL/RANK function as soil factors for bone metastases of epithelial tumors, a finding that provided a mechanistic underpinning for clinical trials to delay bone metastases in prostate and breast cancer. Recently, we developed the idea and for the first time showed that the RANKL/RANK system is an essential molecular link between sex hormones and the development of breast cancer.

Objectives: To capitalize on the breakthroughs in human genetics, I propose to take the next step into the post-genome era of cancer, i.e. to develop systems to rapidly assess the function of candidate genes in breast cancer pathogenesis and metastases. We will utilize the power of fly genetics combined with our unique *Drosophila* libraries to rapidly validate the function of hundreds and even thousands of human candidate breast cancer genes in epithelial transformation and metastases. Once these genes have been validated in fly, we will address their function in mammary cancer and metastases in mice using an entirely novel tool: my laboratory developed haploid embryonic stem cells. This technology allows us now to generate a library of murine ES cell clones that carry homozygous mutations in essentially all protein coding genes. Combining this mutant haploid ES cell library with in vivo mammary cancer and metastases assays, we will

be able to rapidly assess the role of candidate breast cancer genes. In addition to finding essential new cancer pathways using fly and murine haploid ES cell technologies, I propose a project that could fulfill the dream of eradication of breast cancer and could have immediate impact on prevention and treatment of breast cancer, i.e. to further explore the role of RANKL/RANK in mammary stem cell biology, breast cancer initiation, and metastases. Moreover, I propose to take the RANKL/RANK system into human breast cancer patients to provide a novel prognostic marker for breast cancer risk and a molecular rationale for the initiation of clinical trials using already approved RANKL blockade as a tool to prevent the onset of breast cancer.

Innovation and impact: Combining fly genetics and our unique haploid ES cells will allow a functional characterization of human candidate breast cancer genes. The transgenic RNAi library is covering the whole *Drosophila* genome, giving us an opportunity to examine the function of virtually any human candidate cancer gene that has a fly orthologue. Fly genetics will be used to short-list genes that then can be moved to our haploid ES cell system. Haploid ES cells have the potential to revolutionize the discovery and validation of cancer genes. The haploid ES cell library will be made available to other breast cancer researchers and could markedly drive the field in the future because any new candidate gene/pathway could be immediately tested. Further validation of RANKL/RANK as critical molecules in breast cancer initiation, mammary stem cell biology, and metastasis and the recent generation of therapeutics directed to block RANKL will have a direct impact for breast cancer patients. Early detection strategies have been a prime reason for reduction in breast cancer death and our work RANKL has the potential to markedly improve on this. Thus, our experiments might have direct impact on delaying metastases and the prevention of breast cancer.

2. The role of RANKL/RANK in breast cancer

Background. RANK (TNFRSF11A) and RANKL (TNFSF11), a receptor-ligand pair of the tumor necrosis factor (TNF) receptor superfamily, regulate bone physiology (**Figure 1**) by controlling osteoclast development. Soluble or membrane-bound RANKL can be induced by various factors, such as the sex hormone progesterone, Vitamin D3, and parathyroid hormone

related peptide (PTHrP) or cytokines such as tumor necrosis factor alpha (TNF α), interleukin-1, -11 and -17. Osteoprotegerin (OPG) serves as a decoy receptor for RANKL and can inhibit osteoclast development. OPG expression can be regulated by estrogen, thereby linking sex hormones to osteoporosis in postmenopausal women. Mechanistically, the loss of estrogen leads to decreased OPG levels and a coordinate rise in RANKL activity, resulting in increased osteoclast numbers and enhanced bone turnover and osteoporosis, which is a significant health problem in elderly populations. Recently, a second RANKL receptor, LGR4, has been identified that negatively regulates osteoclast differentiation and bone resorption, thereby exerting opposite effects to RANK signaling. Denosumab, a human monoclonal RANKL-blocking antibody, has been developed and approved for the treatment of osteoporosis and skeletal-related events in cancer. Importantly, recent studies have provided new evidence that bone homeostasis is not only regulated by osteoclasts and osteoblasts, but also through bone-embedded osteocytes.

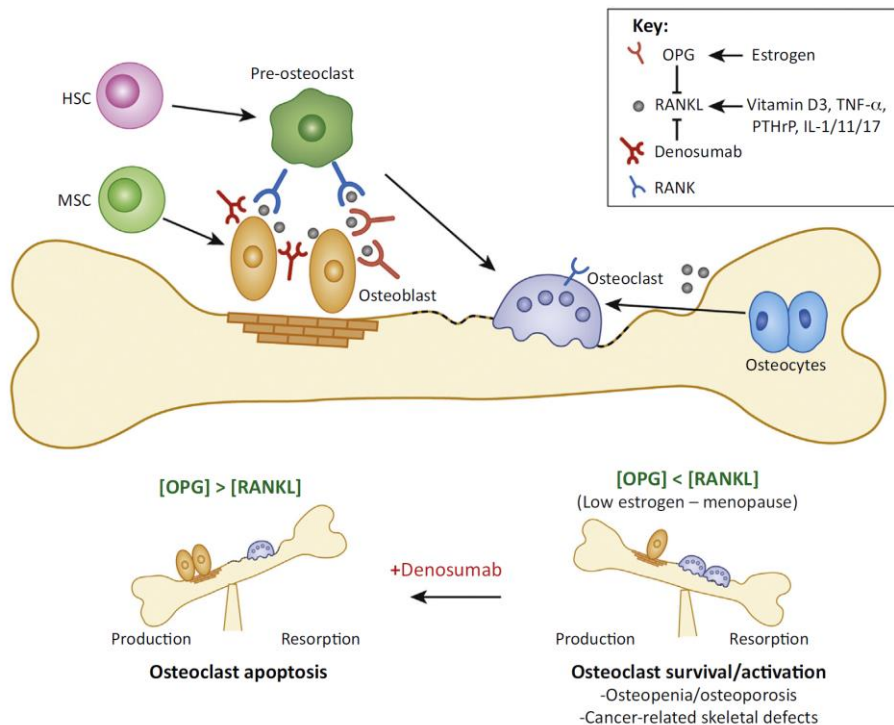


Figure 1. RANKL/RANK pathway controls pathophysiological bone remodeling. Bone resorbing factors, such as PTHrP, IL-1, IL-11, IL-17, and TNF α , act on osteoblasts to induce RANKL, which binds to RANK present on the surface of osteoclast progenitors (pre-osteoclast). Interaction of membrane-bound or soluble RANKL with RANK-expressing pre-osteoclast induces their differentiation into multinucleated osteoclasts as well as activation and survival of mature osteoclasts. Expression of the decoy receptor

osteoprotegerin (OPG) can be induced by estrogen, IL-4 or transforming growth factor β (TGF- β), among other factors. OPG binds to RANKL and hence interferes with RANKL/RANK interaction, thereby inhibiting bone resorption. The relative levels of OPG and RANKL are delicately balanced to ensure healthy bone structure. Under pathological conditions (e.g. cancer-related skeletal defects) or during menopause when estrogen levels, and thus OPG, decline, there is a greater concentration of RANKL which leads to enhanced osteoclast development and ultimately in bone loss. The fully human monoclonal antibody denosumab, binding to RANKL, has been shown in clinical trials to counter such effects.

RANKL/RANK regulate formation of the lactating mammary gland and couple sex hormones to mammary stem cells. The mammary gland consists of luminal and myoepithelial cells that originate from mammary progenitor cells. It was thought that mammary progenitors reside in a quiescent stem cell niche. Mammary progenitor cells are highly dynamic and undergo proliferation and differentiation during each estrous cycle. Mammary stem cell numbers vary at different stages of the estrous cycle. Moreover, pregnancy and aging can significantly affect mammary stem cell numbers, thereby allowing the mammary gland to adapt to various physiological states. Importantly, we were the first to show that RANKL and RANK are essential for the formation of a lactating mammary gland, which was found to be due to controlling the dynamic cycling of the mammary stem cell (MaSC) pool during each estrous cycle. Genetically engineered mice lacking *Tnfsf11* (RANKL) and *Tnfrsf11a* (RANK) display a complete block in the formation of lobuloalveolar milk-secreting structures during pregnancy. Although viable, progeny from *Tnfsf11* and *Tnfrsf11a* knockout mice eventually die because of a lack of milk produced in the mother from the resulting lactation defect. Mammary gland development during pregnancy depends on the presence of several hormones including progesterone, prolactin and PTHrP. Among these, progesterone is the most important for proliferation of adult mammary epithelial cells which, in turn, differentiate into milk-secreting acini. Mechanistically, progesterone induces RANKL expression in hormone-receptor-positive mammary epithelial cells resulting in the proliferation of RANK-expressing mammary epithelial progenitors, a mechanism essential to expand mammary epithelium and form milk-secreting acini in pregnancy (**Figure 2**).

Progesterone is the main upstream regulator of RANK/RANKL signaling in the non-pregnant mammary gland. Progesterone induces RANKL secretion in progesterone receptor (PR)-positive mammary epithelial cells. RANKL then acts through a paracrine mechanism on adjacent

hormone-receptor-negative, RANK-expressing mammary progenitor cells, which subsequently induces their proliferation (**Figure 2**). Moreover, RANKL can act in an autocrine manner to induce the proliferation of RANK-positive luminal cells as RANKL stimulation induces R-spondin, thereby relaying the signal from sex hormones through RANKL/RANK to the Wnt pathway in mammary progenitor cells. The regulation of mammary progenitor cell proliferation by the RANKL/RANK pathway appears to be universal in all mammals that have been investigated. Hence, RANK and RANKL are essential effector molecules that connect progesterone to mammary progenitor cell proliferation during the progesterone-phase of the estrous cycle and in pregnancy.

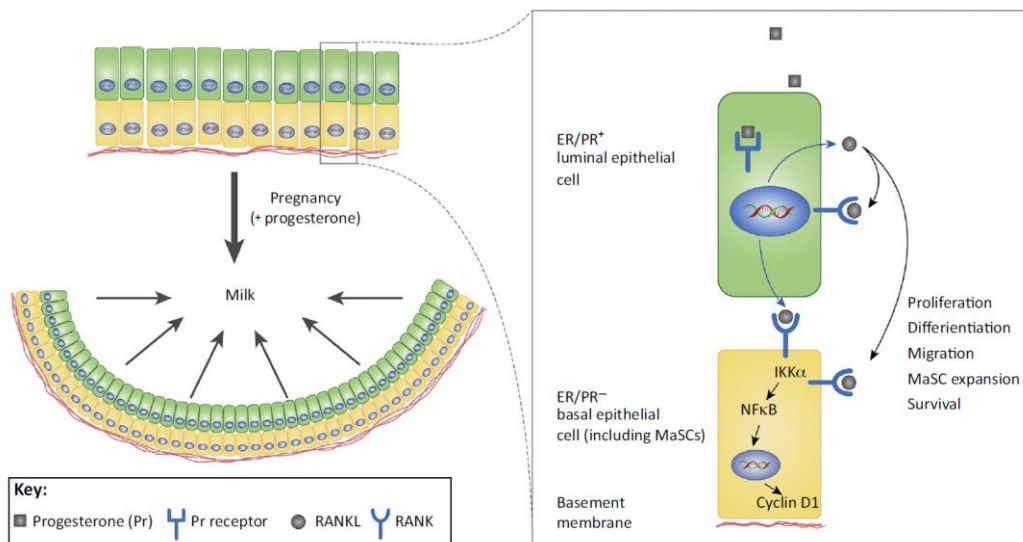


Figure 2. RANKL/RANK-mediated mammary epithelial cell expansion in the estrous cycle and pregnancy. RANK is constitutively expressed on the surface of luminal epithelial cells and basal cells including mammary stem cells (MaSC). Progesterone and progestins induce RANKL expression and secretion in progesterone receptor (PR)-positive luminal epithelial cells. Binding of RANKL to RANK on luminal epithelial cells stimulates further RANKL expression via an autocrine positive-feedback loop. The enhanced levels of membrane and soluble RANKL also act via a paracrine fashion on the PR negative basal cells to induce the IKKα–NFκB–Cyclin D1 signaling axis resulting in proliferation, expansion, differentiation, survival, and migration of MaSCs. In pregnancy, hormones such as prolactin might be also of key importance to induce RANKL expression, essential to form the lactating mammary gland. Of note, the RANKL/RANK system is not required to expand the mammary gland in puberty, a primary function of estrogen.

2.1. RANK/RANKL modulates hormone-driven breast cancer. Breast cancer is the most common female cancer and will affect 1 in 8 women throughout their lifetime. Most breast cancers occur sporadically and mainly affect women during their 6th decade of life. In 2003, two independent studies, namely the Million Women Study and the Women's Health Initiative Study, have shown that women who received estrogen plus progesterone hormone replacement therapy (known as combined HRT) have a significantly higher risk of developing breast cancer compared to women who only received estrogen. Both studies provided strong population-based evidence that progesterone is a crucial risk factor for increased breast cancer incidence, which was further confirmed by an extended post-intervention follow-up 10 years later.

Based on the clinical importance of RANKL inhibition being approved for bone loss for potentially millions of patients, the role of RANKL/RANK in mammary epithelial proliferation in pregnancy in response to sex hormones, we developed the idea that RANKL/RANK might play a role in breast cancer initiation. My group and Gonzalez-Suarez *et al.* were indeed able to show that the RANKL-RANK system is a key regulator of hormone (progesterin)- and oncogene (Neu)-driven mammary cancer. Mechanistically, RANKL promotes the proliferation of mammary epithelial cells, rescues these cells from cell death after DNA damage, and controls tumor stem cell renewal. Therapeutic inhibition of RANKL reduced the incidence of hormone driven breast cancer from 100 to ~ 10% in a mouse model. Thus, 10 years after we proposed that RANKL/RANK might have a role in breast cancer⁶, recent data definitively demonstrated that this system is an essential link between sex hormones and the development of breast cancer.

Results: To further identify downstream activation pathways for RANKL/RANK in breast cancer, we deleted the key signaling molecules TRAF6, IKK α , and NFATc1 using K5Cre and MMTVCre mice to delete these genes in mammary epithelial cells. We also deleted RANK using K5Cre as delete strain, which resulted in a complete block in the formation of a lactating mammary gland and, compared to our previous MMTV-Cre results, significantly more affects the development of hormone-driven mammary cancer, i.e. K5Cre deletes RANK in a key progenitor cell compartment (**Figure 3**). Since K5-Cre also deletes in the thymic epithelium and

thus our results might be due to immune cell development in the thymus, we next tested, as a control, the Cre deleter line *Foxn1* that deletes in the thymic epithelium and hair follicle progenitor cells. However, *Foxn1*-Cre mediated deletion of RANK did not affect the onset of progesterin-driven breast cancer (**Figure 4**). Deletion of TRAF6, but not NFATc1 markedly impaired breast cancer development (**Figures 5 & 6**). K5-Cre driven deletion of *IKK α* resulted in lethality and we therefore could not study this signaling molecule in our in vivo breast cancer model.

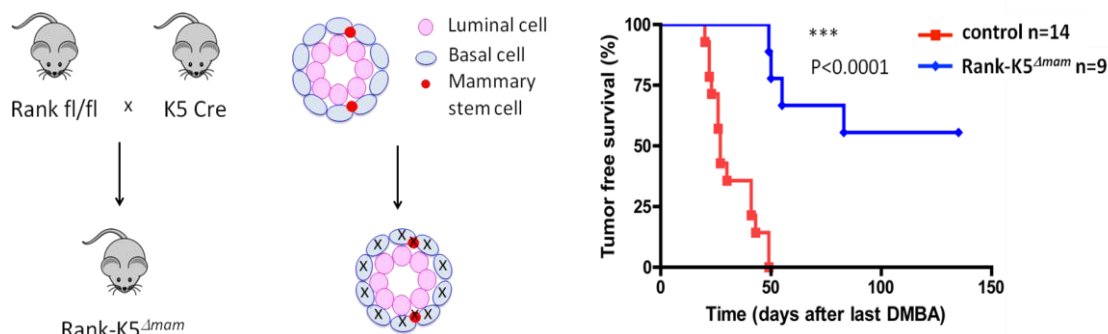


Figure 3. Onset of palpable mammary tumors in K5-Cre *rank*^{flox/Δ} females and age-matched littermate control females treated with the progesterin MPA and the carcinogen DMBA. For MPA/DMBA carcinogenesis, nulliparous, six-week old female mice were s.c. implanted with MPA pellets and treated orally with DMBA as indicated for 8 week. Data are shown as percentage of tumor free mice after the last DMBA challenge.

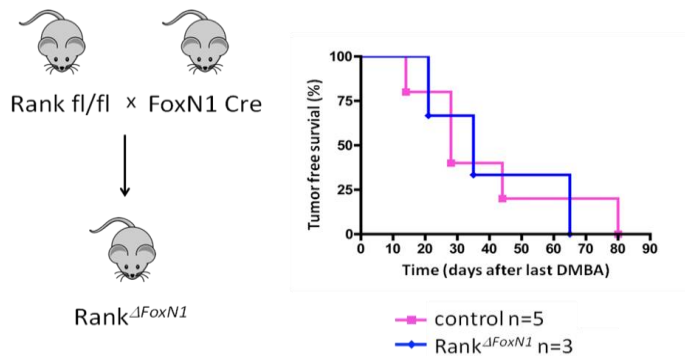


Figure 4. Onset of palpable mammary tumors in FoxN1-Cre *rank*^{flox/Δ} females and age-matched littermate control females treated with the progesterin MPA and DMBA as described in Figure 3. Data are shown as percentage of tumor free mice after the last DMBA challenge.

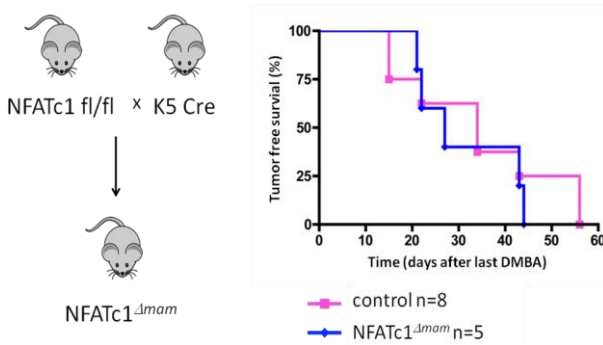


Figure 5. Onset of palpable mammary tumors in K5-Cre *nfatc1*^{flox/Δ} females and age-matched littermate control females treated with the progestin MPA and DMBA as described in Figure 3. Data are shown as percentage of tumor free mice after the last DMBA challenge.

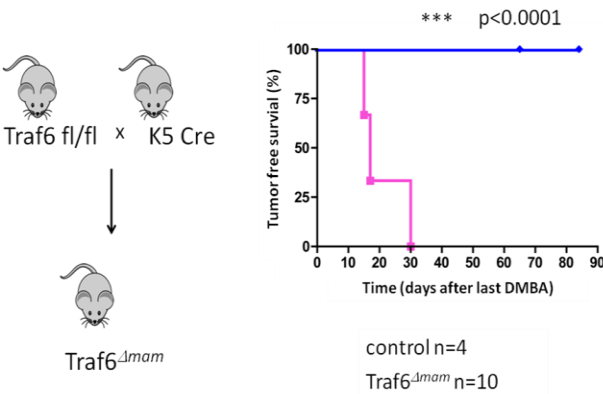


Figure 6. Onset of palpable mammary tumors in K5-Cre *traf6*^{flox/Δ} females and age-matched littermate control females treated with the progestin MPA and DMBA as described in Figure 3. Data are shown as percentage of tumor free mice after the last DMBA challenge.

It has been reported that RANKL might function on Treg cells and thereby drive breast cancer metastases. However, this has never been genetically proven although it is clear that RANKL/RANK can drive metastases. We therefore crossed *RANKL*^{flox} mice to a mammary epithelial cell-deleter line to address what RANKL-expressing epithelial cell type in the mammary gland drive breast cancer. We also knocked-out RANKL in all T cells (Lck-Cre) to test whether RANKL expression in T cells is required in driving mammary cancer. Our data indicate that RANKL has no apparent role on T cells but must be expressed on epithelial cells (**Figure 7**) but not on T cells (**Figure 8**). Our data in epithelial cells would in fact also indicate a non-epithelial (non-T cell source) for RANKL; however, since the K14-Cre line could be in some aspects mosaic, we now need to expand these findings to K5-Cre mice to be able to directly

compare the RANKL data with our results in K5-CreRANK^{flxed} mice. Of note, K14-Cre driven deletion of RANKL indeed abrogates formation of a lactating mammary gland in pregnancy, confirming an essential requirement for epithelial RANK during pregnancy (**Figure 9**). Importantly, our recent data indicate that deleting RANK with K14Cre nearly completely abrogates the development of MPA/DMBA induced mammary tumors!

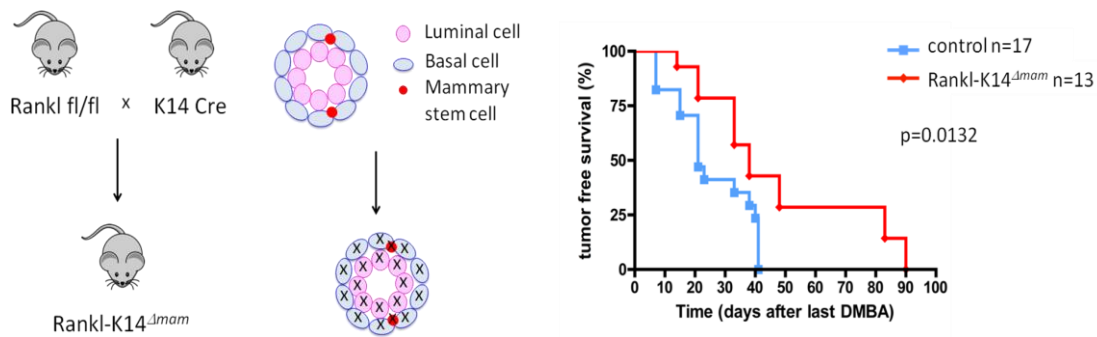


Figure 7. Onset of palpable mammary tumors in K14-Cre *rankl*^{flxed/Δ} females and age-matched littermate control females treated with the progestin MPA and DMBA as described in Figure 3. Data are shown as percentage of tumor free mice after the last DMBA challenge.

