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PRINCIPAL INVESTIGATOR: Josef Penninger

CONTRACTING ORGANIZATION: IMBA – Institut fuer Molekulare Biotechnologie GmbH V
Vienna 1030 Austria

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14. ABSTRACT We combine fly genetics with haploid ES cell mutagenesis and <i>in vivo</i> mouse genetics to functionally characterize human candidate breast cancer genes. Using mouse genetics we have progressed in defining the role of RANKL/RANK in breast cancer. Moreover, we have first results that deletion of RANK might affect the development of BRCA1-mutant breast cancer. If correct this might lead to first cancer prevention trials in BRCA1 carriers using RANKL blockade. Using <i>Drosophila</i> modeling of Ras-driven transformation, we performed a near-genome wide screen for genes that control tumor progression. Using this system we have functionally identified multiple novel cancer genes that also play a role in breast cancer, e.g. the surface receptor TSPAN6 or the chromatin modulator EPC1. Finally, we have progressed in generating a haploid ES cell library for all researchers and have already generated more than 27000 murine ES cell clones targeting more than 10000 different genes. These integrations are repairable and each clone carries a genetic barcode, essential to perform future synthetic lethal experiments or to uncover resistance to defined drugs. Thus, we have successfully initiated all proposed aims with the vision to provide rapid functional annotation of breast cancer genes and, in case of RANKL/RANK, to provide the essential rationale for clinical prevention trials.		

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INTRODUCTION:

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^{1,2,3}. The big challenge is now to determine which of these genes encode fundamental molecular pathways that initiate breast cancer and control metastases. Moreover, it is paramount to rapidly translate such findings into strategies for the prevention, future treatment, and possible eradication of breast cancer. I therefore proposed to integrate unique cancer modeling in *Drosophila melanogaster*, a novel murine haploid ES cell technology and functional mouse genetics to rapidly assess the role of candidate breast cancer genes and to discovery and validate novel genes and pathways with direct relevance to cancer. In addition to finding essential new cancer pathways using fly and murine haploid ES cell technologies, I proposed a project that could fulfill the dream of eradication of breast cancer and could have direct impact on prevention and treatment of breast cancer, i.e. to 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.

BODY:

The following three tasks were proposed

1. **Dissecting the role of RANKL/RANK in mammary cancer.**
2. **Functional validation of breast cancer genes using *Drosophila* genetics.**
3. **Modelling breast cancer and cancer metastases using haploid ES cell technology.**

1. Dissecting the role of RANKL/RANK in mammary cancer.

Background: My group generated the first RANKL mutant mice, providing definitive proof that RANKL (OPGL, TRANCE, or ODF) is the master regulator of bone loss^{4,5}, thus opening new possibilities to develop the first rationale therapies against bone loss in multiple diseases such as osteoporosis, leukemia, AIDS, arthritis, and, importantly, cancer related skeletal events. Intriguingly, we found another essential function for RANKL/RANK: they control the formation of a lactating mammary gland in pregnancy⁶. Our results provided a molecular and evolutionary explanation for gender bias and the high incidence of osteoporosis in females. We have also shown that RANKL and RANK are expressed in primary breast cancers and breast cancer-derived cell lines and that the RANKL/RANK system can function as a soil factor that controls bone metastases of epithelial tumors⁷, a finding that provided, among work from other groups^{8,9}, a mechanistic underpinning for clinical trials to delay bone metastases in prostate and breast cancer. Importantly, inhibition of RANKL can indeed increase survival in a mouse model of breast cancer bone metastasis¹⁰ and blocking RANKL using the already FDA approved blocking antibody (Denosumab) is currently being tested in clinical trials preventing metastases in breast cancer patients.

The Women's Health Initiative and the Million Women Study have shown that hormone replacement therapy (HRT) is associated with an increased risk of incident and fatal breast cancer^{11,12}. In particular, synthetic progesterones (progestins) used by millions of women for HRT and contraceptives markedly increase the risk of breast cancer. Based on the clinical importance of RANKL inhibition being approved for bone loss for potentially millions of patients^{13,14}, the role of RANKL/RANK in mammary epithelial proliferation in pregnancy in response to sex hormones^{6,15}, we developed the idea that RANKL/RANK might play a role in breast cancer initiation. My group¹⁶ and Gonzalez-Suarez *et al.*⁸ were indeed able to show that the RANKL-RANK system is a key regulator of hormone (progestin)- 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 renewal¹⁶. Therapeutic inhibition of RANKL reduced the incidence of hormone driven breast cancer from 100 to ~ 10% in a mouse model⁸. 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 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 results¹⁶, significantly more affects the development of hormone-driven mammary cancer, i.e. K5Cre deletes RANK in a key progenitor cell compartment (Figure 1). 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 progestin-driven breast cancer (Figure 2). Deletion of TRAF6, but not NFATc1 markedly impaired breast cancer development (Figures 3 & 4). 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.

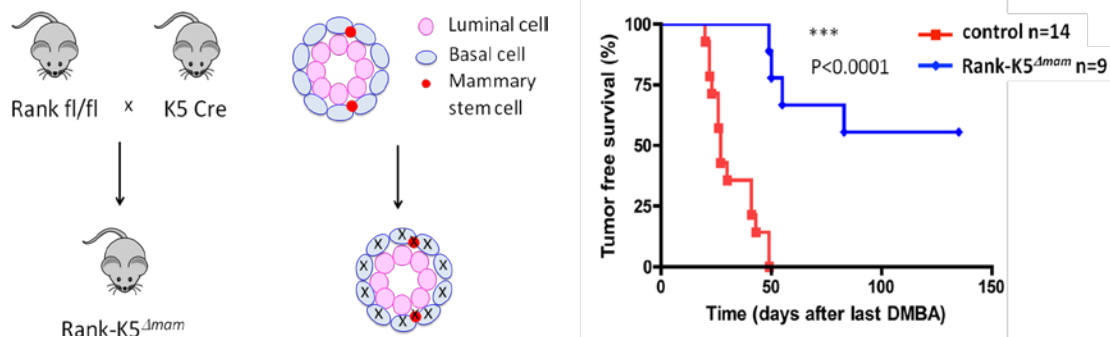


Figure 1. Onset of palpable mammary tumors in K5-Cre *rank*^{flxed/ Δ} females and age-matched littermate control females treated with the progestin MPA and the carcinogen DMBA. For MPA/DMBA carcinogenesis, nulliparous, six-week old female mice were s.c. implanted with MPA pellets and treated orally with DMBA as indicated for 8 week. Data are shown as percentage of tumor free mice after the last DMBA challenge.

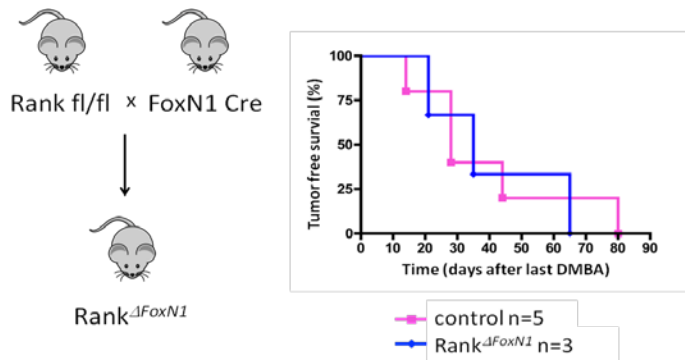


Figure 2. Onset of palpable mammary tumors in *FoxN1-Cre rank^{flxed/Δ}* females and age-matched littermate control females treated with the progestin MPA and DMBA as described in Figure 1. Data are shown as percentage of tumor free mice after the last DMBA challenge.

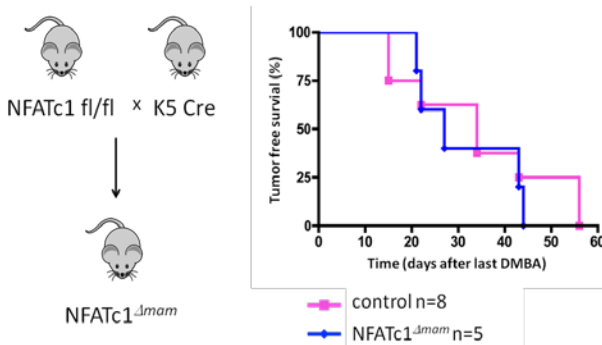


Figure 3. Onset of palpable mammary tumors in *K5-Cre nfatc1^{flxed/Δ}* females and age-matched littermate control females treated with the progestin MPA and DMBA as described in Figure 1. Data are shown as percentage of tumor free mice after the last DMBA challenge.