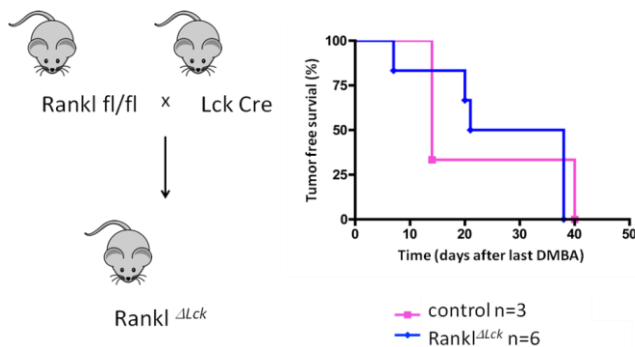


Figure 8. Onset of palpable mammary tumors in Lck-Cre *rankl*^{flxed/Δ} females and age-matched littermate control females treated with the progestin MPA and DMBA as described in Figure 3. Data are shown as percentage of tumor free mice after the last DMBA challenge.

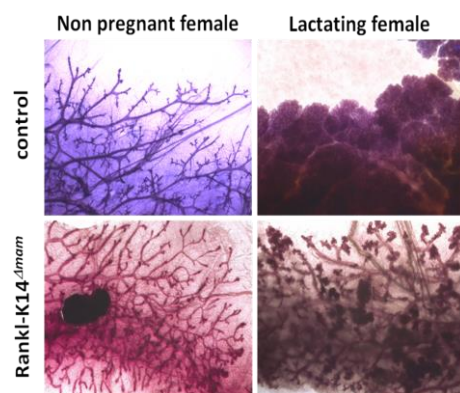


Figure 9. Whole mount images of mammary tissue of nulliparous littermate and lactating control and K14-CreRANK^{flxed} females showing normal alveolar/ductal epithelial structures in non-pregnant females but severe impairment in formation of a lactating mammary gland.

2.2. RANKL/OPG as biomarkers for breast cancer.

Identifying a biomarker which serves as both an indicator for breast cancer risk and at the same time a possible target for prevention is one of the most important yet unsolved needs. RANKL is expressed in primary breast cancers in patients and human breast cancer cells lines and, in mouse studies, the RANKL/RANK system is an important molecular link between progestins and oncogene-driven epithelial carcinogenesis. We therefore hypothesized that serum levels of RANKL and OPG could serve as markers for breast cancer risk providing a molecular rationale for future breast cancer prevention.

Results. In order to test whether deregulation of the RANKL/OPG system is associated with breast cancer in women without known genetic predispositions, we analyzed serum levels in postmenopausal women participating in the prospective UKCTOCS (UK Collaborative Trial of Ovarian Cancer Screening) study. This cohort of women provided a unique opportunity to study changes in serum levels of soluble RANKL and OPG well in advance of breast cancer manifestation. A total of 278 samples from postmenopausal women were analyzed: 40 women donated a serum sample between 5 and 12 months prior to breast cancer diagnosis (median age at sample taken 64.84), 58 women provided sera between 12 and 24 months prior to breast cancer diagnosis (median age at sample taken 60.49 years) and 180 women, who did not develop breast cancer during their follow up (median age at sample taken 62.94 years), served as controls.

We did not observe significant differences in RANKL, OPG or RANKL-to-OPG ratios in controls and women who developed breast cancer 12–24 months after serum sampling. Since we have previously shown that RANKL mediates progesterone driven mammary epithelial proliferation and the RANKL/RANK system is a key mediator of progestin-driven breast cancer in mouse models, we explored a potential connection between progesterone and RANKL/OPG levels in humans. Intriguingly, we observed an increase of RANKL serum levels with increasing progesterone levels in women who developed breast cancer 12-24 months after sample collection (**Figure 10A**) and the opposite trend in controls. In the same cohort, we also observed a non-significant negative correlation of progesterone with OPG serum levels (**Figure 10B**) resulting in

a significant alteration in the RANKL/OPG ratio among women with high serum progesterone (Figure 10C).

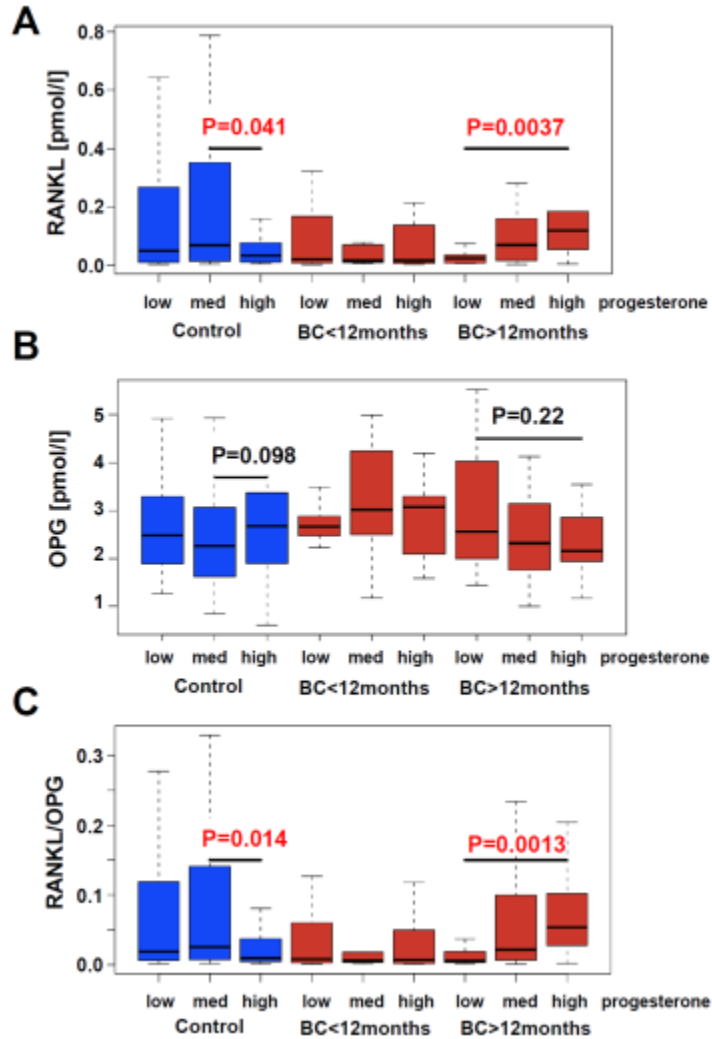


Figure 10. RANKL/OPG serum levels in human breast cancer patients. Analysis of RANKL (A), OPG (B) and the ratio RANKL/OPG (C) in relation to progesterone levels in prospectively collected UKCTOCS serum samples from 180 healthy postmenopausal women who did not develop breast cancer during their follow up and 40 healthy age-matched women who developed estrogen receptor positive breast cancer 5-12 months after their serum was collected as well as 58 healthy age-matched women who did develop ER-positive breast cancer 12-24 months after their serum was collected. Women were grouped according to their serum progesterone levels. Subjects were stratified into breast cancer patients and controls due to self reporting and histological examination. We have tested differences in RANKL, OPG and RANKL/OPG within each group between low versus medium and medium versus high progesterone within each group using the Mann Whitney U test and displayed only the significant p-values ($p < 0.05$).

To assess whether high progesterone and high RANKL indeed define a subgroup of women with increased risk of developing breast cancer, we classified all women - cases and controls considered together - into tertiles according to their serum progesterone levels. Within each group women were classified into low, medium or high RANKL, OPG and RANKL/OPG based on controls within the corresponding progesterone group. Using logistic regression women with high progesterone and high RANKL exhibited a 4.8 (95% CI 1.3 – 22.8; $p=0.0269$) fold risk (**Table 1**).

Table 1. Association of serum progesterone and RANKL with risk of breast cancer.

Progesterone LOW (range 0.03 - 0.19 ng/ml)																
RANKL [pmol/l]			Controls		Breast Cancer < 12 months						Breast Cancer > 12 months					
Tertile	Range		N (%)		N (%)		OR (95% CI)			p-value	N (%)		OR (95% CI)			p-value
1st	0.003	0.0193	21	33.9	7	50.0	1(ref)				6	42.9	1(ref)			
2nd	0.0193	0.179	20	32.3	4	28.6	0.60	0.1	2.3	0.47	8	57.1	1.40	0.4	4.9	0.59
3rd	0.179	3.716	21	33.9	3	21.4	0.43	0.1	1.8	0.26	0	0.0	0.00	NA		0.99

Progesterone MEDIUM (range 0.19 - 0.31 ng/ml)																
RANKL [pmol/l]			Controls		Breast Cancer < 12 months						Breast Cancer > 12 months					
Tertile	Range		N (%)		N (%)		OR (95% CI)			p-value	N (%)		OR (95% CI)			p-value
1st	0.004	0.0255	18	32.1	6	60.0	1(ref)				8	34.8	1(ref)			
2nd	0.0255	0.1745	19	33.9	3	30.0	0.47	0.1	2.1	0.34	9	39.1	1.07	0.3	3.4	0.91
3rd	0.1745	1.5235	19	33.9	1	10.0	0.16	0.0	1.0	0.10	6	26.1	0.71	0.2	2.4	0.59

Progesterone HIGH (0.31 - 12.68 ng/ml)																
RANKL [pmol/l]			Controls		Breast Cancer < 12 months						Breast Cancer > 12 months					
Tertile	Range		N (%)		N (%)		OR (95% CI)			p-value	N (%)		OR (95% CI)			p-value
1st	0.0045	0.0135	20	32.3	5	31.3	1(ref)				3	14.3	1(ref)			
2nd	0.0135	0.0675	21	33.9	5	31.3	0.95	0.2	3.9	0.94	3	14.3	0.95	0.2	5.7	0.96
3rd	0.0675	1.2315	21	33.9	6	37.5	1.14	0.3	4.5	0.84	15	71.4	4.76	1.3	22.8	0.03

All women - cases and controls considered together – were classified into tertiles according to their serum progesterone levels. Within each group, women were classified into low, medium or high RANKL based on controls within the corresponding progesterone group. Using logistic regression odd ratios (ORs) and 95 % Confidence Intervals (CI) have been calculated using the lowest RANKL group as a reference. Cases have been considered separately according to the time to diagnosis (more than one year or less).

Importantly, women within the high progesterone and high serum RANKL/OPG ratio group carried a 5.3 (95% CI 1.5 – 25.4, $p=0.0169$) fold risk to develop breast cancer 12-24 months after diagnosis (**Table 2**). We also grouped the women into RANKL/OPG tertiles across all controls

(not only within a given progesterone tertile) and again women within the high progesterone/high RANKL/OPG group had a 4.2 (95% CI 1.3 – 16.0, $p=0.02$) fold risk for developing breast cancer. Thus, high serum levels of RANKL and high serum progesterone stratify a subpopulation of postmenopausal women without known genetic predisposition at high risk of developing breast cancer 12-24 months before diagnosis.

Table 2. Association of serum progesterone and RANKL/OPG ratio with risk of breast cancer.

Progesterone LOW (range 0.03 - 0.19 ng/ml)*																		
RANKL/OPG ratio			Controls		Breast Cancer < 12 months						Breast Cancer > 12 months							
Tertile ^{\$}	Range		N (%)		N (%)		OR (95% CI) [¶]				P-value	N (%)		OR (95% CI) [¶]				P-value
1st	0.0014	0.0075	21	33.9	7	50.0	1(ref)					9	64.3	1(ref)				
2nd	0.0075	0.0644	20	32.3	4	28.6	0.60	0.1	2.3	0.47		5	35.7	0.58	0.2	2.0	0.40	
3rd	0.0644	2.0472	21	33.9	3	21.4	0.43	0.1	1.8	0.26		0	0.0	0.00	NA	NA	1.0	

Progesterone MEDIUM (range 0.19 - 0.31 ng/ml)*																		
RANKL/OPG ratio			Controls		Breast Cancer < 12 months						Breast Cancer > 12 months							
Tertile [§]	Range		N (%)		N (%)		OR (95% CI) [¶]				P-value	N (%)		OR (95% CI) [¶]				P-value
1st	0.0017	0.0154	19	33.9	6	60.0	1(ref)					8	34.8	1(ref)				
2nd	0.0154	0.091	18	32.1	4	40.0	0.70	0.2	2.9	0.63		9	39.1	1.19	0.4	3.8	0.77	
3rd	0.091	0.4605	19	33.9	0	0.0	0.00	NA		0.99		6	26.1	0.75	0.2	2.6	0.65	

Progesterone HIGH (0.31 - 12.68 ng/ml)*																		
RANKL/OPG ratio			Controls		Breast Cancer < 12 months						Breast Cancer > 12 months							
Tertile ^{\$}	Range		N (%)		N (%)		OR (95% CI) [¶]				P-value	N (%)		OR (95% CI) [¶]				P-value
1st	0.0015	0.0057	21	33.9	6	37.5	1(ref)					3	14.3	1(ref)				
2nd	0.0057	0.0245	20	32.3	4	25.0	0.70	0.2	2.8	0.62		2	9.5	0.70	0.1	4.7	0.71	
3rd	0.0245	0.4672	21	33.9	6	37.5	1.00	0.3	3.7	1.00		16	76.2	5.33	1.5	25.4	0.02	

* All women - cases and controls considered together – were classified into tertiles according to their serum progesterone levels.

\$ Within each progesterone tertile, women were classified into low, medium or high RANKL/OPG based on controls within the corresponding progesterone group.

¶ Using logistic regression odd ratios (ORs) and 95 % Confidence Intervals (CI) have been calculated using the lowest RANKL/OPG group as a reference. Cases have been considered separately according to the time to diagnosis (more than one year or less).

We next analyzed the group of UKCTOCS participants who developed breast cancer within 12 months (BC < 12 months). However, among this cohort high progesterone/high RANKL levels were not associated with an increased incidence of breast cancer (**Figure 10, Table 2**). Intriguingly, we rather observed a progesterone-independent reduction in RANKL and increase in OPG serum levels with breast cancer manifestation resulting in significant decrease in the RANKL-to-OPG ratio compared to UKCTOCS participants who never developed breast cancer (**Figure 11A**). These results indicate that RANKL levels drop whereas OPG levels increase in women close to clinical manifestation of breast tumors. One possible explanation for this phenomenon could be that some women already harbor subclinical disseminated tumor cells that could lead to the observed alterations in serum RANKL/OPG.

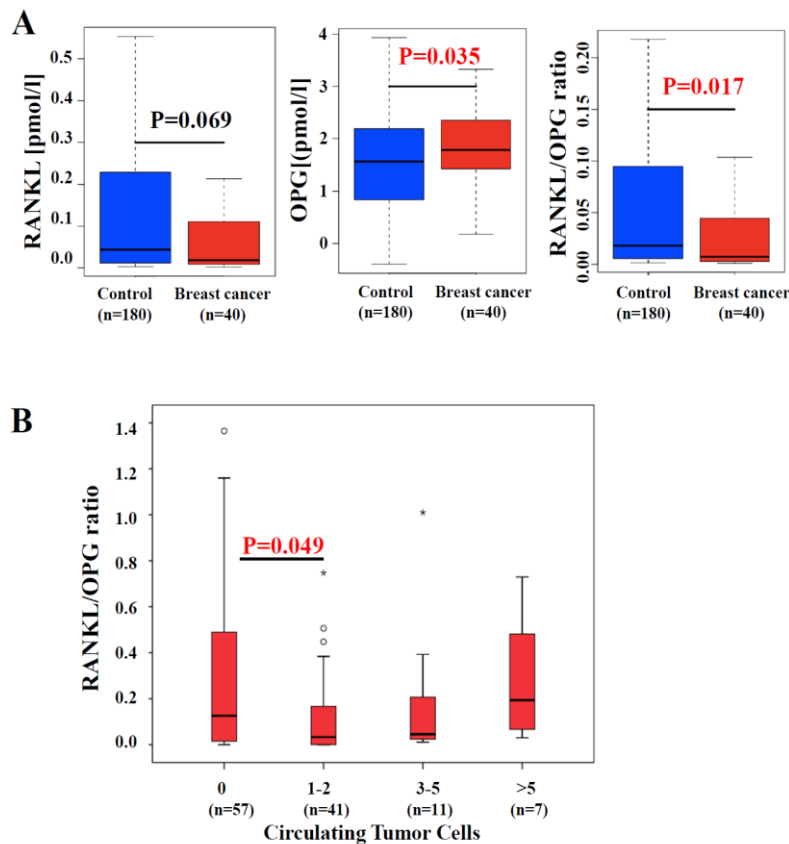


Figure 11. Alterations in RANKL/OPG levels in females close to onset and after manifestation of breast cancer. **A.** Analysis of OPG, RANKL, and RANKL/OPG ratios in prospectively collected UKCTOCS serum samples from 180 healthy postmenopausal women who did not develop breast cancer during their follow up (control) and 40 healthy age-matched women who developed ER positive breast cancer 5-12 months after their serum was collected. P values were calculated using a Mann Whitney U test. **B.** Analysis of RANKL/OPG ratios in 116 women in the SUCCESS trial *after resection* of the local ER positive breast cancer and *before start of systemic treatment* based on the numbers of CTCs. P values were calculated using the Mann Whitney

U test. Abbreviations: CTC, circulating tumor cell; ER, estrogen receptor; OPG, osteoprotegerin; RANKL, Receptor Activator of NF- κ B ligand.

To test this hypothesis, we analysed serum RANKL/OPG in women with breast cancer diagnosis but in the absence of a tumor in the breast – a similar scenario as in the UKCTOCS cohort close to breast cancer diagnosis. We analyzed 116 ER positive breast cancer patients from the SUCCESS trial after surgery and before systemic therapy. **Figure 11B** demonstrates serum RANKL/OPG levels stratified to the number of circulating tumor cells (CTCs) identified in the corresponding blood sample. Intriguingly, women with a small number of CTCs (1-2 CTCs) exhibited a significantly reduced serum RANKL/OPG ratio compared to women without detectable CTCs (**Figure 11B**). Women with a 3-5 CTCs also exhibited reduced serum RANKL/OPG ratios, though this reduction did not reach statistical significance. Of note, the serum RANKL/OPG ratio tends to increase again with an increase in CTCs, albeit the numbers of cases are too small to allow for a firm conclusion. Thus, women with a very low number of CTCs in their blood exhibit a reduced RANKL/OPG ratio suggesting that the alterations we find up to one year in advance of a clinical breast cancer indeed correlate with low numbers of disseminated breast cancer cells.

To substantiate these data in another entirely independent cohort, we determined RANKL and OPG serum levels of participants from the Bruneck study, that allowed us to analyse matched patient samples before and after cancer manifestation. Of 821 subjects with two or more sequential measurements of RANKL and OPG, 697 subjects remained free of cancer during the 15-year follow-up, 19 women developed breast cancer, 16 men developed prostate cancer, and 89 other cancer types (all malignancies except for squamous-cell skin cancer). In line with the UKCTOCS study, levels of RANKL declined with the manifestation of breast cancer ($P=0.006$; paired t-test), whereas RANKL serum concentrations were not altered in subjects with other types of new-onset cancer and those remaining free of neoplastic disease (**Figure 12A**). In parallel, OPG levels rose with the diagnosis of breast cancer ($P=0.037$; paired t-test), whereas no such increase was observed in patients with other types of cancer or subjects free of neoplastic disease (**Figure 12A**). As a consequence, the RANKL-to-OPG ratio significantly decreased in individuals with new-onset breast cancer (**Figure 12B**). In males, no significant differences in OPG and RANKL levels were found in cancer-free individuals or those with new onset prostate cancer, which is also related to sex hormones, or other types of cancer. Our data in two different human prospective breast cancer

cohorts indicate that alterations in RANKL/OPG ratios are significantly associated with breast cancer manifestation.

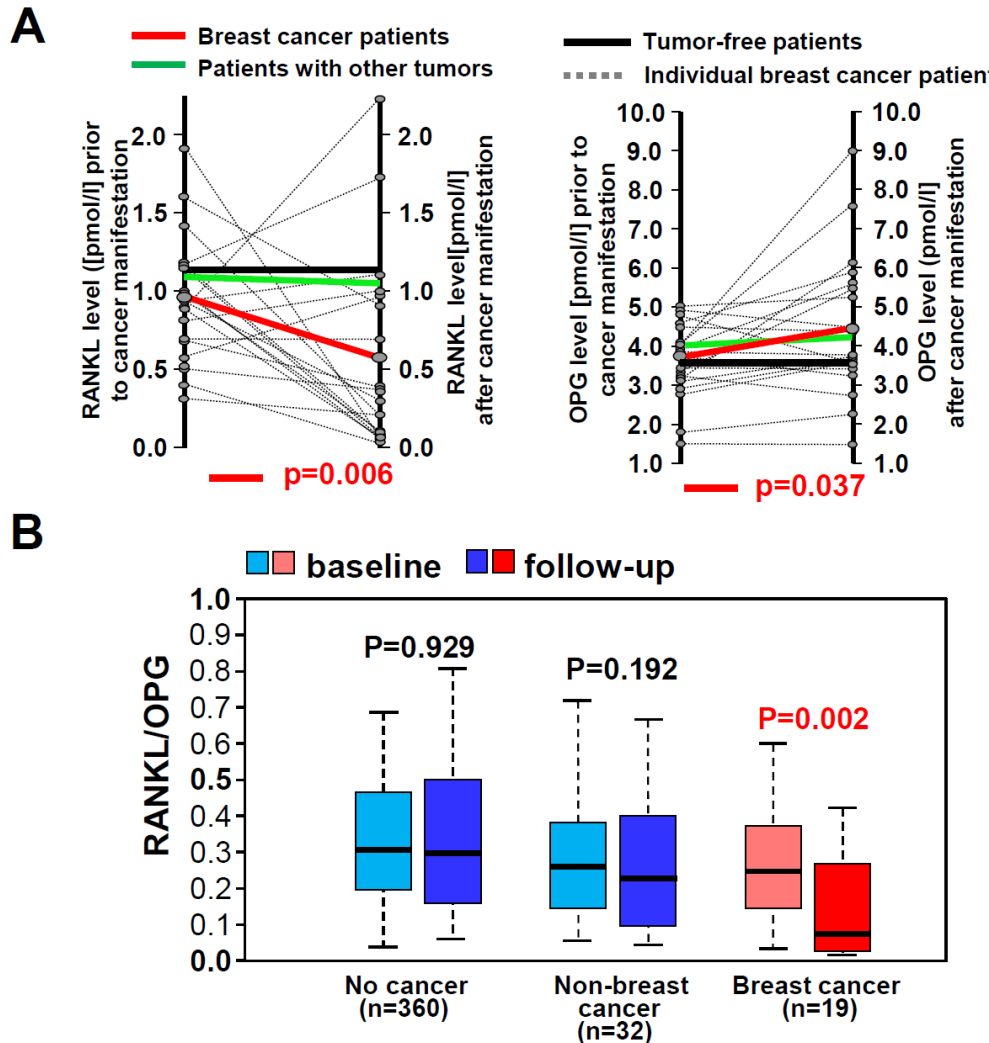


Figure 12. Alterations in RANKL/RANKL levels in females close to onset and after breast cancer.

A. Individual changes in serum levels of OPG and soluble RANKL in 19 female subjects from our longitudinal Bruneck cohort before and after manifestations of breast cancer (dotted black lines). Mean changes are shown for subjects with incident breast cancer (red lines), other types of new-onset cancer (n=32; green lines) and women free of neoplastic disease during the 15 year follow-up (n=360; black lines). **B.** Box plots of RANKL-to-OPG ratios assessed prior to (hatched) and after (filled boxes) cancer manifestation in women from the prospective Bruneck study indicate median ratio levels and inter-quartile ranges. In individuals who remained free of cancer, ratios given are those assessed at the time intervals corresponding to those in cancer patients.

Discussion. We and other groups have previously demonstrated a central role for the osteoclastogenic molecule RANKL in breast cancer development using genetic mouse models. We were now able to show that increased progesterone and RANKL serum levels stratify a subgroup of postmenopausal women without known genetic predispositions that exhibit a ~5 fold increased risk of developing breast cancer 12-24 months before cancer diagnosis. This indicates that either increased free RANKL or high RANKL and high progesterone serum levels, as seen in the case of our postmenopausal UK cohort, could be useful biomarkers to predict future breast cancer more than one year in advance of breast cancer diagnosis. These data are in line with our genetic mouse models that the RANKL/RANK/OPG system drives the initiation and progression of breast cancer. Our findings that an increased RANKL/OPG ratio > 12 months prior to breast cancer development is consistent with data from meta-analysis showing that sex steroid hormone levels well in advance of breast cancer diagnosis are more significantly associated with breast cancer risk compared with hormone levels analyzed closer to diagnosis.

When we analyzed the serum of postmenopausal women shortly before breast cancer diagnosis or matched sera from women before and after clinical manifest breast cancer, we observed reduced RANKL and increased OPG serum levels. A similar phenomenon was shown for serum RANKL levels and osteoporosis: low serum RANKL levels are associated with a 10-fold higher risk of non-traumatic fractures in postmenopausal women. Moreover, increased OPG is associated with enhanced bone loss in postmenopausal women not on hormone replacement therapy and increased OPG levels have been observed in patients with bone metastasis. The mechanisms of these serum changes in breast cancer need to be further investigated and might reflect compensatory mechanisms against developing microtumors and/or redistribution/sequestration of RANKL/OPG within different body compartments. Although women in our study do not have clinical manifest bone metastasis, it has been demonstrated that tumor cells can disseminate systemically from earliest epithelial alterations in transgenic mice and even from very early breast cancers in women and that these disseminated tumor cells can form micro-metastases in bone marrow and lungs. Likewise, it is well established that breast cancer cells interfere with osteoblasts (being a major source for RANKL) and induce osteoblast

necrosis and apoptosis. Hence the effect we see on RANKL and the RANKL/OPG ratio in women who get clinical manifest breast cancer close to serum collection may be triggered by an effect of epithelial/tumour cells altering RANKL/OPG in the bone marrow. Of note, recently it has been reported that blocking of RANKL reduces the reoccurrence of breast cancer in women receiving adjuvant Tamoxifen therapy.

One key issue for the management of cancer is to accurately predict the risk in order to triage women into screening or preventive strategies. In breast cancer, population-based mammography screening is currently used to identify women with early breast cancer. Our data suggest that the apparent alterations in RANKL/OPG in postmenopausal women with high progesterone levels could be used as a future biomarker to identify a subgroup of women at high risk of breast cancer. Moreover, it has been recently shown that women with high genetic breast cancer risk due to *BRCA1* mutations appear to be exposed to increased RANKL. Our data also indicate that serum RANKL/OPG ratios correlate with CTCs and that a low number of CTCs – even in the absence of a local breast cancer – is associated with a reduced serum RANKL/OPG ratio. The mechanism of this phenomenon is not yet clear, but it is attractive to speculate that unknown soluble serum factors which are associated with a low number of CTCs may suppress RANKL expression and/or alter processing of membrane bound RANKL into soluble RANKL. These studies now need to be confirmed in additional cohorts. Since an antibody to block RANKL (Denosumab) has already been approved for treatment of osteoporosis patients and treatment of skeletal related events in solid tumors, our data in the presented prospective human cohorts further support the notion that the same antibody could potentially be used to prevent the development of breast cancer in high risk women. Similarly, higher concentrations of soluble RANKL are positively associated with an increased risk of estrogen receptor-positive (ER⁺) but not ER⁻ breast cancer. Moreover, a recent clinical study revealed that RANK expression is increased in hormone receptor negative breast cancer and correlates with a worse recurrence-free survival and risk of bone metastasis.

2.3. RANKL inhibition as a preventative strategy in BRCA1/2 mutation carriers.

Although most breast cancers are sporadic in origin, approximately 5-10% are inherited through gene mutations. Among these inherited breast cancer cases, the majority are caused by mutations in the tumor suppressor genes *Breast Cancer 1 (BRCA1)* or *Breast Cancer 2 (BRCA2)*. The risk for developing breast cancer is increased in *BRCA1/2* mutation carriers as the remaining, non-mutated *BRCA* allele may be inactivated via somatic mutation or epigenetic silencing, however, it is still uncertain why *BRCA1/2*-mutation carriers predominately develop breast or ovarian cancers, over other forms. Recent evidence indicates that estrogen controls the survival of *BRCA1*-deficient cells via a PI3K/NRF2-regulated pathway, which may partially explain the occurrence of hormonally-driven tumors in patients harboring *BRCA1/2* mutations. Such hormonal influence on *BRCA*-driven breast cancers is seen in various mouse models in which activation of estrogen signaling synergizes with *Brca1* loss to promote tumorigenesis. For instance, in mammary tissue, *BRCA1* appears to be involved in the regulation of both estrogen and progesterone receptors. Stimulation of *Brca1*-mammary gland specific knockout mice with exogenous progesterone resulted in an increased proliferation of mammary epithelial cells. Direct effects of progesterone on *Brca1*-associated breast cancer were also demonstrated by studies using *Brca1;p53* deficient mice, a mouse model that reflects the frequent occurrence of *TP53* mutations in *BRCA1*-mutated tumors. The administration of the progesterone antagonist, Mifepristone, markedly blocked tumor development in this mouse model. Thus, both mouse and human studies indicate that sex hormones play a significant role in the pathogenesis of *BRCA1*-mutated breast cancer.

As RANK and RANKL are key molecules downstream of sex hormones in the mammary gland, we speculated that the RANKL/RANK pathway also plays a role in the etiology of *BRCA1* mutation-driven breast cancer.

Results: One key question is whether RANKL inhibition could be one day used for prevention of breast cancer. To accomplish this, we however need to provide direct evidence that inhibition of RANKL and its receptor RANK can indeed delay the occurrence of mammary cancer in high risk

mammary cancer scenarios. To directly assess the role of RANKL/RANK in *Brca1* mutation-mediated tumorigenesis, we deleted *Brca1* plus *p53* in basal mammary epithelial cells and mammary progenitor cells using K5Cre mice to induce spontaneous mammary cancer as previously reported^{18,19}. This line was then crossed into *Rank*^{fl^{ox}/fl^{ox}} mice to examine spontaneous tumorigenesis in the presence or absence of RANK expression. Importantly, at 4 month of age we observed spontaneous and widespread epithelial hyperplasia (**Figure 13a**) as well as low and high grade mammary intraepithelial neoplasias (MIN) and also invasive carcinomas in *K5Cre;Brca1;p53* double mutant mice (n=8) (**Figure 13b**). The mammary glands of age-matched females with concomitant ablation of *Rank* (n=8) appeared largely normal displaying a significantly lower number of low grade and high grade MINs and we never detected any carcinomas (**Figure 13b**). We observed marked DNA damage in both double and triple mutant mice as determined by γ H2Ax immunostaining. These data show that genetic deletion of *Rank* in basal mammary epithelial cells markedly abrogates the development of intraepithelial neoplasias and invasive carcinomas as a consequence of *Brca1;p53* mutations.

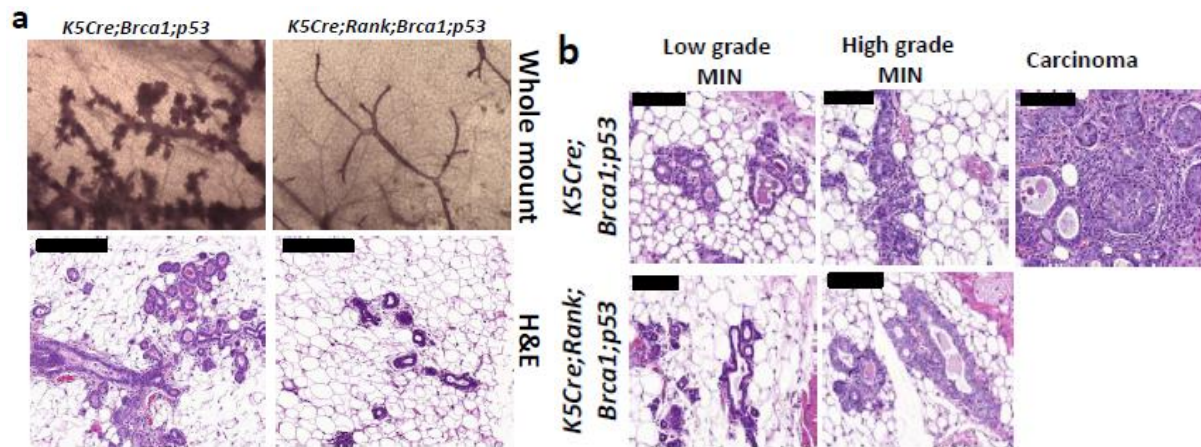


Figure 13. Ablation of *Rank* in basal mammary epithelial cells markedly decreases tumor formation in *Brca1/p53* mutant female mice. **a**, Representative whole mount images (haematoxylin staining, magnifications 52x) and paraffin sections (H&E staining, scale bar represents 200μm) of mammary glands from 4 month old *K5Cre;Brca1;p53* double and *K5Cre;Rank;Brca1;p53* triple knockout littermate mice. **b**, Representative images (H&E staining, scale bars represent 100μm) of low grade MINs, high grade MINs and adenocarcinomas in mammary glands from 4 month old *K5Cre;Brca1;p53* and *K5Cre;Rank;Brca1;p53* mutant littermates.

The occurrence of skin cancer commonly observed in the *K5Cre;Brca1;p53* double^{18,19} as well as *K5Cre;Rank;Brca1;p53* triple mutant mice precluded further analysis of mammary tumorigenesis

beyond the 4 month time-point. We therefore switched the Cre deleter line and introduced all three conditional alleles onto a WapCre^C mouse background. Of note, in this mouse line, the Whey-acid protein (Wap)rTACre activity is independent of doxycyclin and pregnancy and the Cre activity present in both luminal and basal epithelial cells in the mammary gland. WapCre^C-mediated *Rank* deletion had no apparent effect on formation of the mammary gland during puberty. While all WapCre^C;*Brca1*;*p53* mutant females developed spontaneous palpable tumors starting around day 100 after birth, concomitant *Rank* deletion in the mammary epithelium resulted in a delayed tumor onset. The median day of onset for WapCre^C;*Brca1*;*p53* mice was 158 days, whereas the median day of onset for WapCre^C;*Rank*;*Brca1*;*p53* triple mutant mice was 184 days. Importantly, while all WapCre^C;*Brca1*;*p53* double mutant females developed spontaneous mammary tumors, 25% of WapCre^C;*Rank*;*Brca1*;*p53* triple mutant littermates never developed any tumors which was also reflected by the overall survival rates (**Figure 14, left panel**), even when we followed these females throughout their lifetimes, sometimes up to 2 years of age. Once mammary tumors developed in both double and triple mutant mice, their growth curves appeared to be similar. Thus, *Rank* inactivation using WapCre^C-driven deletion delays the onset and in some cases even completely prevents the development of *Brca1*;*p53* mutation-driven spontaneous mammary cancer.

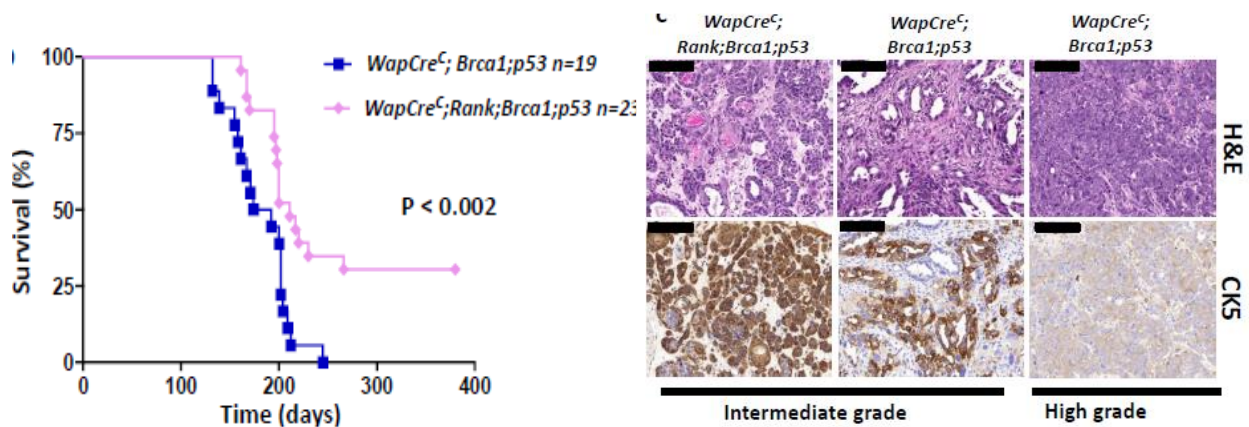


Figure 14. *Rank* deletion reduces the incidence of *Brca1* and *p53* mutation-driven mammary tumorigenesis. **Left panel,** Overall survival rates in WapCre^C;*Brca1*;*p53* and WapCre^C;*Rank*;*Brca1*;*p53* mice. Data are shown as percentage of overall mouse survival. **Right panel,** Representative histological sections of mammary tumors isolated from WapCre^C;*Brca1*;*p53* and WapCre^C;*Rank*;*Brca1*;*p53* mice showing different histological grades (H&E) and altered, grade-dependent Cytokeratin 5 immunostainings (CK5). Scale bars represent 100µm.

Histopathologic analysis showed that mammary tumors from both genotypes exhibit typical signs of intermediate grade cancers; however high grade cancers were only observed in mammary tumors from *WapCre^C;Brca1;p53* double mutant females (**Figure 14, right panel**). All intermediate grade tumors that developed in double and triple mutant mice expressed Cytokeratin5⁺ (CK5) indicative of a basal cell subtype. However, in high grade tumors from *WapCre^C;Brca1;p53* double mutant females CK5 expression was in most cases lost, further supporting the notion of marked epithelial dedifferentiation of such mammary cancers (**Figure 14, right panel**). CK5 positive low to intermediate grade foci and CK5 negative high grade foci were often found within the same tumor. We failed to detect metastases into the lung in either double or triple mutant mice that had developed tumors. Of note, whereas RANK was expressed in all mammary tumors analysed in *WapCre^C;Brca1;p53* females, RANK expression was absent in tumors from *WapCre^C;Rank;Brca1;p53* triple mutant females as determined by immunohistochemistry or qPCR. Thus, loss of RANK not only delays tumor onset, but also affects progression to high tumor malignancy.

Ki67 immunostaining of mammary tumors from *WapCre^C;Brca1;p53* double mutant and *WapCre^C;Rank;Brca1;p53* triple mutant females showed comparable proliferation of tumor cells in both genotypes, a finding that might explain that established tumors grow at a comparable “speed”. Finally, immunostaining for γ H2Ax showed that loss of *Brca1* and *p53* resulted in massive DNA damage in intermediate as well as high grade tumors, irrespective of the presence or absence of RANK expression). Importantly, we also observed marked DNA damage in the mammary epithelium of a two year old *WapCre^C;Rank;Brca1;p53* triple mutant female that had never developed any tumor (**Figure 15**). Although there was substantial DNA damage, the mammary glands appeared histologically normal and only very few mammary epithelial cells proliferated as determined by very low Ki67 positivity. Analysis of mammary epithelial cells (MECs) isolated from the tumor-free 2 year old *WapCre^C;Rank;Brca1;p53* female confirmed efficient deletion of *Brca1*, *p53* as well as *Rank*. These data indicate that loss of *Rank* protected from mammary tumorigenesis despite the presence of DNA damage due to the inactivation of *Brca1*.

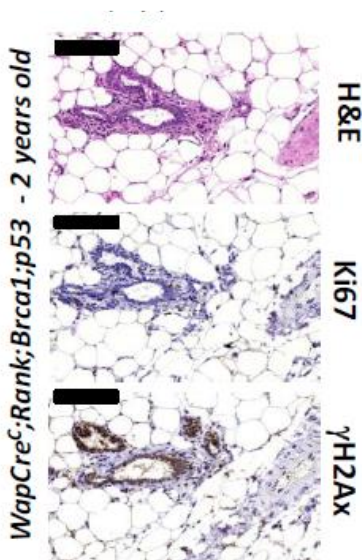


Figure 15. *Rank* deletion can prevent from *Brca1* and *p53* mutation-driven mammary tumorigenesis. H&E staining, Ki67 and γ H2Ax immunostainings of mammary glands from a 2 year old tumor free WapCre^C;Rank;Brca1;p53 mutant mouse, showing apparently normal mammary gland tissue, low Ki67 positivity and DNA damage indicated by γ H2Ax positivity. Scale bars represent 100 μ m.

Since RANK controls key mitogenic pathways that may influence stem/progenitor cells and its deletion delays onset and in some cases even prevents *Brca1*;p53-mutation driven mammary tumors using two different Cre deleter mouse lines, we speculated that RANKL/RANK might affect proliferation and expansion of mammary progenitor cells on the *Brca1*;p53 mutant background. To assess if RANKL/RANK control expansion of *Brca1*;p53 mutant mammary progenitor cells, we performed ovariectomy (ovx) on females of all genotypes followed by sham treatment (no hormones) or reconstitution with estrogen and progesterone (E+P) to control for sex hormone levels. As previously reported, sex hormone treatment resulted in a marked expansion of Lin⁻CD24⁺CD49^{fhi} basal mammary progenitor cells in both control as well as WapCre^C;Brca1;p53 double mutant mice; however, this expansion was markedly reduced in the absence of RANK expression (**Figure 16a,b**). Sex hormone-driven proliferation in ovx females was further determined by *in vivo* BrdU labelling. WapCre^C-driven deletion of *Brca1* and *p53* resulted in markedly increased numbers of cycling mammary epithelial cells; this sex hormone-induced proliferation of mammary epithelial cells was significantly reduced in *Rank* mutant females). Importantly, deletion of *Rank* also markedly abrogated the *in vitro* colony formation capacity of basal Lin⁻CD24⁺CD49^{fhi} mammary progenitors from WapCre^C;Brca1;p53 mutant mice; the progenitor capacity within the ER⁺PR⁻ alveolar progenitor Sca1⁻ compartment was also lower in the absence of RANK). Similar to our data using WapCre^C deleter mice, sex hormone treatment resulted in expansion of the basal Lin⁻CD24⁺CD49^{fhi} progenitor compartment in ovariectomized

K5Cre;Brca1;p53 double knockout mice as compared to sham operated mice, while this expansion was not observed in the absence of RANK. Thus, RANKL/RANK controls sex-hormone induced expansion and activity of *Brca1;p53* mutant basal progenitor cells.