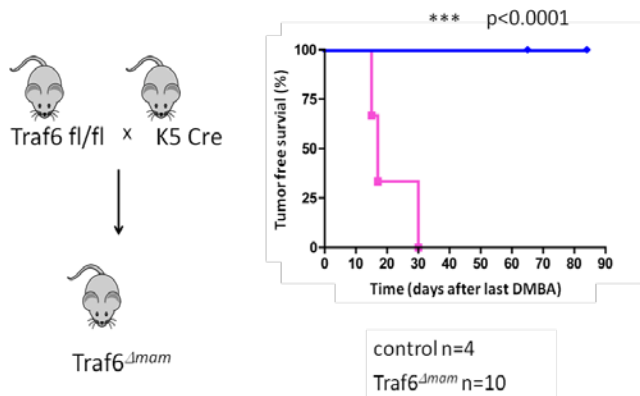


Figure 4. Onset of palpable mammary tumors in *K5-Cre traf6^{flxed/Δ}* females and age-matched littermate control females treated with the progestin MPA and DMBA as described in Figure 1. Data are shown as percentage of tumor free mice after the last DMBA challenge.

It has been reported that RANKL might function on Treg cells and thereby drive breast cancer metastases⁹. However, this has never been genetically proven although it is clear that RANKL/RANK can drive metastases. We therefore crossed RANKL^{fl^{ox}} mice¹⁷ 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 5) but not on T cells (Figure 6). Our data in epithelial cells would in fact also indicate an 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^{fl^{oxed}} 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 RANKL during pregnancy (Figure 7).

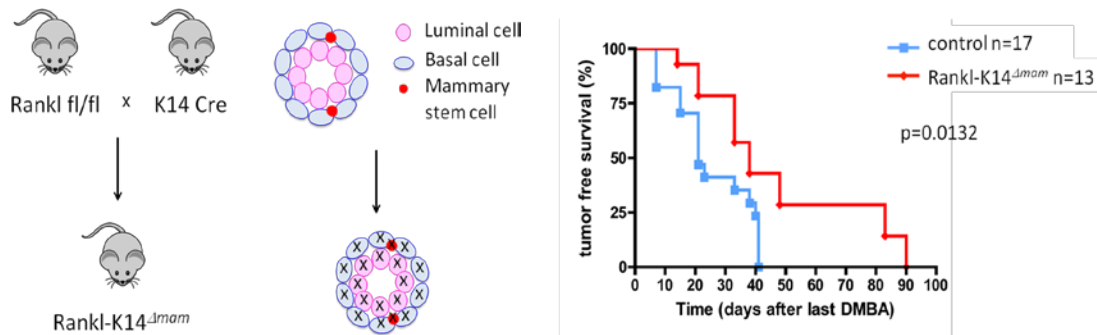


Figure 5. Onset of palpable mammary tumors in K14-Cre *rankl*^{fl^{oxed}/Δ} females and age-matched littermate control females treated with the progestin MPA and DMBA as described in Figure 1. Data are shown as percentage of tumor free mice after the last DMBA challenge.

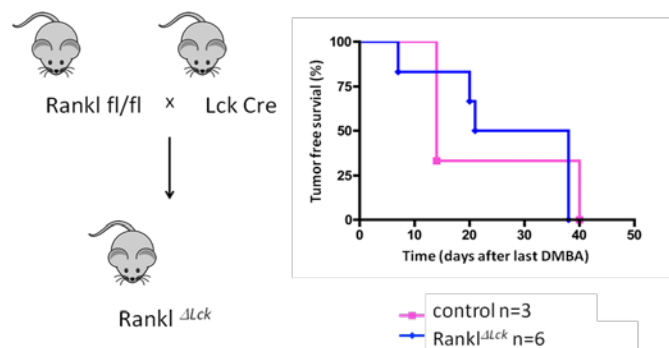


Figure 6. Onset of palpable mammary tumors in Lck-Cre *rankl*^{fl^{oxed}/Δ} females and age-matched littermate control females treated with the progestin MPA and DMBA as described in Figure 1. Data are shown as percentage of tumor free mice after the last DMBA challenge.