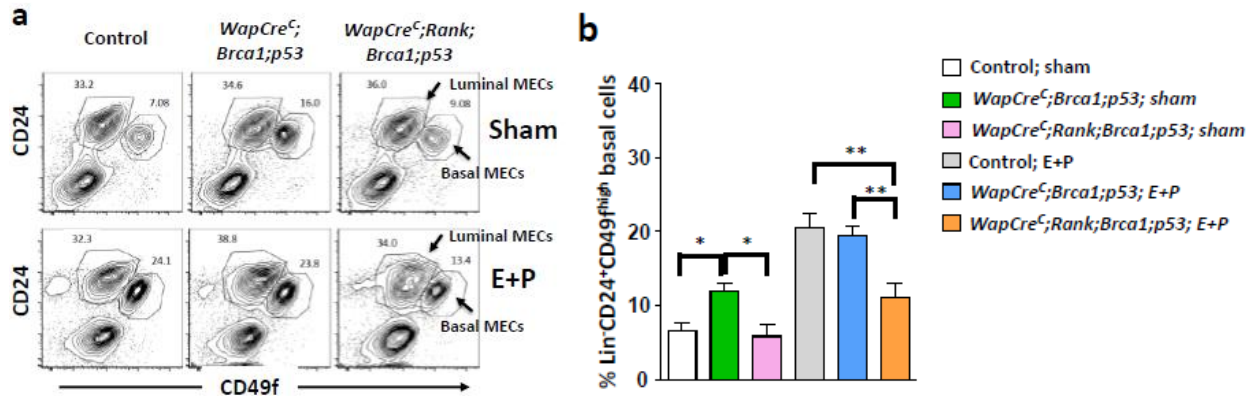


Figure 16. RANK mediates proliferation and expansion of *Brca1;p53* mutant basal mammary epithelial cells in response to hormonal stimulation. **a**, Representative FACS profiles of luminal (CD24⁺CD49^{fl}) and basal (CD24⁺CD49^{hi}) mammary epithelial cell populations (MECs) in ovariectomized *WapCre^C*-negative control, *WapCre^C;Brca1;p53* double knockout and *WapCre^C;Rank;Brca1;p53* triple knockout female mice treated with 17 β -estradiol plus progesterone (E+P) or left untreated (sham). **b**, Quantification of CD24⁺CD49^{hi} basal mammary epithelial cells in ovariectomized *WapCre*-negative control, *WapCre^C;Brca1;p53* double and *WapCre^C;Rank;Brca1;p53* triple knockout mice treated with E+P or without hormone treatment (sham). Data are from 10-12 old mice, at which age we never observed any evident tumors, and represent mean $\pm$ SEM of at least n=3 mice/group. * p<0.05; **p<0.005; Student's t-Test.

Discussion. We provided key and direct evidence for a crucial role of RANK/RANKL in *Brca1* mutation-driven breast cancer. Genetically modified mice that carry *Brca1* and *p53* mutations exhibited excessive proliferation and malignancy in their mammary glands at the age of 4 months. Concurrent inactivation of *Tnfrsf11a* in these mice almost completely abrogated the occurrence of malignancy. Similarly, all *Brca1;p53;Wap^CCre* mice showed the occurrence of tumors but only 25% of littermate *Tnfrsf11a*-deleted mice developed any tumors in their life time. Most importantly, pharmacologic RANKL-blockade markedly abrogated the occurrence of pre-neoplastic lesions in this model of *Brca1* mutation-driven mammary tumorigenesis as only 1 out of 13 *Brca1* mutant mice showed mammary epithelial neoplasia when RANKL was inhibited whereas nearly all vehicle-treated control mice developed pre-neoplastic lesions. *Brca1*-

mutations correlated with an increase in mammary progenitor cell numbers, which is RANKL/RANK dependent. The *in vitro* colony forming capacity of human mammary epithelial progenitor cells from heterozygous *BRCA1*-mutation carriers was also significantly inhibited by treatment with the monoclonal RANKL blocking antibody denosumab (**Figure 17**).

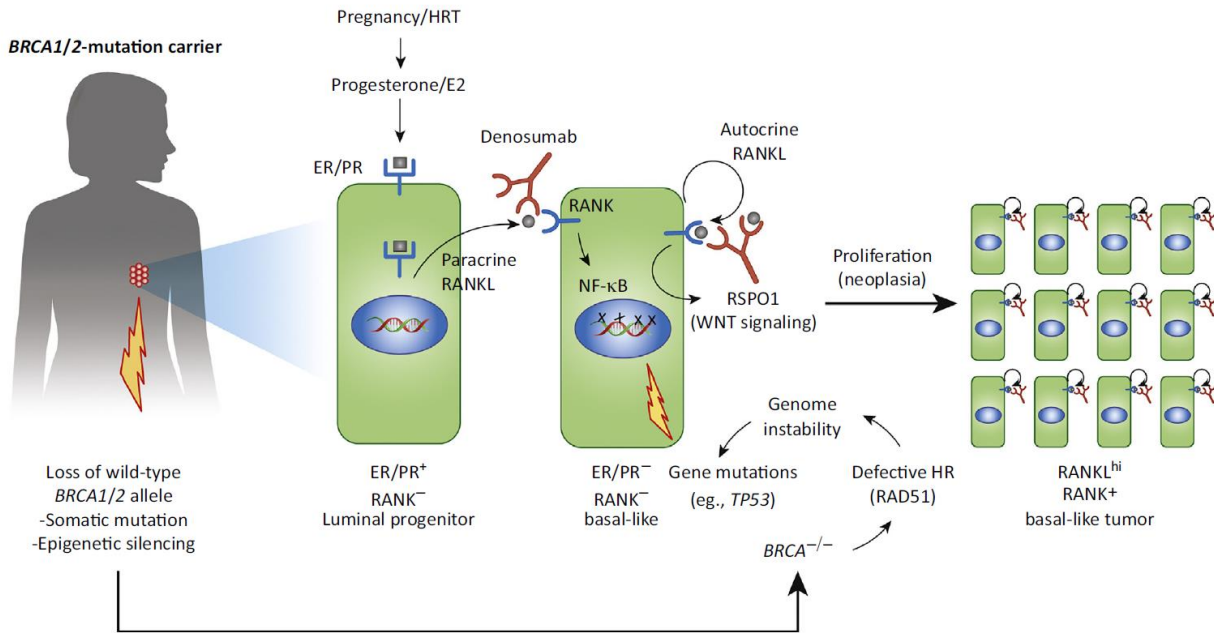


Figure 17. RANKL/RANK-mediated proliferation of *BRCA1* mutant mammary progenitor cells. RANK is constitutively expressed on the surface of a subset of basal mammary epithelial progenitor cells. Women carrying a heterozygous *BRCA1* mutation are prone to spontaneous loss of the wild type allele due to somatic mutation or epigenetic silencing. Loss of *BRCA1* leads to genomic instability, DNA damage and genetic mutations (e.g. *TP53*). Natural progesterone, or synthetic progestins, lead to increased RANKL expression in *PR*⁺ luminal breast epithelial cells which then activates RANK-dependent cell proliferation of adjacent progenitor cells which might be further enhanced via RANKL-mediated WNT signals via R-spondin-1 (RSP01). Altogether, the genotoxic stress and amplified proliferation cues, culminate in the development of breast cancer. Based on these findings and the essential evolutionary role of RANKL/RANK to drive expansion and proliferation of mammary progenitor cells, drugs that target RANKL such as denosumab may prevent breast cancer in *BRCA1/2* mutation carriers and possibly other high-risk breast cancer groups.

Finally, high RANK expression in pre-malignant lesions and breast cancer samples of patients carrying a *BRCA1*-mutation was observed. In addition, SNP data from the Collaborative Oncological Gene-environment Study (iCOGS), which includes approximately 15,200 *BRCA1* and 8,200 *BRCA2* mutation carriers, identified six SNPs in the *Tnfrsf11a* locus (*Tnfrsf11a* codes

for RANK) that were significantly associated with breast cancer risk in the overall series of *BRCA1* mutation carriers. SNPs that correlated with enhanced risk to develop breast cancer for *BRCA2* mutation carriers have also been uncovered, suggesting that these findings could extend to other high breast cancer risk groups. Taken together, multiple independent studies strongly indicate that RANKL/RANK act on both murine and human mammary progenitor cells, and that RANKL/RANK are indeed critical regulators for the initiation, as well as progression, of *BRCA1* mutation-driven mammary cancer.

Our results show that genetic inactivation of RANK has a marked effect on the onset of mammary tumors and also survival of female mice that carry *Brca1;p53* mutations. It has been recently shown that RANK expression is markedly increased in triple negative breast tumors from *BRCA1* carriers compared with other subtypes. RANKL acts in a paracrine fashion on the membranous RANK of ER/PR-negative epithelial cells of the breast to expand the stem and progenitor cell populations. Our data show that RANKL/RANK also regulates the expansion and functional capacity of progenitor cells on a *Brca1/p53* mutant background. Since RANKL is induced by progesterone, these data could explain why sex hormones, in particular progesterone, play an important role in *BRCA1* mutation-driven tumorigenesis in humans and mouse models. Future mechanistic studies are required to test the hypothesis that the abnormal, sustained accumulation of hormone receptor negative cells in *Brca1* deficient mammary glands is due, in part, to a dysregulated RANKL/RANK-driven expansion of progenitor cells. Taken together these data show that RANKL/RANK link sex hormones to progenitor cell expansion and tumorigenesis in inherited breast cancer and provide a viable strategy to possibly prevent breast cancer in patients with *Brca1* mutations.

Summary. RANK and RANKL were first identified as key regulators of bone metabolism that control osteoclast differentiation and activation. In the last two decades, many studies focusing on this pathway have revealed that RANK and RANKL are also involved in various physiological or pathological conditions, such as regulation of the immune system, development of lymphoid organs, bone metastasis regulation, and the development of lactating mammary glands and the initiation and progression of sex hormone-driven mammary cancer.

In the mammary gland, RANKL is induced by the sex hormone progesterone (and possibly other factors such as prolactin), which then acts in a paracrine fashion on hormone receptor-negative RANK expressing epithelial cells, which ultimately induces the expansion of mammary progenitor cells. Our studies supported by DoD have provided direct genetic and pharmacological proof that the RANKL/RANK pathway plays a vital role in the progression of familial *BRCA1*-mutated associated breast cancer. Mechanistically, RANKL can regulate the expansion of RANK-positive luminal progenitor cells, which in concert with other oncogenic stresses such as *BRCA1* deficient-mediated DNA damage or altered cell death following genotoxic stress can result in the development of mutant cell clones, ultimately manifesting in breast cancer. Importantly, safety of pharmacological RANKL inhibition has been established in thousands of women that have received the RANKL blocking antibody although adverse effects such as pain, anemia, diarrhea, skin problems and in some cases, serious infection was observed after long term administration of denosumab. Thus, targeting the RANKL/RANK pathway by using an approved drug is a feasible strategy for the prevention of breast cancer in *BRCA1*-mutant carriers and might be effective for treatment of other forms of breast cancer. If the data on RANKL inhibition in preclinical mouse mammary tumor and *in vitro* human mammary progenitor assays hold true, RANKL blockade could prevent breast cancer in humans. A multinational phase III clinical trial using denosumab to prevent the development of breast cancer in *BRCA1*-mutation carriers is currently being initiated, with the first woman set to receive the RANKL blocking antibody in November of 2018. Thus, what we set out to accomplish, to develop novels for future prevention of breast cancers is not being actively tested in women with high risk and might indeed in the near future lead to a radical new breast cancer prevention strategy.

Of note: In a multicenter, randomized, double blind and placebo-controlled trial carried out in Austria and Sweden over 6 years, it was found that breast cancer patients receiving aromatase inhibitor therapy and treated with Denosumab had a significantly delayed onset for the first clinical fracture and an overall lower number of fractures without severe adverse events.

Therefore, targeting the RANKL/RANK pathway has already benefitted thousands of breast cancer patients.

2.4. RANKL/RANK link female sex hormones to other cancers – the surprising case of lung cancer. Lung cancer is the leading cause of cancer deaths. Besides smoking, epidemiological studies have linked female sex hormones to lung cancer in women, however the underlying mechanisms remain unclear. RANKL inhibition with a monoclonal Ab called Denosumab has been approved for osteoporosis and skeletal-related events in cancer, and has been linked to delayed re-occurrence of breast tumours in an adjuvant setting. During the clinical trials for bone metastasis, a post-hoc exploratory analysis surprisingly found that Denosumab significantly improved survival of lung cancer patients with established metastasis. Expression of estrogen and progesterone receptors also have been reported in lung cancer cells and numerous studies have indicated more rapid progression of lung cancer in women, suggesting that sex hormones might affect the development of primary lung tumours. Since the RANKL/RANK system is known to be regulated by sex hormones, we hypothesized that it might have a direct role in primary lung cancer.

We were now able to show that pharmacologic RANK inhibition reduces tumour growth in patient derived lung cancer xenografts (PDX). Clonal genetic inactivation of RANK in mouse lung epithelial cells markedly impairs the progression of KRas^{G12D}-driven lung cancer, resulting in a significant survival advantage (**Figure 18**). Mechanistically, RANK rewires energy homeostasis in human and murine lung cancer cells and promotes expansion of lung cancer stem-like cells, which is blocked by inhibiting mitochondrial respiration. Most importantly, our data also indicates survival differences in KRas^{G12D}-driven lung cancer between male and female mice, and we showed that female sex hormones can promote lung cancer progression via the RANK pathway (**Figure 19**). We also showed that RANK is frequently and highly expressed on primary human lung tumors and that a molecular signature of high RANKL and high RANK expression and low expression of OPG and LGR4, the natural endogenous inhibitors of the

RANKL/RANK pathway, is associated with worse prognosis for lung cancer patients, in particular in females, mirroring our genetic mouse data.

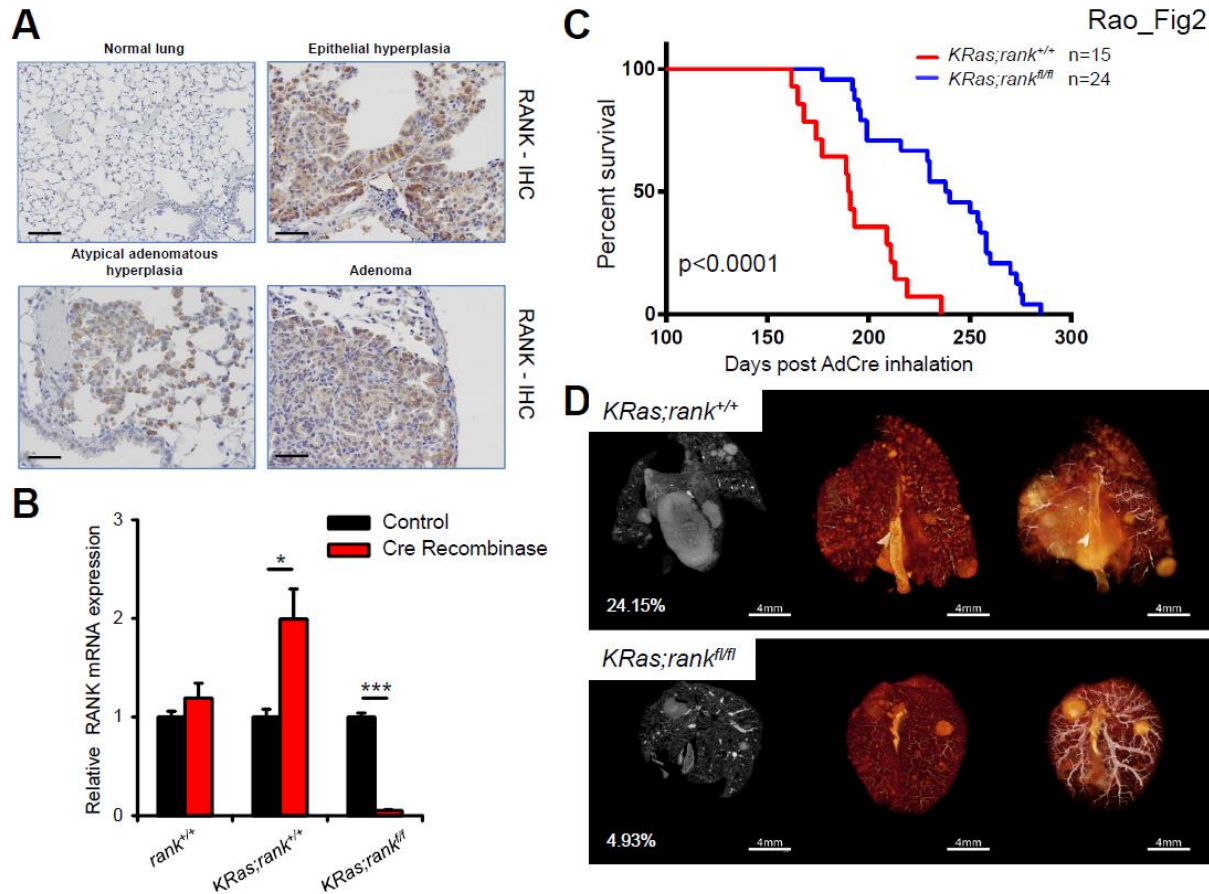
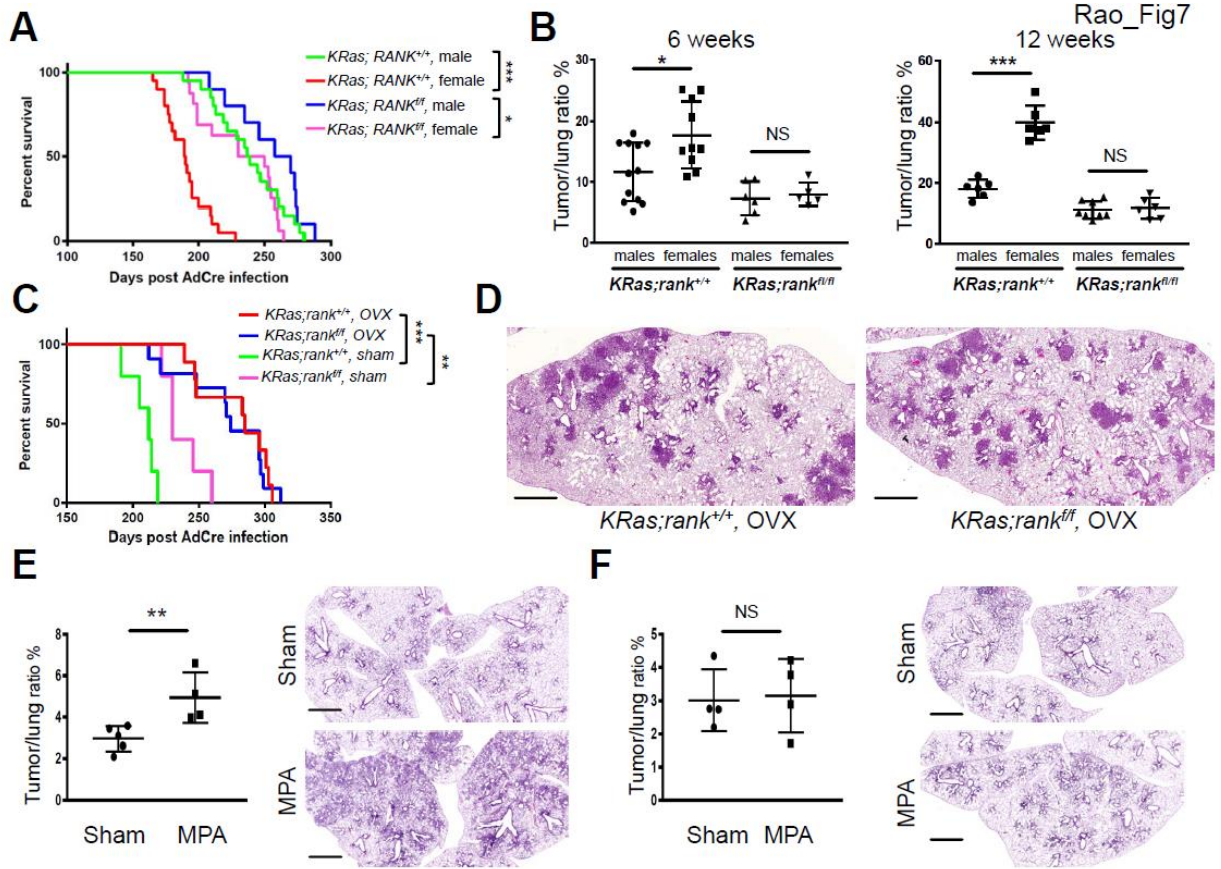


Figure 18. Loss RANK prolongs survival of *Kras*^{G12D}-driven lung cancer. (A) Increased RANK expression during progression of lung tumors. Analysis of RANK expression by IHC during lung tumor progression in *Kras*^{G12D} mice. Representative images for RANK expression in atypical adenomatous hyperplasia (AAH), epithelial hyperplasia (EH) and adenoma of AdCre-infected mice were shown. Normal lung parenchyma is shown for a mouse not infected with AdCre (normal lung). AAH and EH were from mice 11 weeks post-AdCre infection; adenomas from 14.5 weeks post-infection. Scale bars, 100 μ m. (B) qPCR analysis of RANK mRNA levels in primary pneumocytes purified from *KRas*;*rank*^{+/+} and *KRas*;*rank*^{fl/fl} mice (experiment was performed with 3 technical replicates and repeated 3 times independently). Samples without AdCre infection in every genotype were set to 1, respectively. * $p < 0.05$, *** $p < 0.001$ (Unpaired, two-sided t-test). (C) Kaplan Meier survival curves for *KRas*;*rank*^{+/+} (n=15, median survival=191 days) and *KRas*;*rank*^{fl/fl} (n=24, median survival=239 days) littermate mice injected intranasally with AdCre (2.5×10^7 PFU). $p < 0.0001$ (log rank test) between *KRas*;*rank*^{fl/fl} and littermate controls. (D) Representative microCT images of lung tumors of *KRas*;*rank*^{+/+} and *KRas*;*rank*^{fl/fl} littermate mice assayed 25 weeks after AdCre inhalation. Three different images of the same lung are shown and the percentages of tumor-to-lung volumes indicated.



In summary, our human population data and experimental genetic mouse models suggest a scenario where RANK is induced in both males and female lung epithelial cells by activating oncogenic Kras and in both male and female lung tumor cells couple to mitochondrial respiration. RANKL and OPG levels are markedly regulated by female sex hormones, RANKL being regulated via progesterone, which we recapitulate in our experiments. Thus, RANKL/OPG regulation by female sex hormones can create a milieu where RANK is being hyperactivated by the presence of increased RANKL, explaining how female sex hormones could drive lung tumorigenesis. Since a drug, that blocks RANKL has already been approved for human use with a very good safety profile, our findings are of direct clinical relevance and offer a feasible, targeted approach for the treatment of primary lung cancer.

3. Modelling cancer pathways using haploid stem cell technologies.

Background. Genetic screens have markedly contributed to the understanding of basic physiology and disease pathways. A drawback of genetic screens in cell culture is that one needs to derive cells from homozygously mutated animals or target a gene twice since mammalian cell culture systems are unable to undergo sexual reproduction cycles. Some organisms such as yeast or males of social insects are haploid, i.e. they carry a single set of chromosomes, while haploidy in mammals is exclusively restricted to mature germ cells. A single copy of the genome provides the basis for genetic analyses where any recessive mutation of essential genes will show a clear phenotype due to the absence of a second gene copy. Most prominently, haploidy in yeast, has been utilized for recessive genetic screens that markedly contributed to our understanding of normal development, basic physiology, and disease. Somatic mammalian cells carry two copies of chromosomes (diploidy) that obscure genetic analysis. Near haploid leukemic cells however have recently been developed as a high throughput screening tool. Although deemed impossible, we generated mammalian haploid

embryonic stem cells from parthenogenetic mouse embryos, thereby opening haploid screening technology to a wide range of biological questions. Haploid stem cells open the possibility of combining the power of a haploid genome with pluripotency of embryonic stem cells to uncover fundamental biological processes in defined cell types at a genomic scale.

3.1. Generation of the largest murine stem cell biobank using repairable mutagenesis.

Approaches to functionally analyze the mammalian genome include ENU mutagenesis, gene targeting, RNA interference and CRISPR-mediated genome editing. Although powerful, these approaches suffer from various caveats, such as poor knockdown efficiency and off-target effects. Additionally, clonal variability within populations can compromise comparisons and reproducibility. Thus, reversible mutations enabling direct comparison of phenotypes within a single clone are pivotal to study genetic dependencies.

To generate a conditional mutagenesis system at a genome-wide scale and at the clonal level, we applied insertional mutagenesis with genetically bar-coded lentiviral-, retroviral- and transposon [*Tol2* and *Sleeping beauty (SB)*]-based vectors in haploid murine embryonic stem cells (mESCs, **Figure 20a**), which enable recessive genetics. We analyzed several parthenogenic haploid mESC lines and chose to pursue AN3-12 cells. Insertional mutagenesis permits the integration of invertable splice acceptors, resulting in conditional alleles, as well as high-throughput direct identification of integration sites. To map insertion sites and complex internal bar-codes ($>10^7$) by inverse PCR, an optimized universal sequencing strategy was established. A combination of strategies was used to avoid genomic biases of the insertional mutagenesis systems (**Figure 20a,b**), yielding genome-wide mutagenesis (**Figure 20c**). Of note, *Tol2* outperformed the classical viral delivery systems and even *in silico* mutagenesis at <1 million integrations. The mutagenesis systems also generated anti-sense and intergenic integrations at high frequency due to cryptic transcriptional start sites, however this is not expected to affect disruption of transcription in sense orientation. Thus, we utilized various delivery systems to obtain unbiased, genome-saturated, and conditional mutagenesis.

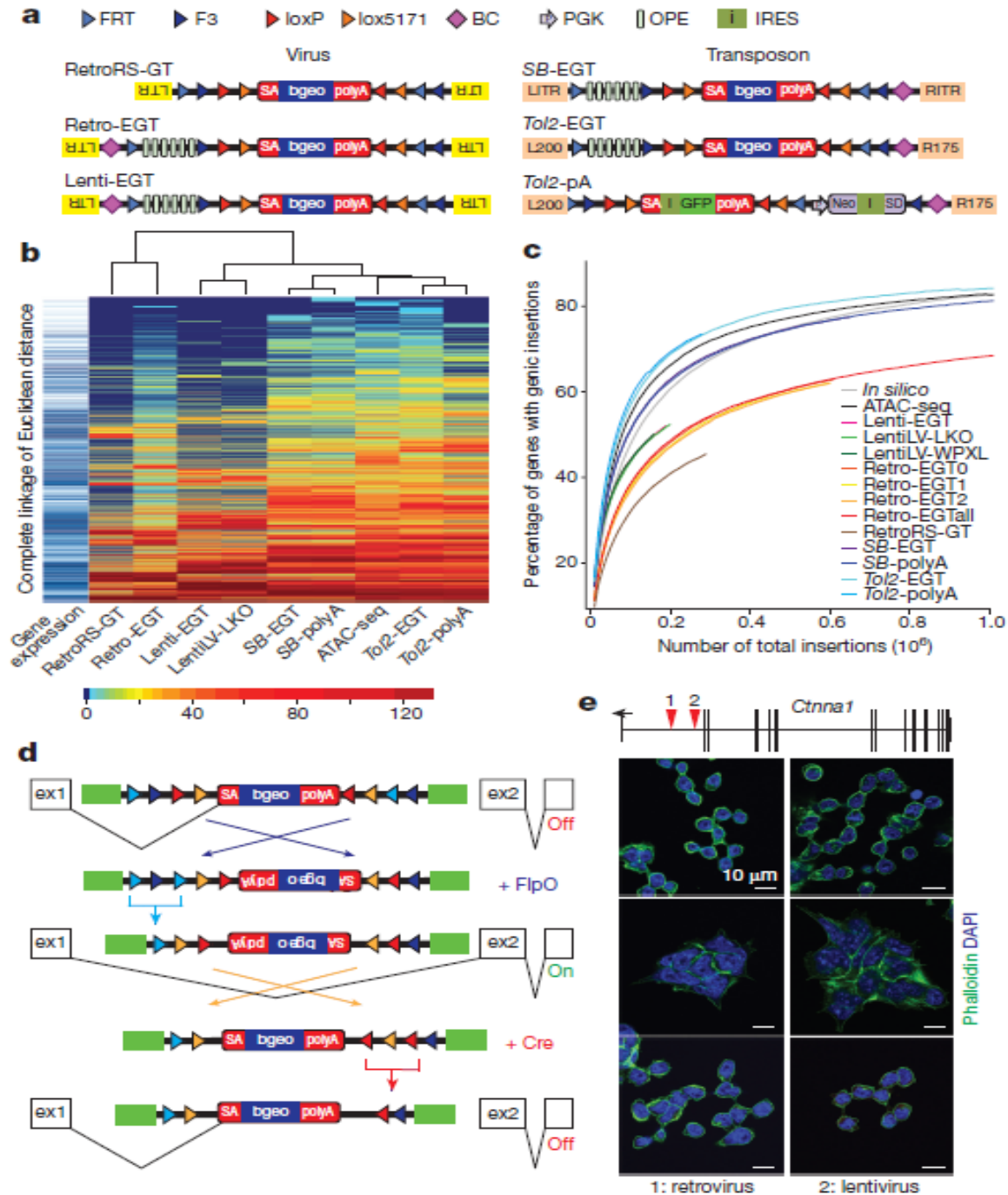


Figure 20. A repairable mutant mES cell library. **a)** Schematic representation of insertional mutagenesis vectors: Splice acceptor sites (SA) are revertible using non-compatible loxP/lox5171 and FRT/F3 sites (triangles). G418 resistance is conferred by beta-Geo (bgeo) transcribed from the revertible cassette (gene trap vectors, GT) or Neo independently from a PGK promoter (polyA trap, pA), stabilized by a splice donor (SD). Six osteopontin enhancer (OPE) elements (enhanced gene trap; Lenti-ETG, Retro-EGT, and Tol2-EGT vectors) enhance expression of beta-Geo via Oct4/Pouf51 binding. RetroRS carries a spacer sequence between loxP sites and lacks OPEs. Purple diamonds indicate internal barcodes (BC). LTR, long terminal repeats; L200/R175 and

LITR/RITR, terminal repeats of *Tol2* and *SB*. **b)** Heat map representing numbers of integrations per gene per 1 million integrations. Gene expression levels are shown (blue=highly expressed, white=not expressed). Color code shows numbers of integrations. **c)** Saturation of mutagenesis systems compared to random *in silico* mutagenesis. Y-axis, total numbers of insertions versus % of genes with integrations. **d)** Schematic representation of splice acceptor inversions. **e)** Loss of mESC adhesion in clones with integrations in intron 1 of *Ctnna1*. Inversion of the gene trap restores cell adhesion, subsequent reversion again disrupts adhesion. Phalloidin images polymerized actin; DAPI visualizes nuclei. Size bars, 10 μ m. One representative experiment out of 2 biological replicates is shown.

A key advantage of Haplobank is the ability to compare each mESC clone with its sister clone carrying the conditionally inverted splice acceptor. As a proof-of-principle, we analyzed two mESC clones containing sense integrations within *Ctnna1*, which encodes alphaE-catenin and is critical for mESC adhesion. mESCs with sense integrations exhibited reduced *Ctnna1* expression and impaired cell adhesion, which were both restored by FlpO-mediated reversal of the integrated mutagenesis vectors. Further, Cre-mediated reversal back into the sense orientation again disrupted alphaE catenin-mediated cell adhesion (**Figure 20d,e**). Additionally, we evaluated mESC lines carrying non-disruptive, anti-sense integrations in presumed essential genes. We infected these mESC lines with a pool of retroviruses that encode Cre and mCherry, or GFP only. If a gene is essential, Cre-mediated reversion of the integration to the disruptive sense orientation should specifically deplete mCherry⁺ cells from the mCherry⁺/GFP⁺ cell pool over time, as detected by flow cytometry. Indeed, we confirmed the essential role of several genes for mESC survival. Thus, our system allows for a direct functional annotation of essentiality, instead of screening for the absence of mutations; moreover, one can directly examine the penetrance and timing of lethal phenotypes.

Next, we developed a high-throughput pipeline to create a biobank of reversible mutations in AN3-12 haploid mESCs. Starting from haploid mESC pools that contained between 10⁷-10⁸ distinct mutations for each mutagen, we arrayed, processed, banked, and mapped over 100,000 individual mESC clones. In total, we generated sense and anti-sense clones that target 16,970 of ~ 24,000 annotated mouse genes (genome release mm10), covering over 70% of the protein-coding genome (**Figure 21**). Integrations display a 5' bias in genes and in coding sequence, resulting in truncations that are likely to generate loss-of-function alleles. All pools of mutated cells and clones were deposited to *Haplobank*, and all mapping information, expression levels, and quality control measures can be accessed at www.haplobank.at (**Figure 22**). This resource represents a comprehensive library of

mESC clones carrying hemi/homozygous, twice reversible, bar-coded integrations, combining the power of stem cells with tunable mutagenesis.

Category	Retro-GT	Lenti-GT	Tol2-GT	Tol2-pA	Sense	Antisense	Sum
Clones with mapped insertions	19448	26972	14296	39635	-	-	100351
Clones with insertions in genes	9992	18293	8738	29295	43527	33039	66318
Clones with single insertions in genes	7421	15948	5050	12879	24889	17902	41298
Number of genes targeted	6238	7265	7267	14408	13989	13055	16970
Number of genes in single insertion clones	3705	6158	3778	7258	9109	7851	11703

Figure 21. Haplobank clones. Numbers of clones available with respect to different mutagens, orientation of the inserted gene trap to gene transcription, as

well as the number of different genes hit. A gene is defined as the genomic region between the transcriptional start and stop sites. www.haplobank.at (Please access as User: BetaForReviewer; password: ready2GoLive).

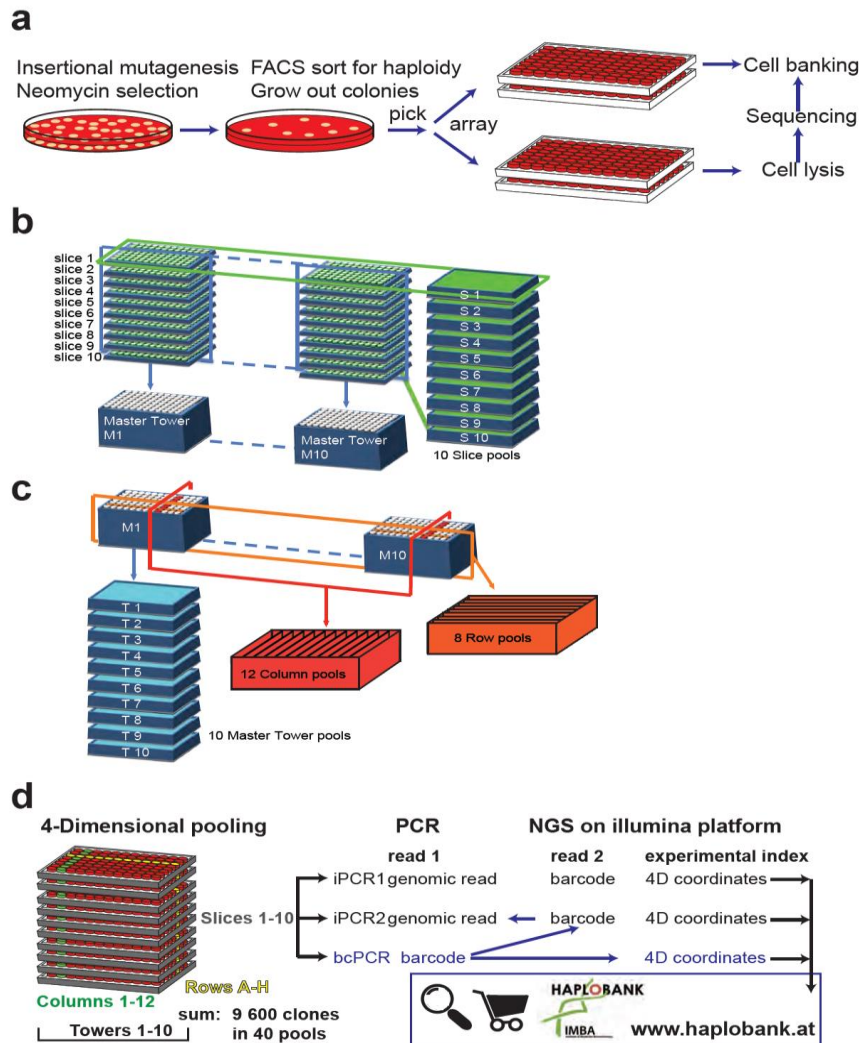


Figure 22. Generation of the mutant ES cell library. **a**, Schematic work flow for generation of the mutant haploid ES cell library. Single cell derived clones were manually picked 10 or 11 days after seeding, expanded in 96 well plates, and either frozen in quadruplicates or further processed for mapping of the integration sites. **b**, Schematic illustration of the first step of 4D pooling. Each plate was pooled into the respective slice tray as well as a master-plate, uniting identical well coordinates of all plates. **c**, Schematic illustration of the second step of 4D pooling. Each master-plate was pooled into a master tower pool, a plate with lamella uniting columns, and a plate with lamella uniting columns, thereby generating pools for rows and columns over all samples. **d**, 4-Dimensional pooling of 9600 clones in 8 rows, 12 columns, 10 slices, and 10 towers resulting in 40 pools. Subsequent to iPCR to introduce experimental indices, pools were combined and deep sequenced. Amplification of internal barcodes confirmed clonal identity and

mapping in 4 dimensions. All mapped clones were deposited to Haplobank (www.haplobank.at).

In summary, the Haplobank resource contains over 100,000 individually mutagenized and bar-coded mESC lines targeting 16,970 protein coding genes. Haplobank complements a collection of 3,396 reversibly targeted genes in a near haploid human leukemia cell line. Our proof-of-principle experiments uncovering genes required for rhinovirus infection and angiogenesis establish the power of Haplobank in forward and reverse genetic screens, respectively. The strong variability between independent clones revealed the importance of assessing mutant and repaired clones side-by-side, and addresses an increased demand for rigor and reproducibility. Thus, Haplobank clones and revertible technologies enable high-throughput, reproducible functional annotation of the genome. This work, supported by DoD and as outlined in the first proposal, has now been published in Elling et al. Nature 2017.

3.2. From haploid genetics to a key regulator of blood vessel formation and metastases in breast cancer.

Using the power of this haplobank library we modeled blood vessel development from our stem cells. We indeed managed to develop such model starting from haploid ES cells that can be differentiated into embryoid bodies and then further into 3D blood vessel organoids following addition of VEGF-A in collagen matrix (**Figure 23**). These organoids contain endothelial cells, pericytes, and a basal membrane, and form perfect lumens. Moreover, we can transplant the organoids and also the embryoid bodies to study blood vessel formation in vivo mouse models. Of note, since the mutations in the haploid ES cells are repairable, we can directly compare the function-side, thereby avoiding potential pitfalls of clonal effects in different cell lines. This tissue engineering approach now allows us to test the role of multiple known and predicted modifiers of angiogenesis, in particular to assess the functions of possible modulators of tumor angiogenesis.

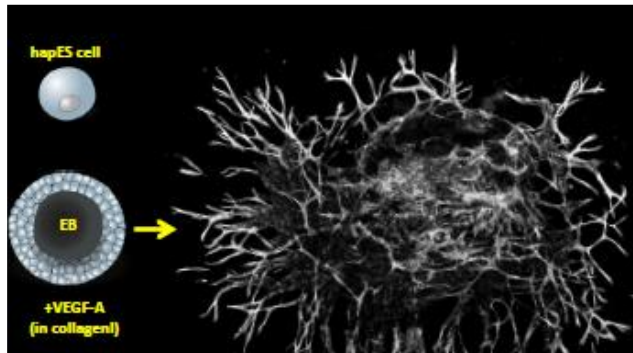


Figure 23. Schematic outline of blood vessel development from haploid ES cells (hapES cell) and embryo bodies (EB). The image shows a CD31⁺ endothelial 3D organoid.

As tumor growth depends on the formation of a neovascular supply, inhibition of tumor-induced neo-angiogenesis represents a potential strategy to treat cancer. Current anti-angiogenic therapies blocking tyrosine kinase receptors (TKR) such as VEGFR falter at the onset of resistance owing to upregulation of additional angiogenesis factors. Study and validation of these alternative neo-angiogenesis regulators as therapeutic targets is key, either alone or in combinatorial therapies. One promising candidate is apelin, the ligand for the G-protein-coupled APJ receptor and a potent angiogenic factor frequently upregulated in human cancer. Apelin is a secreted peptide specifically upregulated in cancer cells and tumour endothelial cells, which makes it an attractive target in cancer with potentially low toxicity. Importantly, when we analysed *Apelin* mutant and repaired haploid ES cells for sprouting in our 3D blood vessel model, we found that VEGF-A induced blood vessel growth requires the expression of Apelin (**Figure 24**).

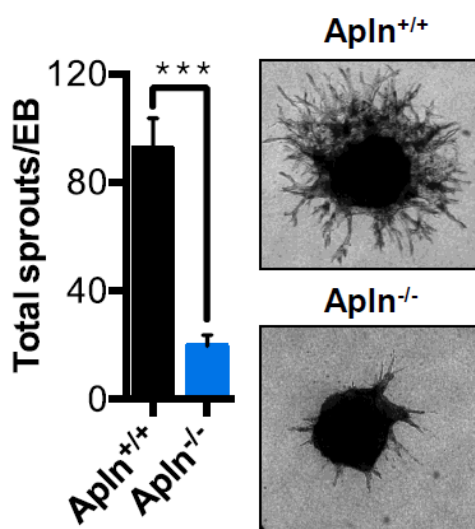


Figure 24. Targeting Apelin decreases tumour angiogenesis and disfavours VEGF-induced vessel sprouting. **Left panel,** Percentage of CD31-positive area in NeuT;ApIn^{+/+} (n=3) and NeuT;ApIn^{-/-} (n=3) age-matched mammary tumours (P<0.05; T test). **Right panel,** Total number of sprouts per ApIn^{+/+} (n=14) or ApIn^{-/-} (n=13) VEGF-stimulated embryoid body (EB) 5 days after initiation of the vessel sprouting (P<0.005; T test). Representative bright field images are shown.

Unbiased meta-analysis of multiple datasets using the Kmplot and Prognoscan databases showed that high levels of apelin expression are significantly associated with poor prognosis in breast cancer (**Figure 25A,B**). To study the potential for improving prognosis through blockage of apelin expression in mammary cancer, we intercrossed apelin-deficient (*Apln*^{-/-}) mice with MMTV-*NeuT* transgenic mice to generate MMTV-*NeuT*;*Apln*^{-/-} and MMTV-*NeuT*;*Apln*^{+/+} mice (termed *NeuT*;*Apln*^{-/-} and *NeuT*;*Apln*^{+/+}). The *Apln*^{-/-} mouse is a whole body knockout and therefore mimics the effect of targeting apelin in the whole body. Apelin-null mice showed a significant prolonged survival compared with their wild type counterparts after tumour onset (**Figure 25C**), showing that the apelin signalling pathway is a promising target for cancer treatment. Accordingly, age-matched apelin-null littermates presented a decreased percentage of tumour burden in the mammary glands (**Figure 25D**).

Similar results were found in a mouse model of metastatic mammary cancer (E0771 cells) where apelin was interfered and cells were orthotopically injected into the mammary glands of C57B6/J *Apln*^{-/-} mice (**Figure 25E**). To extend these results, we then analysed age-matched NeuT-positive tumours and found that tumours without apelin had a significantly decreased microvessel density (**Figure 25F**). Together, these data show that blocking apelin signalling is an effective strategy to reduce tumour angiogenesis and increase survival in mice with mammary and lung cancer, both cancers in which high apelin levels are correlated with a worse prognosis in humans.