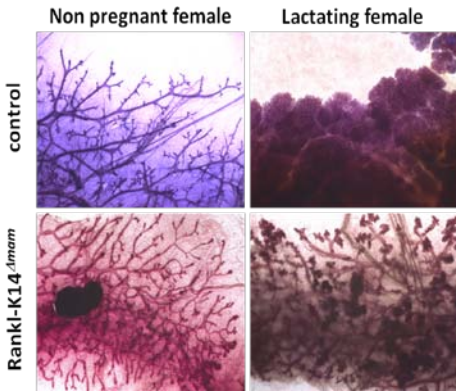


Figure 7. Whole mount images of mammary tissue of nulliparous littermate and lactating control and K14-CreRANK^{flxed} females showing normal alveolar/ductal epithelial structures in non-pregnant females but severe impairment in formation of a lactating mammary gland.

To analyse the role of RANKL/RANK in human breast cancer, we assayed sera from premenopausal women at high risk of developing breast cancer due to *BRCA1* mutations. Our first data show that women with *BRCA1* mutations exhibit deregulated RANKL and reduced OPG (the natural inhibitor of RANKL) levels during their menstrual cycle, resulting in an increased exposure to RANKL (Figure 8). Thus, the RANKL/RANK/OPG system appears to be deregulated in females with increased risk of breast cancer and with diagnosed breast cancer.

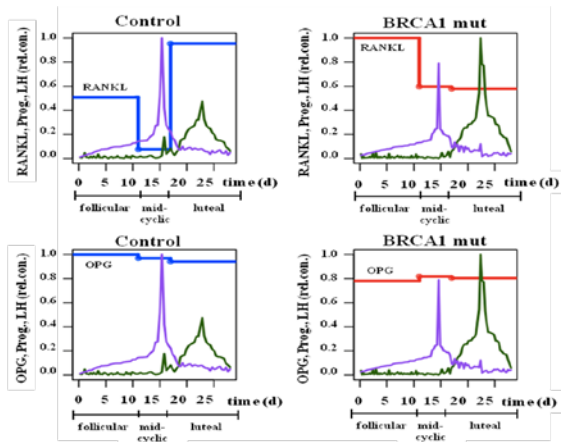


Figure 8. Relative RANKL and OPG (Osteoprotegerin) serum levels in *BRCA1* wild type controls (blue) and *BRCA1* mutation carriers (red) are shown in relation to progesterone (green) and LH (purple) levels in the three defined phases of the menstrual cycle (follicular, mid-cyclic and luteal phase).

To definitively assess the role of RANKL/RANK in *BRCA1*-driven breast cancer, we now deleted RANK in the mammary epithelium using a mouse model of *BRCA1*/p53-driven breast cancer¹⁸. Importantly, in this mouse model it has been reported that blocking of progesterone receptors results in delayed mammary tumor formation¹⁸. To perform these experiments we needed to introduce 4 different mutant alleles into one mouse background, a tour-de-force of mouse breeding (Figure 9, left panel). Finally we derived such mice, and our first, preliminary

results indeed indicate that loss of RANK in mammary epithelial cells delays the onset of mammary epithelial hyperproliferation in mice that carry p53 and BRCA1 mutations. If these data can be confirmed in larger cohorts of mice, including survival results, one could indeed think about initiating phase II/III breast cancer prevention trials in humans BRCA1 carriers using the already FDA-approved anti-RANKL antibody.