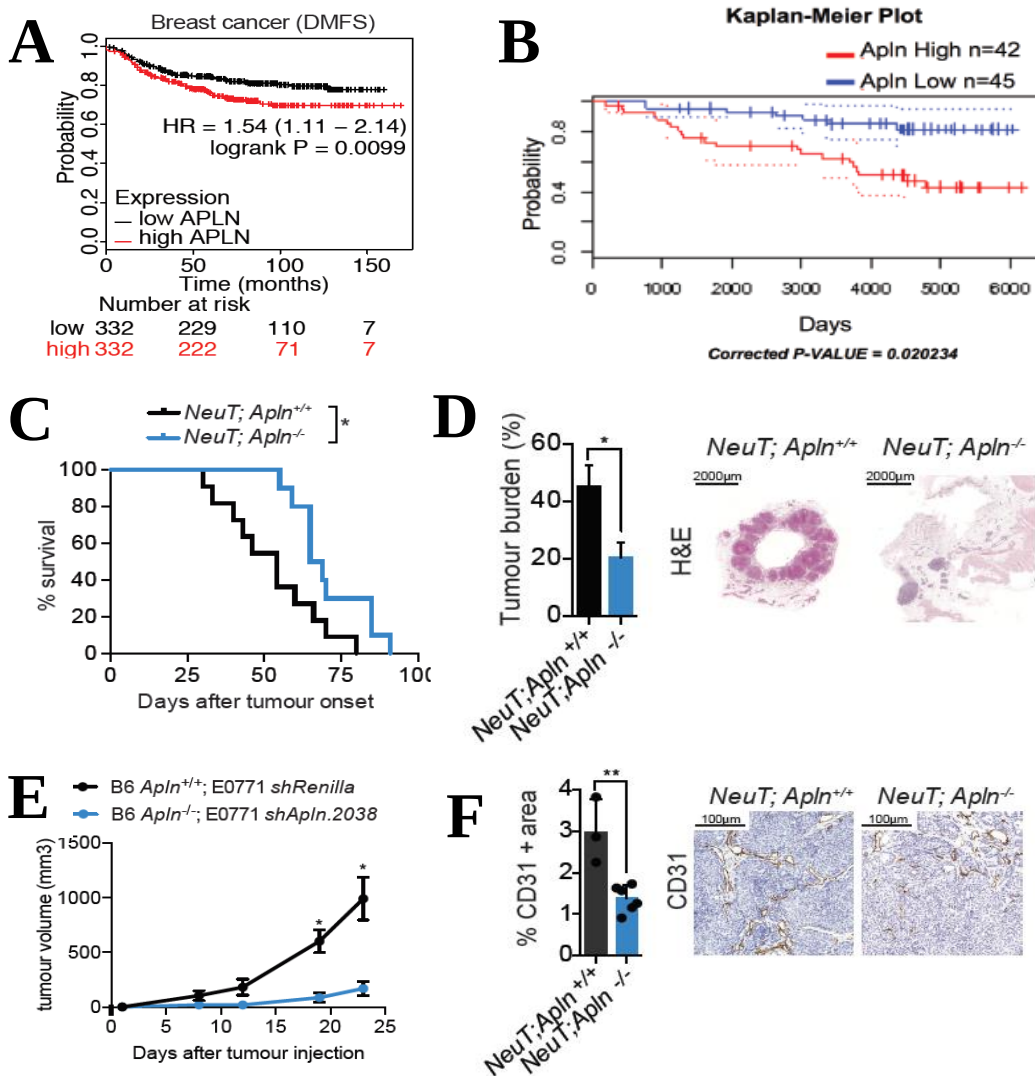


Figure 25. Apelin targeting improves survival and reduces angiogenesis in breast cancer. **A**, Kaplan Meier plot for high and low apelin-expressing human breast cancer patients. Patients were split by the median. Affymetrix probe ID 222856_at. DMFS=distant metastasis free survival. **B**, Kaplan Meier plots for high and low apelin-expressing groups in human breast cancer from Prognoscan filtered by significant corrected P-value and Cox P-value. Dataset GSE6532-GPL570, distant metastasis free survival. **C**, Kaplan Meier plot for survival in *NeuT;Aplin^{+/+}* (n=11) and *NeuT;Aplin^{-/-}* (n=10) mice with mammary cancer after tumor onset (*P<0.05; Log rank test). Mice were sacrificed when the tumour size was 1-1.5cm³, following the ethical guidelines. **D**, Percentage of tumor burden in 4 weeks age-matched *NeuT;Aplin^{+/+}* (n=6) and *NeuT;Aplin^{-/-}* (n=9) mammary glands (*P<0.05; t test). Data are shown as means $\pm$ s.e.m. Representative H&E images are shown. **E**, Tumor volume followed over-time from E0771 cells control (*shRenilla*) or apelin-interfered (*shAplin.2038*) orthotopically-injected in C57B6/J *Aplin^{+/+}* or *Aplin^{-/-}* mice. Data are means $\pm$ s.e.m (n=3; *P<0.05, multiple t tests). **F**, CD31-positive area in *NeuT;Aplin^{+/+}* (n=3) and *NeuT;Aplin^{-/-}* (n=6) 4 weeks age-matched mammary tumours. Data are means $\pm$ s.d. (**P<0.005; t test). Representative images are shown.

To elucidate the mechanism by which apelin ablation reduces tumour burden and angiogenesis, we used the E0771 orthotopic mammary cancer model to specifically downregulate *Apln* and *Aplnr* in cancer cells only. When injected in C57B/6J wild type mice (B6 hereafter), only *Apln*-interfered (*shApln*) E0771 cells (**Figure 26A**) showed significantly reduced cell growth (**Figure 26B,C**) and vessel density (**Figure 26D**) compared with control *shRenilla*-expressing (*shRen*) E0771 cells. Apelin receptor interference (*shAplnr*) did not show any reduction in tumour growth or vessel density compared with *shRen*-expressing control cells (**Figure 26A-D**), pointing towards an exclusive paracrine action of apelin. Accordingly, the morphology and proliferation of E0771 cells, control or interfered for apelin or apelin receptor, *in vitro* was unaffected (**Figure 26E,F**), even when the cells were stimulated with an active form of apelin (AplnPyr13) or when they were under hypoxic conditions.

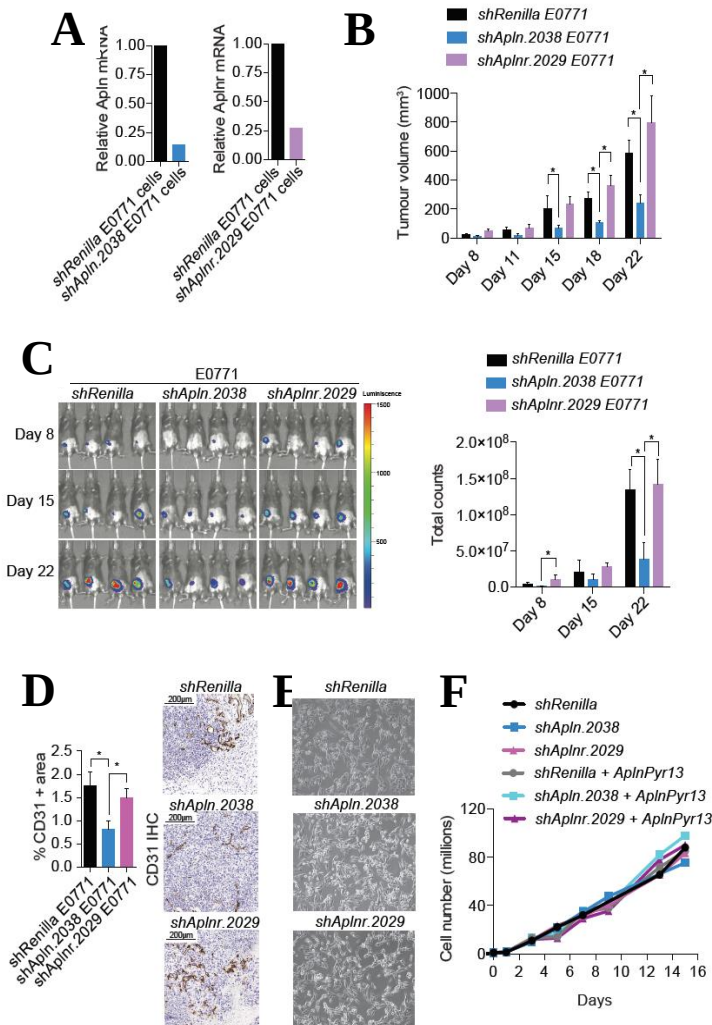


Figure 26. Apelin control mammary tumor growth in the E0771 orthotopic breast cancer model. **A**, RT-qPCR of apelin and apelin receptor in E0771 control cells (*shRenilla*) and apelin or apelin receptor-interfered cells (*shApln.2038* and *shAplnr.2029*, respectively). **B**, Tumour volume followed over-time from E0771 cells control (*shRenilla*), apelin-interfered (*shApln.2038*) and apelin receptor-interfered (*shAplnr.2029*) orthotopically-injected in C57B6/J mice. Data are mean $\pm$ s.e.m (n=3-4; *P<0.05, multiple t tests). **C**, Bioluminescence image and quantification of mammary tumors from orthotopically-injected E0771 cells *shRenilla*, *shApln.2038* or *shAplnr.2029* in C57B6/J mice at day 8, 15 and 22 post-injection. Data are means $\pm$ s.e.m (n=4, *P<0.05, multiple t tests). **D**, CD31+ area of E0771 tumours at day 12 post injection. Representative staining images are shown. Data are shown as means $\pm$ s.e.m (n=6-7; *P<0.05; t test). **E**, Representative images of E0771 cells *shRenilla*, *shApln.2038* or *shAplnr.2029* in culture (20x). **F**, Growth curve of E0771 cells *shRenilla*, *shApln.2038* or *shAplnr.2029* in absence or presence of apelin peptide (AplnPyr13).

The orthotopic injection of apelin-interfered E0771 breast cancer cells into *Apln*^{+/+} or *Apln*^{-/-} mice showed a tendency towards major reduction of tumor growth in the latter compared with the former (**Figure 27A**), suggesting that the blockage of apelin production not only in tumour cells but also in the tumor microenvironment would be therapeutically beneficial, although tumour apelin seems to play the predominant role in tumour progression. Orthotopic injection of E0771 *shApln* cells into *Apln*^{-/-} mice not only reduced vessel density but also vessel leakage (**Figure 27B,C**), which is a major undesirable effect of current RTKI anti-angiogenic therapy.

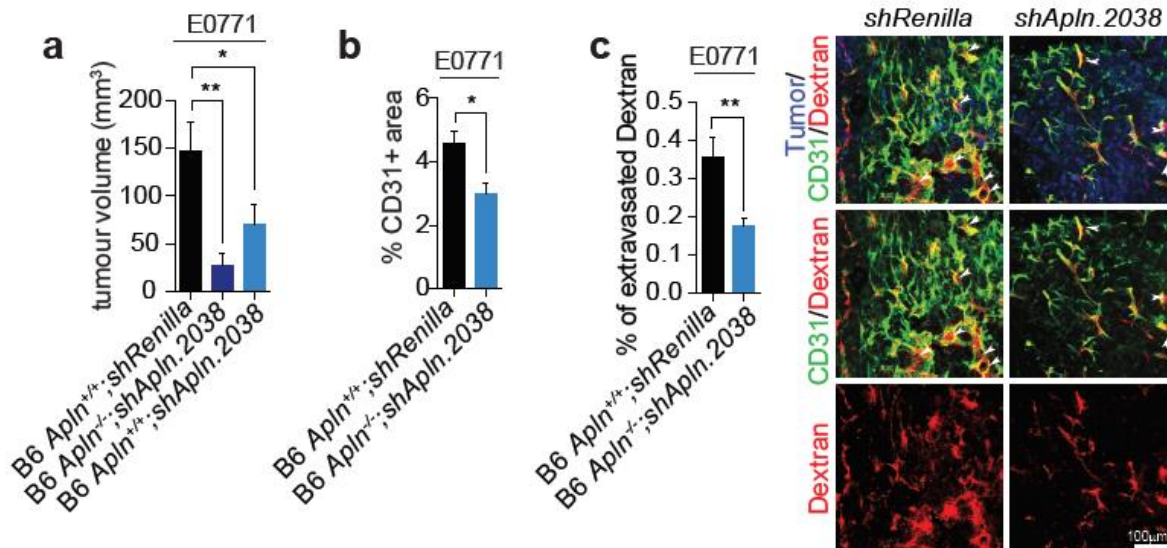


Figure 27. Apelin controls breast cancer growth and tumor vessel leakage. **A**, Tumor volume of *shRenilla* or *shApln.2038* E0771 tumours at day 12 post orthotopic injection in C57B6/J *Apln*^{+/+} or *Apln*^{-/-} mice. Data are means $\pm$ s.e.m (n=6-12; *P<0.05; **P<0.005; one-way ANOVA to *shRenilla*). **B**, Percentage of CD31+ area of age-matched mammary tumors (day 23) from E0771 cells control (*shRenilla*) or apelin-interfered (*shApln.2038*) orthotopically-injected in C57B6/J *Apln*^{+/+} or *Apln*^{-/-} mice. Data are means $\pm$ s.e.m (n=3; *P<0.05, t test). **C**, Percentage of extravasated Dextran in age-matched mammary tumours (day 19) from E0771 cells control (*shRenilla*) or apelin-interfered (*shApln.2038*) orthotopically-injected in C57B6/J *Apln*^{+/+} or *Apln*^{-/-} mice, respectively. Representative immunofluorescence stainings of Dextran (red) and CD31+ vessels (green) are shown. Data are means $\pm$ s.e.m (n=9-12; **P<0.005; t test).

Despite the promise of anti-VEGF and multitargeted pan-VEGF RTKI anti-angiogenic therapy, resistance remains one of the key issues related to its failure. To study whether apelin blockade could complement current RTKI anti-angiogenic therapies, we administered the multitargeted pan-VEGF RTKI sunitinib (Sutent) to *NeuT*;*Apln*^{-/-} and *NeuT*;*Apln*^{+/+} mice upon mammary

tumor onset. Sunitinib treatment has been widely studied in preclinical and clinical settings and is well-known as a potent inhibitor of tumour angiogenesis and tumour growth, especially in sustained treatment. Apelin ablation combined with sunitinib treatment significantly increased survival from tumor onset (**Figure 28A**). Age-matched samples showed a significant reduction of tumoural tissue in the combinatory situation (**Figure 28B**). To normalize the data, we size-matched NeuT+ mammary tumors at an early stage (20–70mm³) using MRI, which were untreated or treated with sunitinib until the day of sacrifice and followed over time. MRI analysis showed that sunitinib treatment decreases the rate of tumor growth. *NeuT;Apln*^{-/-} tumors presented a similar growth rate. Sunitinib-treated *NeuT;Apln*^{-/-} tumors showed a pronounced decrease in growth, which was confirmed by mitotic count and PHH3 immunostaining 6 weeks after tumors were size-matched – or 4 weeks after in the case of some *NeuT;Apln*^{+/+} sacrificed early owing to the large size of their tumors, when all tumors except for the sunitinib-treated *NeuT;Apln*^{-/-} tumours presented similar growth rates (**Figure 28C,D**).

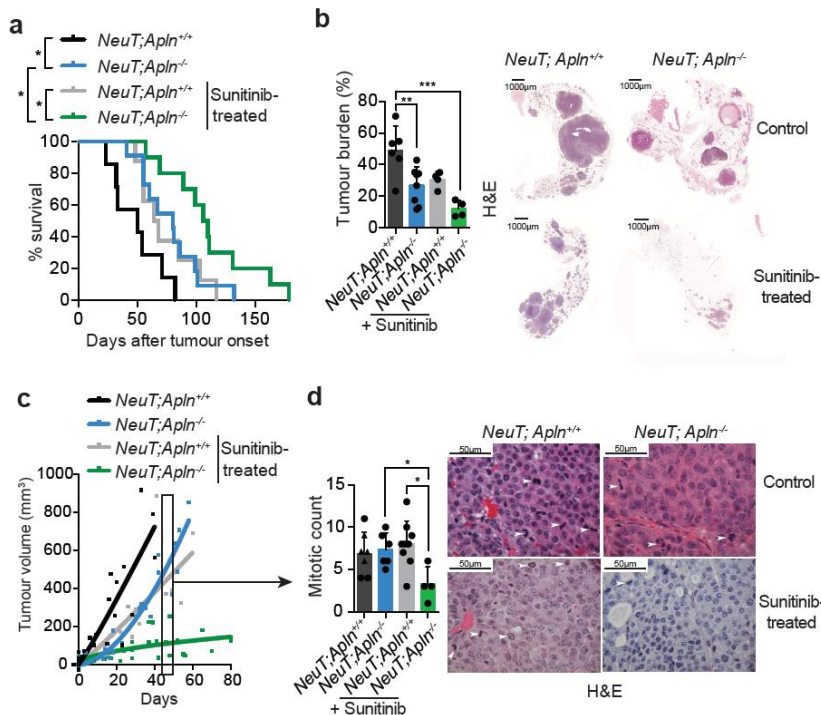


Figure 28. Combination of apelin and RTK blockage improves survival in breast cancer.

A, Kaplan Meier plot for survival in *NeuT;Apln*^{+/+} and *NeuT;Apln*^{-/-} mice with mammary cancer, control or treated with sunitinib malate (60mg/kg per dose) three times a week after tumour onset (n=7-11; *P<0.05; Log rank test). Mice were sacrificed when the tumour size was 1-1.5cm³, following the ethical guidelines. **B**, Percentage of proliferative area in 4 weeks age-matched *NeuT;Apln*^{+/+} and *NeuT;Apln*^{-/-} mammary glands from control mice or mice treated with sunitinib. Data are means ± s.d. (n=4-8; **P<0.005; ***P<0.0005; One-Way ANOVA to *NeuT;Apln*^{-/-} sunitinib).

NeuT;Apln^{+/+}). Representative H&E images are shown. **C**, Tumour volume of NeuT+ mammary tumors size-matched at 20-70mm³ and followed over-time by MRI analysis (n=5-7). Lines are nonlinear fit of tumor growth. **D**, Mitotic count of 6 weeks age-matched tumors from *NeuT;Apln*^{+/+} and *NeuT;Apln*^{-/-} mice, control or treated with sunitinib. Some control mice had to be sacrificed after 4 weeks owing to the tumour size following ethical guidelines (triangular shapes). Data are means ± s.d. (n=4-9; *P<0.05; One-Way ANOVA to *NeuT;Apln*^{-/-} sunitinib). Representative H&E images are shown.

Although anti-angiogenic treatment initially reduces tumour growth, in the long term, it increases other malignant tumor features. We visualized the dynamics of current RTKI anti-angiogenic therapy and apelin targeting on tumor vascularity over time using MRI. First, size-matched early stage tumors were imaged by MRI after 2, 4 and 6 weeks and the relative tumour blood volume (rTBV) was assessed. Whereas control *NeuT;Apln^{+/+}* tumors show an increase in blood volume over time, sunitinib treatment reduces vascularity especially at the mid-term point (4 wks) (**Figure 29A**). This analysis was confirmed by CD31 immunostaining of microvessel density in 4 wk age-matched tumors (Figure 10B). *NeuT;Apln^{-/-}* tumors also exhibited reduced blood volumes following similar kinetics to sunitinib-treated *NeuT;Apln^{+/+}* tumors, although this was only significant in CD31 vessel density. Sunitinib-treated *NeuT;Apln^{-/-}* tumors showed a trend towards reduced vessel density and blood volume compared to control *NeuT;Apln^{+/+}* tumors but, unlike sunitinib therapy alone, it was consistently maintained over time (**Figure 29A,B**). This data shows that both absence of apelin and blockage of the VEGF pathway by RTKI can reduce tumor angiogenesis and that a combination of both have extended effects.

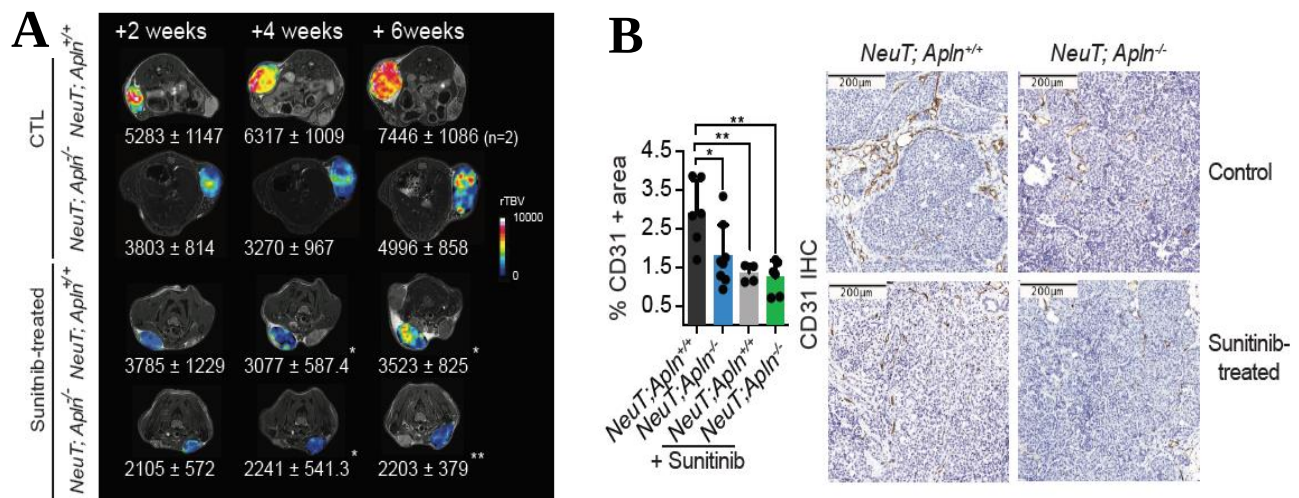


Figure 29. VEGFR and Apelin cooperate in establishing the tumor vasculature. **A**, MRI analysis of relative tumor blood volume (rTBV) over-time after NeuT+ mammary tumors were size-matched at 20-70mm³. Data are shown as means ± s.e.m from 4 different tumors. In *NeuT;Apln^{+/+}* only 2 tumors were measured after 6 weeks as some mice had to be previously sacrificed owing to the tumor size following ethical guidelines. Significance to *NeuT;Apln^{+/+}* is shown (*P<0.05; **P<0.005; one-way ANOVA). **B**, Percentage of CD31+ area in *NeuT;Apln^{+/+}* and *NeuT;Apln^{-/-}* 4 weeks age-matched mammary tumors, control or treated with sunitinib. Representative CD31 stainings are shown. Data are means ± s.d. (n=4-8; *P<0.05; **P<0.005; one-way ANOVA).

In addition to the impact of anti-angiogenesis therapy on the primary tumour, metastases have been shown to increase with the use of VEGF pathway inhibitors, including sunitinib therapy, although a reduction in primary tumour growth occurs at the same time. We therefore evaluated the number of lung metastatic foci in mice with NeuT+ tumours. To match this result with the vascular MRI data, we analysed the lungs from tumour-bearing mice after 6 weeks after the tumours were size-matched. As in previous studies, we observed that sustained sunitinib treatment significantly increases the number of metastases, but apelin targeting dramatically reduced their number (**Figure 30**), thereby overcoming one of the key features of resistance to anti-VEGF or multimodal RTKI anti-angiogenic treatment.

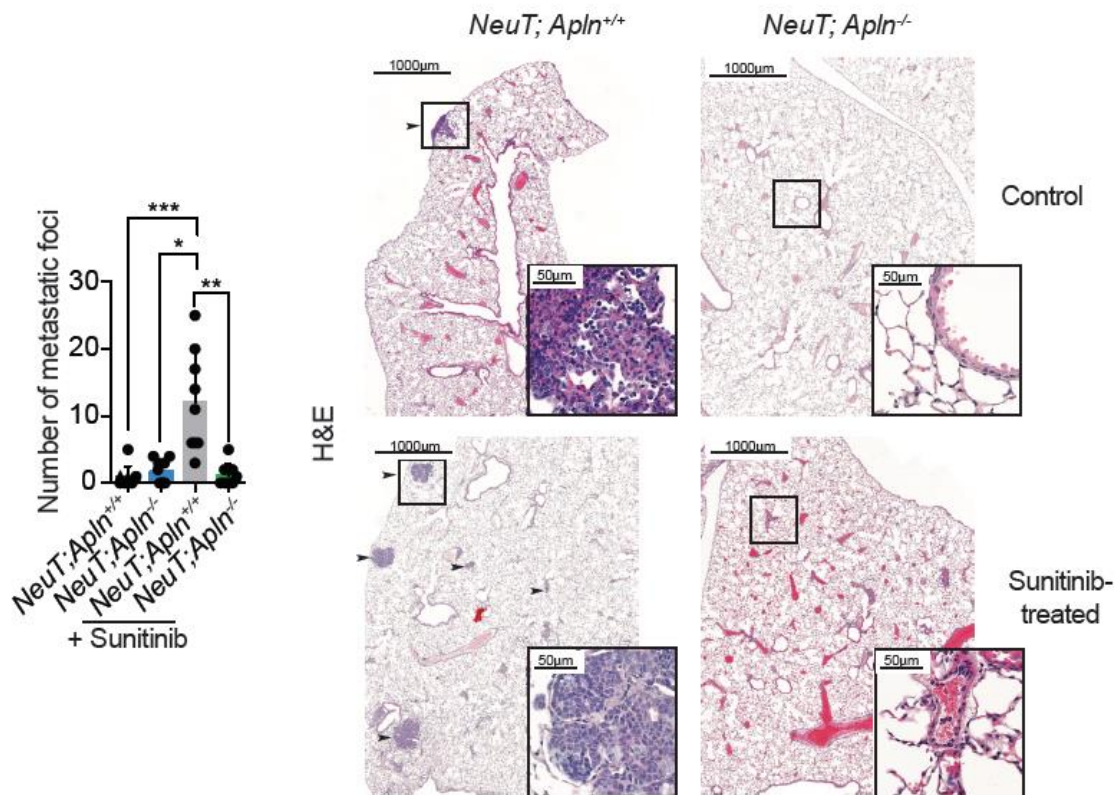


Figure 30. Apelin targeting subdues anti-angiogenic RTKI-induced metastasis. Number of metastatic lung foci from NeuT+ mammary tumors 6 weeks age-matched after tumour onset. Data are shown as means $\pm$ s.d (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$; Kruskal-Wallis test; $n = 3$, three longitudinal levels per lung). Right panels show representative images of lung metastases in the indicated mice and treatments.

We confirmed and extended this result using the E0771 orthotopic mammary cancer model, which showed similar results in tumour growth upon sunitinib treatment (**Figure 31A**).

Histological analysis of metastatic foci in the lungs of mice sacrificed with large size-matched tumours showed that tumour apelin blockage significantly reduced anti-angiogenic sunitinib-treatment-induced metastasis (**Figure 31B,C**). Taken together, these results confirm that targeting apelin reduces the metastatic potential of tumors treated with RTKI anti-angiogenic therapy.

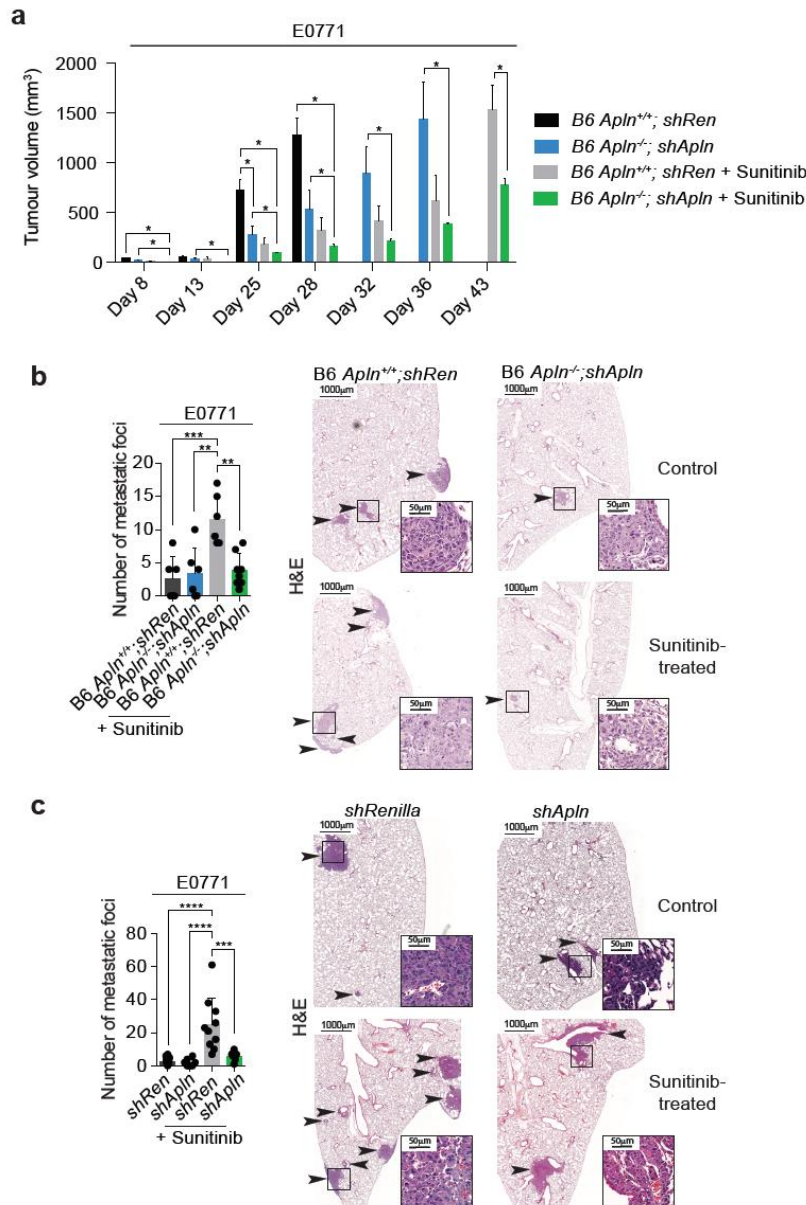


Figure 31. Targeting apelin reduces metastases of breast tumors treated with anti-VEGF therapy. **A**, Volume of mammary tumors from E0771 control (*shRen*) and apelin-interfered (*shApln.2038*) cells orthotopically-injected in C57B6/J *Apln*^{+/+} or *Apln*^{-/-} mice, as indicated. Data are shown as means $\pm$ s.e.m. (n=3-4; *P<0.05, multiple t tests). **B**, Number of metastatic lung foci from 1.5cm³ size-matched E0771 control (*shRen*) and apelin-interfered (*shApln.2038*) tumours orthotopically-injected in C57B6/J *Apln*^{+/+} or *Apln*^{-/-} mice, as indicated. Data are shown as means $\pm$ s.d. (**P<0.005; ***P<0.0005; One-way ANOVA; n=3, two longitudinal levels per lung). **C**, Number of metastatic lung foci from 1.5cm³ size-matched E0771 control (*shRen*) and apelin-interfered (*shApln.2038*) tumours orthotopically-injected in C57B6/J mice. Data are shown as means $\pm$ s.d. (**P<0.005; ***P<0.0005; One-way ANOVA; n=3, two longitudinal levels per lung).

Discussion. Cancer switches on angiogenesis. Initial attempts to switch off tumor angiogenesis therapeutically were thwarted by resistance and side effects. Our findings unveil a new strategy combining RTK inhibition (i.e. of the VEGFR pathway) with GPCR inhibition (i.e. of the apelin signalling pathway) to diminish tumor growth, microvessel density, vessel abnormality and thus tumor malignancy and therapy-induced metastasis. We have shown here that both pathways, namely VEGF and Apelin, control angiogenesis and vessel permeability. As the apelin signalling pathway is mostly relevant during developmental angiogenesis and is specifically upregulated in tumors, the toxic effects of the pathway blockage are predicted to be minimal. Toxicity is a relevant issue often associated with anti-cancer treatments including anti-angiogenic therapy. Evidence for a lack of toxicity is the fact that *Apln*^{-/-} mice are viable and healthy in adulthood, which shows that apelin signalling blockage is compatible with life. Our transcriptome results in sprouting endothelial cells suggest that apelin is also necessary to regulate TNF and TGFβ1-target genes. This finding could also explain why apelin and VEGF targeting present different kinetics of microvessel density and leakage. In addition, and more strikingly, blockage of apelin in combination with RTKI potentiates its anti-angiogenic effects and significantly reduces the malignant features associated with therapy resistance. Apelin targeting in addition to VEGF RTKI would not only reduce the growth and malignant progression of the primary tumor through maintenance of a less abnormal tumor vasculature, but would also decrease the impact on metastasis, which is usually the main cause of death in breast cancer.

Taken together, our data indicates that apelin is a potential novel target for cancer treatment. In combinatory strategies, apelin targeting could address the limitations of current anti-angiogenic therapy by prolonging tumor vessel normalization and, as a consequence, maintaining a diminished malignancy of the tumor for a longer period of time. This would not only increase the therapeutic window but it is also a common key feature to overcoming resistance to any tumor therapy.

3.3 Sugar modifications in breast cancer.

The Haplobank also allowed us to explore pathways and developed and confirm novel technologies that have been implicated for decades in cancer development, namely protein glycosylation.

Glycosylation, the covalent attachment of simple or complex sugar structures onto proteins, is the most abundant post-translational modification, affecting virtually all aspects of biology, from bacteria to humans. Over 50% of human proteins are subject to these dynamic sugar modifications, which alter their activities in fundamental biological processes, such as cell adhesion, nuclear transport, signal transduction, intracellular trafficking, protein localization, virus infections, host-pathogen interactions, or essential immune functions. Moreover, despite its key importance in biology, the identification and functional validation of the glycosylation of complex glycoprotein interactions has remained largely unexplored. Whereas massive inroads have been made into genomics, metabolomics, or protein and lipid research, glycosylation remains largely unexplored at the proteome scale due to limited technologies for profiling the complex glycoproteome, thus remaining one of the key frontiers of life sciences.

Current technologies to examine such sugar modifications are often either limited to the identification and quantification of de-glycosylated peptides, require laborious generation of sample-specific spectral libraries, or rely on dedicated instrumentation and data-acquisition regimes in conjunction with specialized proprietary commercial data analysis tools. We developed a novel approach and computational tools for the interrogation of glycopeptides from complex biological samples. We also developed methods and algorithms that for the first time not only enable the comprehensive identification of complex sugar structures and their mapping to sites within the corresponding proteins at the proteome scale, but also for their relative quantification (**Figure 32**). Our novel comparative and high-throughput glycoproteomics

technologies enable genome-wide insights into protein glycosylation and sugar modifications in complex biological systems.

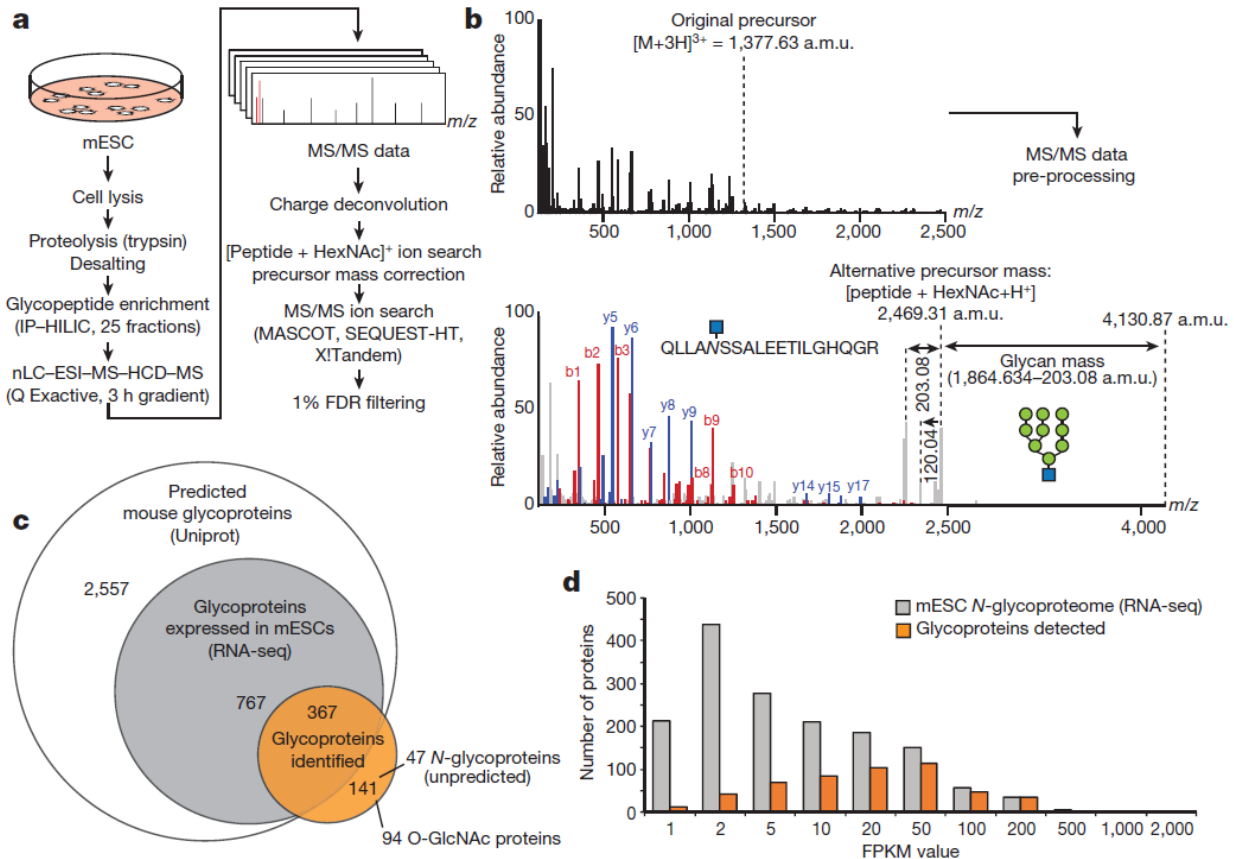


Figure 32. Glycoproteomics. **a**, Glycoproteomic workflow combining proteomics platforms with a new algorithm for the identification of intact glycopeptides from complex biological samples. **b**, MS/MS raw data pre-processing by charge deconvolution and precursor mass correction allows for the automated identification of glycopeptides, on the basis of low-abundant peptide fragment ions in the lower mass-range of glycopeptide MS/MS spectra (a.m.u., atomic mass unit). **c**, Coverage of experimentally identified glycoproteins (orange circle; Supplementary Table 10) among all predicted mouse glycoproteins (Uniprot, white) and the glycoproteins expressed (on the basis of RNAseq, grey) in mESC. **d**, Transcript abundance of all glycoproteins expressed (RNA-seq) in our mESC clone (grey), compared with the glycoproteome experimentally detected (orange). Data are representative of two independent mESC glycoproteomics experiments with similar results.

As an example of what we can do now: Using our novel approach, we were able to generate a first draft of the glycoproteome of murine and human cells. In these experiments, we nearly doubled the number of experimentally confirmed glycoproteins (in the entire literature),

identified previously unknown glycosylation sites and uncovered multiple novel glycosylated proteins, including evolutionary conserved and species-specific sugar modifications in murine and human stemness factors (**Figure 33**).

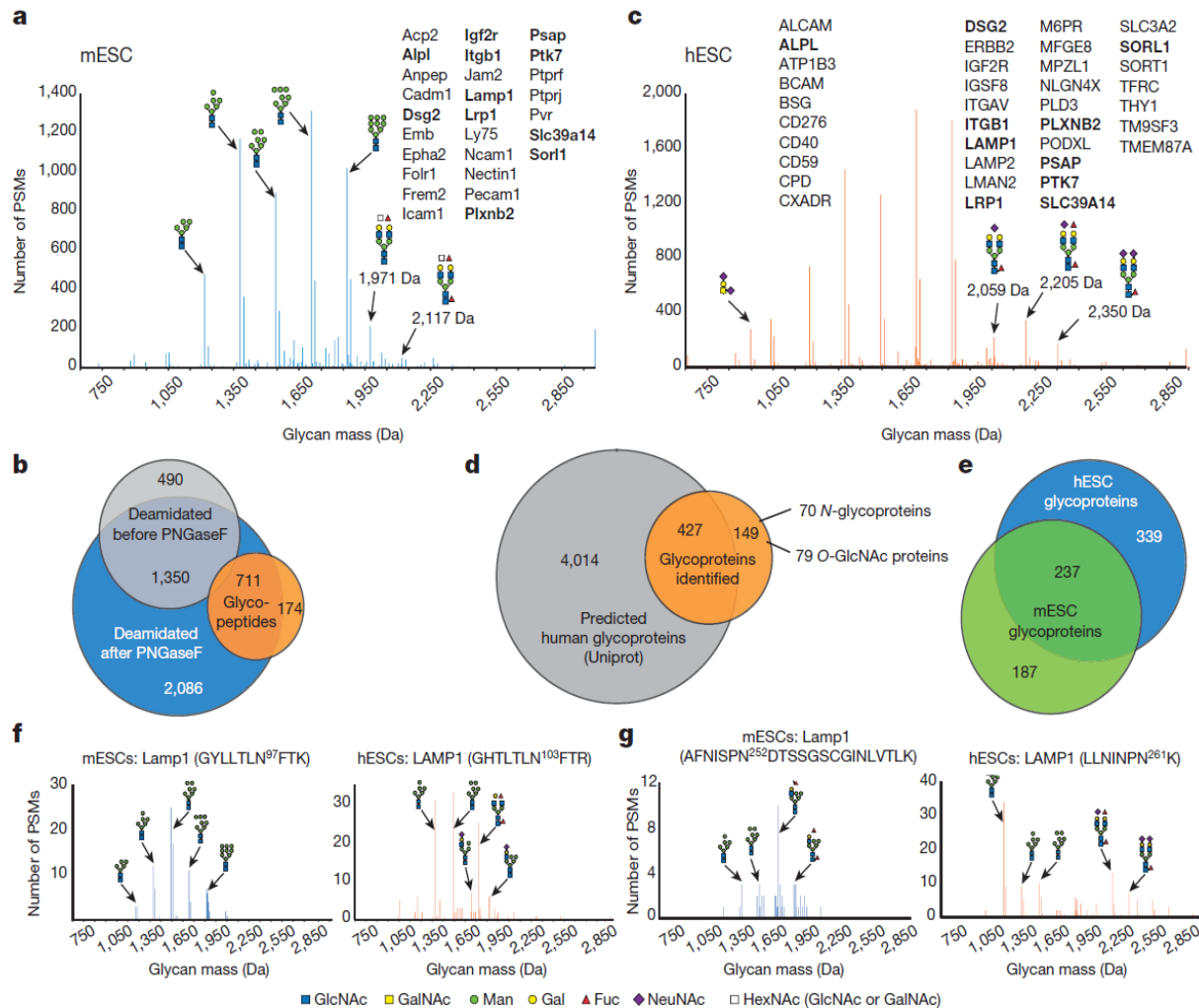


Figure 33. The glycoproteomes of human and mouse embryonic stem cells. Glycan-mass histogram of all glycopeptide-derived peptide spectrum matches (PSMs) identified (FDR < 1 %) from **(a)** murine ESC and **(c)** human ESCs. Bin-width 1 Da. Indicated proteins show either species-specific or evolutionarily conserved (bold) glycoproteins carrying stemness-related glycan-structures (labelled by their corresponding mass). **(b)** Deglycosylation and deamidation of mESC derived glycopeptides upon PNGaseF treatment (blue) confirmed the identity of the N-glycopeptide sequences identified by our approach (orange). Note that chemical deamidation of peptides was observed before PNGaseF treatment (grey) and further increased upon extended incubation times under alkaline conditions. **(d)** Coverage of experimentally identified glycoproteins (orange) among all hypothetical (Uniprot) human glycoproteins (grey). Note, similar to mouse, multiple human glycoproteins, experimentally identified by our approach, were not predicted by Uniprot. **(e)** Cross-species comparison of glycoproteins identified in human ESCs (blue) and

murine ESCs (green). **(f and g)** Exemplary comparison of glycan profiles at orthologous N-glycosylation sites of mouse Lamp1 and human LAMP1. Glycan-mass histograms of all glycopeptide PSMs (FDR < 1 %), specific for the N-glycosylation sites, are indicated. Bin-width is 1 Da. In all figures, depicted glycan structures were inferred from their mono-saccharide compositions and based on known glycobiological rules. Glycan structures are represented according to the consensus pictogram symbols for different mono-saccharides.