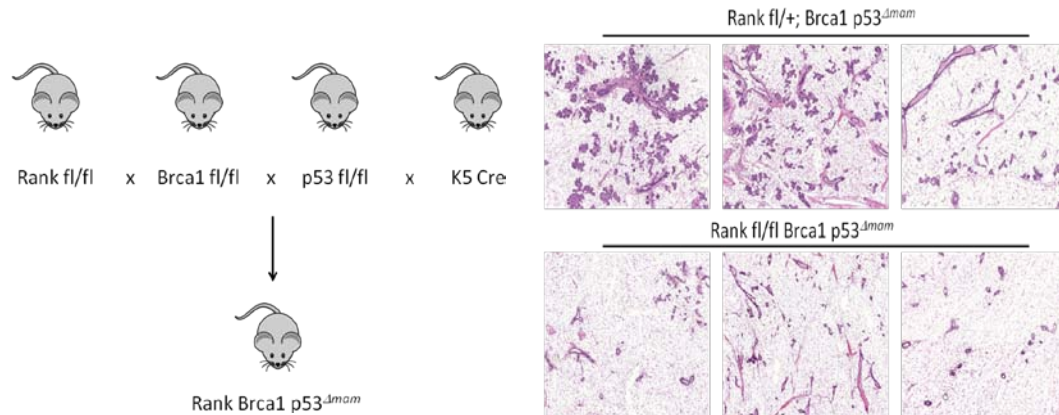


Figure 9. Breeding scheme (left panel) and H&E analyses (right panels) of mammary tissue of three different nulliparous littermate mice that are mutant for BRCA1 and p53 and carry either one allele of RANK (Rank fl/+) or are mutant for RANK (Rank fl/fl). Note epithelial hyperproliferation in 2 out of 3 “control” mice that still express RANK.

2. Functional validation of breast cancer genes using *Drosophila* genetics.

Background: Many developmental pathways and signaling cascades implicated in cancer have been originally identified in *Drosophila melanogaster*¹⁹⁻²¹ and oncogenic signaling pathways such as Ras and Notch are conserved from fly to human²². Moreover, in landmark studies, metastasis has been successfully modeled in epithelial cells of fly larvae^{23,24}. Thus, fundamental mechanisms of oncogenic transformation and metastases appear to be highly conserved among flies and humans. Researchers at our campus, initially started at IMBA, have created the now largest global RNA interference library in the world representative of nearly all expressed (~14,500) *Drosophila* genes, to generate a system where every fly gene can be inactivated via RNAi *in vivo* using tissue specific promoters²⁵. This fly library is a unique resource in the world. We are therefore in a unique position to functionally assess the role of genes in cancer cell growth, invasion, and metastasis in a whole organism and near genome-wide levels.

Results: We have modeled hyperplasia in larval epithelial cells using activated Ras^{V12} which allowed us to identify hundreds of novel genes that modulate Ras-induced transformation. Expression of the human orthologs of our primary fly hits were significantly ($p < 0.00002$) downregulated in thousands of human tumors making fly a viable model system to study human cancer. Of note, Ras is amplified in triple negative breast tumors (P. Humbert, pers. communication).

Among the fly genes we found and functionally validated in different oncogene models, we focused on one of the strongest hits, namely TSPAN6. TSPAN6 is a surface molecule of yet unknown function. We therefore first made TSPAN6 whole body knock-out mice. These mice are viable and have no overt macroscopic phenotype. Importantly, when we challenged the mice with MPA plus DMBA to drive breast cancer, we observed markedly enhanced onset of first palpable tumors and secondly reduced overall survival of the mice (Figure 10).

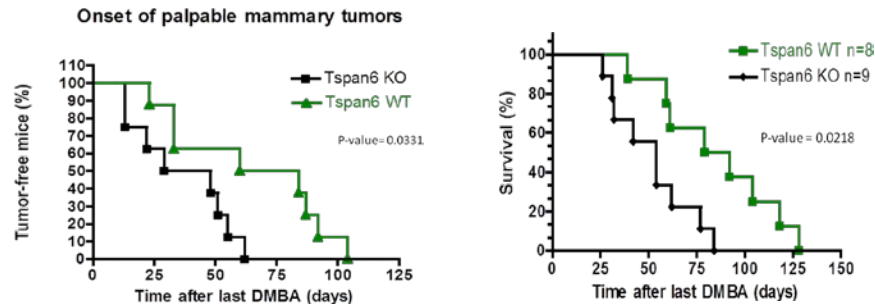


Figure 10. Onset of palpable mammary tumors (left panel) and overall survival (right panel) in TSPAN6 expressing and TSPAN6 mutant age-matched littermate control females treated with the progestin MPA and the carcinogen DMBA. For MPA/DMBA carcinogenesis, nulliparous, six-week old female mice were s.c. implanted with MPA pellets and treated orally with DMBA as indicated for 8 week. Data are shown as percentage of tumor free mice after the last DMBA challenge.

Since we performed our experiments in whole body mutant mice, it was possible that other cell types than the epithelium might also contribute to the increased mammary tumor development. We therefore generated a mouse carrying a TSPAN6floxed, crossed these mice with K5Cre lines to delete TSPAN6 in epithelial tissues (Figure 11) and finally challenged females with MPA and DMBA to induce hormone-driven mammary tumors. Similar to the whole body mutant mice, deletion of TSPAN6 in the mammary epithelium markedly enhanced mammary tumor onset and significantly reduced the survival of the female mice. Thus, TSPAN6 appears to function as a novel tumor suppressor for breast cancer.

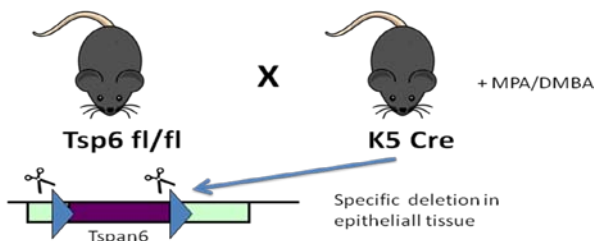


Figure 11. Breeding scheme to generate mice with TSPAN6 ablation in the mammary epithelium. For cellular topography of K5-Cre driven gene deletion please see Figure 1.

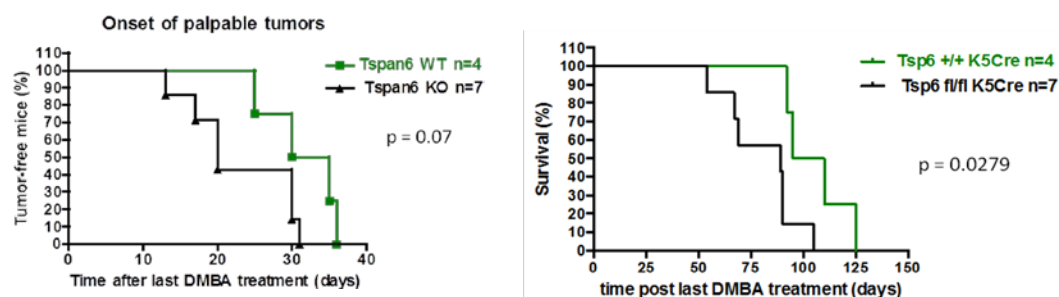


Figure 12. Onset of palpable mammary tumors (left panel) and overall survival (right panel) in K5-Cre TSPAN6fl/fl and TSPAN6 expressing control littermates treated with the progestin MPA and the carcinogen DMBA. Data are shown as percentage of tumor free mice after the last DMBA challenge.

3. Modelling breast cancer and cancer metastases using haploid ES cell technology.

Background: Over the last century, genetic screens have markedly contributed to our understanding of basic physiology and disease²⁶. 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 cycles²⁶. RNAi-mediated gene knock-down can partially overcome the limitation to study gene function²⁷. While having lead to a number of breakthroughs, RNAi suffers from sometimes inefficient and transient knock-down or off target effects²⁸. Stable knock-down can be achieved by shRNA, however, even less efficient²⁹. Furthermore, the magnitude of the gene knock-down by RNAi varies within cell populations and cells with inefficient knock-down of genes promoting proliferation or survival exhibit a growth advantage, especially in *in vivo* models³⁰.

Results: We have recently developed a new technology, namely haploid ES cells. These haploid ES cell lines were derived in my laboratory from parthenogenic mouse blastocysts, give rise to teratomas and can differentiate into all germ layers *in vitro* and *in vivo*³¹. We next developed gene trap vectors for genome-wide mutagenesis that are conditional and can be reverted to wild type via Cre-recombinase as well as back to a null allele via Flippase thereby allowing immediate confirmation of the candidate gene³² (Figure 13, left panel). After mutagenesis at the haploid stage, these cells duplicate the genome within 1-2 weeks to become diploid, i.e. we can evaluate the effects of mutations in a close to normal genetic setting. Moreover, each clone carries its own genetic barcode which should allow us to perform quantitative near-genome-wide screens in the future. Since each system has defined hotspots of integration, we have also developed multiple insertional mutagenesis vectors, based on retrovirus and lentivirus backbones as well as two different transposon systems, namely *Sleeping Beauty* and *Tol2* (Figure 13, right panel). Of note, we have also developed the *PiggyBac* transposon system but have not used it yet. Using these systems we performed mutagenesis assays to determine insertional hotspots; importantly, as predicted each system has its hotspots, however, when we combine all the mutagenesis system our data indicate that we have been able to develop systems for near-genome saturated mutagenesis (Figure 14). Thus, we have developed systems that will allow us to perform genome-wide, unbiased insertional mutagenesis generating clones that can be repaired and carry a barcode that, in the future, can be used for quantitative readouts at the genome level.