The covalent attachment of sugar structures onto defined amino-acid side-chains of proteins, i.e. the generation of protein-linked sugar-structures, is intimately linked to and often the basis for complex cellular processes, including protein expression, folding, trafficking and localization, as well as the cells metabolic state or binding to growth factors. As a consequence, aberrations in any of these processes can alter protein glycosylation and thereby alter the function of cells and entire organ systems. In cancer, it has been known for decades that the development and progression of the tumors as well as metastases to tissues require a plethora of changes in both, the cellular repertoire as well as the relative abundance of sugar-modified protein structures. Cancer cells, in many cases, revert to prior stages of development. This important biological property of cancer cells is also reflected in their expression embryonic glycosylation profiles, a group of developmentally encoded sugar modifications. Importantly, these sugar modifications allow cancer cells to signal and outgrow their “normal” neighboring cells, to start to migrate, to invade blood vessels and to move to secondary sites and to form metastases (e.g. more than a million cancer patients/every year develop metastases). Thus, protein glycosylation can affect all aspect of cancer development. As a consequently, protein glycosylation is increasingly recognized as a prime target for novel immune-based anti-cancer therapies. Our novel glycoproteomics approach will now allow us to address long-standing key questions in cancer development. Specifically, we can now characterize of protein-glycosylation in primary and metastatic breast cancer. The comparative analysis of such samples will provide a detailed characterization of the changes in protein glycosylation upon tumor formation and metastasis in breast cancer, site-specifically.

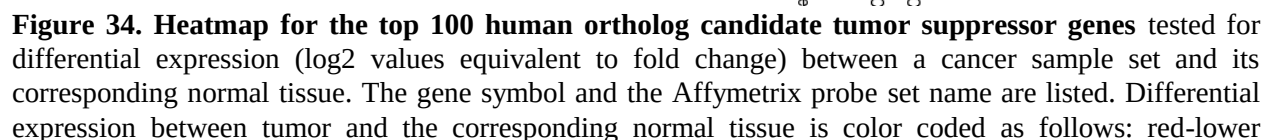
Our first preliminary experiments using 4T1 breast tumor cells show marked changes of tumor cells at the primary tumor site in the mammary gland and the metastatic tumor cells from the

lung, the latter cells adapting a glycosylation pattern of the host organ, the lung. Our new glycoproteomics technology should empower us to take a fresh look at and to gain entirely novel insights into an age-old problem – why and how tumors metastasize.

4. Genetic approaches in *Drosophila* to identify novel cancer genes and tumor suppressors.

Background: Many developmental pathways and signaling cascades implicated in cancer have been originally identified in *Drosophila melanogaster* and oncogenic signaling pathways such as Ras and Notch are conserved from fly to human. Moreover, epithelial tumor cell metastasis has been modeled in fly larvae. Thus, fundamental mechanisms of oncogenic transformation and metastases are highly conserved among flies and humans. Researchers at our campus have created the largest global RNA interference library in the world representative of nearly all expressed *Drosophila* genes; in Vienna at the host institution we had local access to a library of *Drosophila* where every fly gene can be inactivated via RNAi *in vivo* using tissue specific promoters. We used this library to perform a near genome-wide screen to identify genes involved in the growth, invasion, and metastasis of epithelial cells.

Results: In an attempt to identify genes involved in cancer development and metastases, we designed a high through-put *in vivo* RNAi screening system for genes involved in progression of oncogenic Ras-driven epithelial tumors. An activating point mutation in codon 12 of Ras, Ras^{V12}, is one of the most frequent mutations found in human patients. Using our experimental set-up we performed a large scale *Drosophila* screen to identify novel genes that modulate *dRas*^{V12}-induced epithelial tumorigenesis at a systems level. Female *Drosophila* carrying the *dRas*^{V12} oncogene were crossed to male flies containing *UAS-RNAi* transgenes resulting in progeny that expresses *dRas*^{V12} and the respective RNAi transgenes specifically in the developing eye-antennal disc epithelium. We tested 10,720 *UAS-RNAi* lines corresponding to evolutionary conserved 6675 genes, which allowed us to identify hundreds of novel genes that modulate Ras-induced



expression in tumors, orange -no significant difference, white and yellow- increased expression in tumors.

The fly orthologs of these top one hundred candidate human genes were then retested in a secondary screen in *Drosophila* using the *ey-GAL4 UAS-dRasV12* system. In this system, expression of oncogenic *dRas^{V12}* in the eye epithelium results in a rough adult eye phenotype due to epithelial hyperplasia that can be then scored for *dRasV12* cooperating genes. Together with *dRasV12* overexpression, 80% of genes retested in this setting showed increased hyperplasia of the eye epithelium (**Figure 35**). These results collectively validate the top 100 candidate *dRasV12* cooperative tumor suppressors and point towards evolutionary conservation of genes that control epithelial tumor progression. Importantly, we also identified multiple novel candidate tumor suppressors.

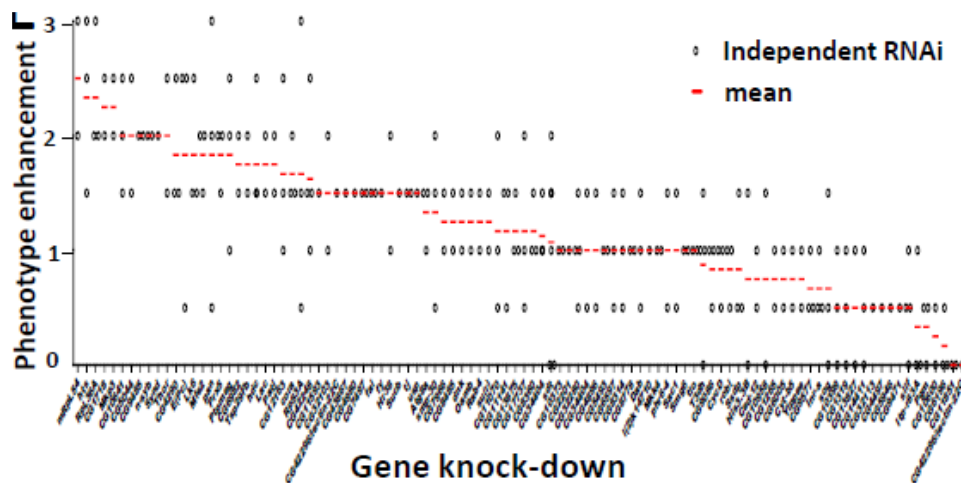
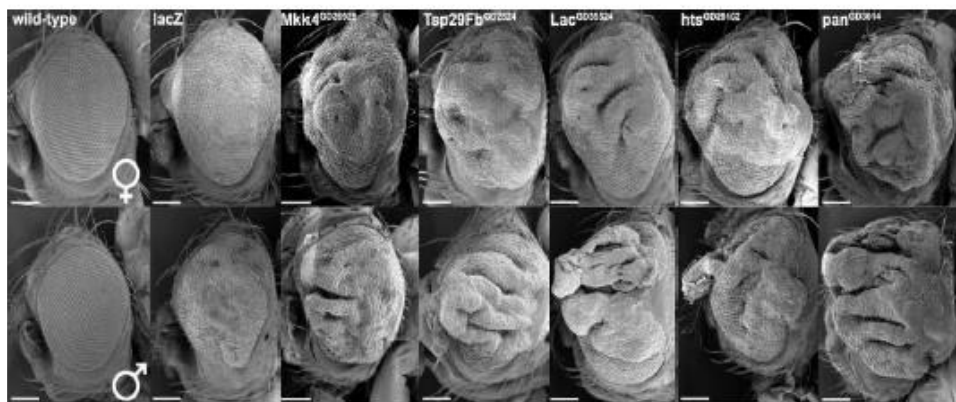


Figure 35. Validation screens in flies. **Top panel,** Representative scanning electron micrographs of *dRasV12*-cooperative eye overgrowth shown for both females and males in a secondary RNAi screen for 100 genes with the best differential expression score in human tumors. Genotypes from top left: wild-type, *ey>dRasV12/lacZ*, *ey>dRasV12/mkk4-RNAi*, *ey>dRasV12/Tsp29Fb-RNAi*, *ey> dRasV12/Lac--RNAi*, *ey> dRasV12 /hts--RNAi*, *ey>dRasV12/pan--RNAi* (scale bar = 100µm). **Bottom panel,** Enhancement of *dRasV12*-driven eye overgrowth by RNAi lines representing the top 100 genes down regulated in human tumours. 0 = no enhancement, 1 = enhancement, 2 = strong enhancement, 3 = very strong enhancement. The diamond indicates scores for individual RNAi lines tested. The red line represents the mean of phenotype scores when gene knockdown effect was tested with multiple RNAi lines.

25 of our validated hits could not be assigned to known signaling pathways and genetic networks, suggesting that these may be novel, functionally yet uncharacterized cancer genes. Among these 25 candidates, the strongest phenotypes in the secondary screen were observed for the three following *Drosophila* genes: *CG1064*, *smp-30* and *Tsp29Fb* corresponding to the human genes *EFHD1*, *RGN*, and *TSPAN6*, respectively. When comparing invasive phenotype scores observed in our primary screen assay, downregulation of *EFHD1* and *Tsp29Fb* revealed the strongest cooperation with *dRas^{V12}* as evidenced by secondary foci formation and dissemination of single GFP⁺ tumor cells. Among these three candidates, *EFHD1* and *RGN* have been previously implicated to have tumor suppressor functions. Functional involvement of *Tsp29Fb*/*TSPAN6*, a member of the tetraspanin family coding for transmembrane-spanning proteins, in tumor growth and progression has not been reported thus far. In humans and mice, *TSPAN6* mRNA is expressed in multiple epithelial tissues including the mammary gland. Therefore, we focused on the functional characterization of fly *Tsp29Fb* and its mammalian ortholog *TSPAN6*.

To explore the role of *TSPAN6* in mammalian cancer, we generated *TSPAN6* whole body knock-out mice as well as *TSPAN6^{flxed}* mice using homologous recombination. These mice are viable and have no overt macroscopic phenotype. Our functional data show that *TSPAN6* restrains growth and metastasis of human and murine Ras-mutant epithelial cancer cells and clonal inactivation of *TSPAN6* in mice markedly enhanced *Kras^{G12D}*-driven initiation and malignant cancer progression. Mechanistically, both fly *Tsp29Fb* and mammalian *TSPAN6*

control Ras and EGFR activation and affect morphology, migration, and fate of epithelial cells. The whole-body and epithelial-specific TSPAN6 mutant females were also challenged with MPA and DMBA to induce hormone-driven mammary tumors. Importantly, we observed markedly enhanced onset of first palpable tumors and reduced overall survival of the mice (**Figure 36**). Similar to the whole body mutant mice, tissue specific deletion of TSPAN6 in the mammary epithelium markedly enhanced mammary tumor onset and significantly reduced the survival of the female mice (**Figure 37 and 38**). Our evolutionary approach to identify novel players in cancer progression allowed us to identify a novel gene, TSPAN6, that affects epithelial architecture and restrains tumor growth and metastasis.

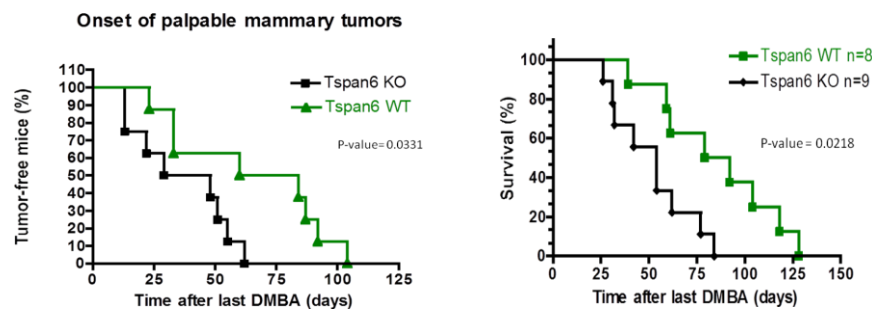


Figure 36. Onset of palpable mammary tumors (left panel) and overall survival (right panel) in TSPAN6 expressing and TSPAN6 mutant age-matched littermate control females treated with the progestin MPA and the carcinogen DMBA. For MPA/DMBA carcinogenesis, nulliparous, six-week old female mice were s.c. implanted with MPA pellets and treated orally with DMBA as indicated for 8 weeks. Data are shown as percentage of tumor free mice after the last DMBA challenge.

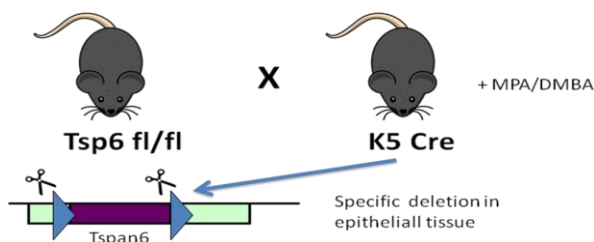


Figure 37. Breeding scheme to generate mice with TSPAN6 ablation in the mammary epithelium.

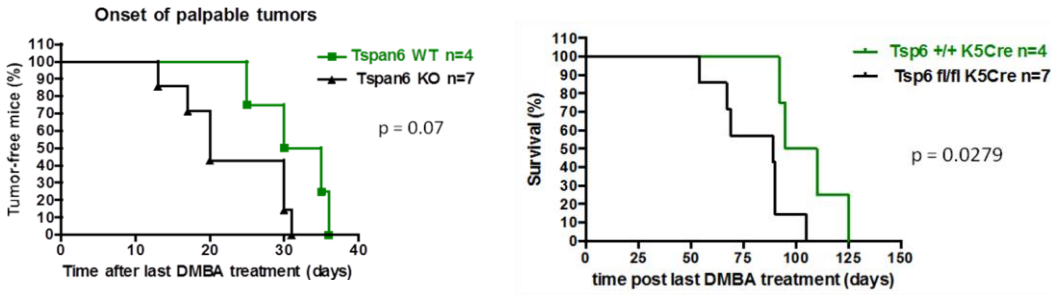


Figure 38. Onset of palpable mammary tumors (left panel) and overall survival (right panel) in K5-Cre TSPAN6fl/fl and TSPAN6 expressing control littermates treated with the progestin MPA and the carcinogen DMBA. Data are shown as percentage of tumor free mice after the last DMBA challenge.

SNP chip analysis of human tumor cell lines revealed that the *TSPAN6* locus in the Xq22.1 genomic region is frequently deleted in breast cancer cells: 24/59 cell lines analyzed showed chromosomal deletions encompassing the *TSPAN6* locus. We also observed deletions spanning the *TSPAN6* locus in primary breast cancer. Importantly, typical for tumor suppressors, we detected defined focal deletions and break-points at the genomic region containing the *TSPAN6* locus in primary human breast tumors. Thus, *TSPAN6* is commonly down-regulated in multiple human breast cancers and the genomic region encompassing the *TSPAN6* locus is frequently deleted in breast tumor cell lines and primary human breast cancer. Since the biological mechanisms of tumor development and progression in breast cancer differ according to their molecular phenotype, we evaluated *TSPAN6* mRNA expression in three major molecular subtypes of breast cancer, i.e. ER-HER2- (“basal-like”), ER-HER2+ (“HER2-overexpressing”), and ER+HER2- (“luminal”) subtypes. Intriguingly, whereas *TSPAN6* mRNA levels were not prognostic in patients with ER-HER2- and ER-HER2+ tumors (not shown), we observed a significant association of *TSPAN6* expression with metastases-free survival in patients diagnosed with ER+HER2- breast cancer. Among patients with ER+ breast tumors, *TSPAN6*^{low} mRNA expression is associated with advanced tumor grades, poorer survival, and enhanced risk of distant metastases. *TSPAN6*^{low} expression also predicts poor clinical outcome even in luminal-A patients with lymph node negative disease.

Thus, TSPAN6 appears to be a novel prognostic biomarker for ER+ breast cancer and TSPAN6^{high} expression defines a subpopulation of ER+ breast cancer patients with very good clinical prognosis. Thus, TSPAN6 appears to function as a novel tumor suppressor for breast cancer.

5. RESEARCH ACCOMPLISHMENTS:

Year 1:

- Fine mapping of RANKL/RANK pathways in mammary cancer development
- Human serology studies on BRCA1 carriers
- Generation of mice to assess the role of the RANKL/RANK system in BRCA1-driven mammary cancer
- Generation of a systems map for genes that cooperate with Ras in epithelial transformation
- Genetic validation of TSPAN6 as a novel tumor suppressor gene in breast cancer
- Development of efficient insertional mutagenesis vectors
- Barcoded gene traps for future quantitative biology studies
- Generation of first mutant ES cells clones

Year 2:

- Continued elucidation of RANKL/RANK activation pathways in mammary cancer development
- Generation of mice to assess the role of the RANKL/RANK system in BRCA1-driven mammary cancer
- Demonstration that RANK has a key role in BRCA1 mutant mammary cancer
- Mechanistic elucidation on RANK-driven mammary tumor pathways in a BRCA1/p53 mutant background
- Generation of a systems map for genes that cooperate with Ras in epithelial transformation
- Cross-validation of our fly hits with thousands of human tumors
- Identification of TSPAN6 as a prognostic biomarker for a subtype of breast cancer
- Experimental validation of TSPAN6 as a novel tumor suppressor gene in breast cancer

- Development of efficient insertional mutagenesis vectors and set-up of near-genome wide screening system using haploid stem cells
- Generation of mutant ES cells clones with intragenic insertions covering ~ half of entire protein coding mouse genome.

Year 3:

- Assessing the role of the RANKL/RANK system in BRCA1-driven mammary cancer
- Demonstration that RANK has a key role in BRCA1 mutant mammary cancer using two different genetic model systems
- RANK controls hormonal expansion of BRCA1/p53 mutant mammary stem cells
- Near genome wide fly screen to identify novel regulators of epithelial tumorigenesis and metastases.
- Cross-validation of our fly hits with thousands of human tumors and secondary validation of the top 100 tumor suppressor candidates
- Identification of TSPAN6 as a prognostic biomarker for a subtype of breast cancer
- Generation of two different TSPAN6 mutant mouse lines
- Experimental validation of TSPAN6 as a suppressor of hormone-induced breast cancer
- Development of efficient insertional mutagenesis vectors and set-up of near-genome wide screening system using haploid stem cells
- Development of tissue engineered 3D blood vessel organoids from haploid ES cells
- Identification of Apelin as a key regulator of tumor neo-angiogenesis in breast cancer

Year 4:

- Serum analysis of RANKL, OPG and Progesterone in different breast cancer cohorts
- In postmenopausal women without known genetic predisposition, high RANKL and progesterone serum levels stratify a subpopulation of women at high risk of developing breast cancer 12-24 months before diagnosis (5.33-fold risk, 95%CI 1.5-25.4; $P=0.02$).
- In women with established breast cancer, RANKL/OPG ratios change dependent on the presence of circulating tumor cells (CTCs).
- In a prospective human breast cancer cohort, alterations in RANKL/OPG ratios are significantly associated with breast cancer manifestation.
- The RANKL/RANK/OPG system is deregulated in post-menopausal women at high risk for breast cancer and in women with circulating tumor cells.

- Continued analysis of of Apelin as a regulator of tumor neo-angiogenesis in breast cancer
- Demonstration that Apelin is involved in VEGFR-mediated blood vessel growth
- Targeting Apelin reduces the metastatic potential of tumors treated with current RTKI anti-angiogenic therapy
- Identification of Apelin as a potential novel target for cancer treatment

Year 5:

- Serum levels of RANKL/OPG are potentially indicative of predisposition and progression of breast cancer in humans.
- Demonstration that RANK has a key role in BRCA1 mutant mammary cancer using two different genetic model systems.
- RANK controls hormonal expansion of BRCA1/p53 mutant mammary stem cells.
- RANKL/RANK link sex hormones to lung cancer providing a first molecular explanation on gender specific outcomes in lung cancer.
- Generation of mutant ES cells clones with repairable mutations covering ~ 17000 mouse genes, the largest mutant mouse embryonic stem cell bank in the world.
- Development of a novel technology platform for quantitative read-out of glycosylated proteins.

6. Publications 2012-2018

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<http://www.researcherid.com/rid/I-6860-2013>

<http://orcid.org/0000-0002-8194-3777>

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7. List of personnel receiving pay:

Domagoj Cikes
David Hoffmann
Ivona Kozieradzki
Shang Wan Liu
Josef Penninger
Shuan Rao
Hiroshi Shiraishi
Verena Sigl
Daniel Wenzel
Reiner Wimmer