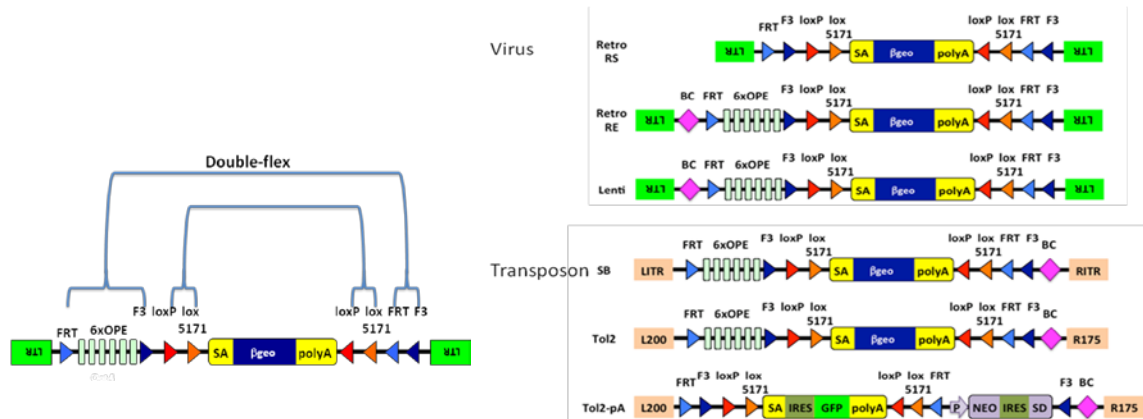


Figure 13. Schematic representation of mutagenesis vectors we developed. The double flex (twice revertable) system is indicated in the left panel. Right panels show the insertional mutagenesis vectors we have developed in the last year based on retro and lentiviral LTRs (long terminal repeats), and two different Transposons (Sleeping beauty = SB; and Tol2). OPE sites allow integration into coding genes not expressed in ES cells³². SA, splice/acceptor sites. BC, unique genetic barcode added to each clone.

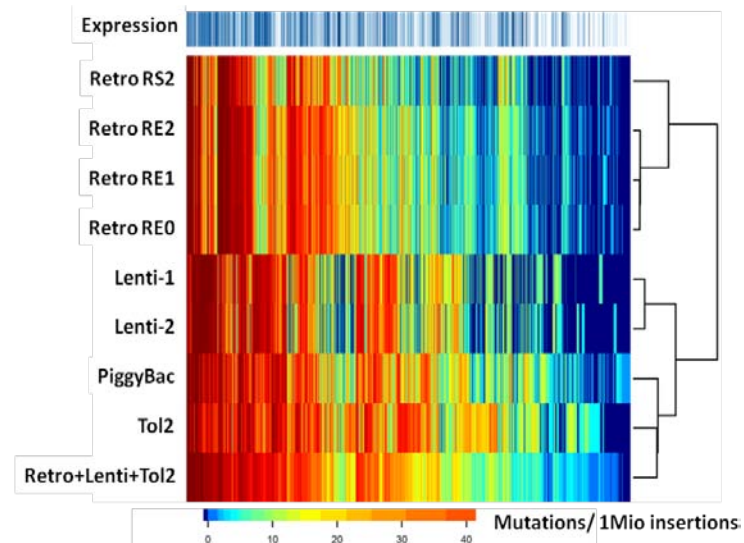


Figure 14. Genome wide estimates of integration hotspots using the indicated mutagenesis vectors. The color scheme indicates numbers of integrations in genes based on 1 Million insertions. Of note we now routinely generate between 50-100 Million integrations in a single experiment using about 1 billion sorted haploid ES cells as the starting point. Importantly, when combined we nearly hit every protein coding gene in the genome. We have not yet analyzed the hit rates for microRNAs or ncRNAs.

Using our murine haploid embryonic stems, we have now started to establish a cell bank with the vision to develop a library of ES cell clones where nearly every expressed gene carries a homozygous loss of function mutation. We initially proposed to derive, isolate, expand, freeze and map ~ 50,000 independent mutant clones as an initial step towards the goal of a genome wide collection of null alleles, not knowing about the frequencies of insertional mutagenesis, insertional hotspots etc. Amazingly, the system actually works better than anticipated. We have

already picked and mapped 27 479 individual ES cell clones, 17867 of which carry a single integration. 11226 of these integrations are intragenic where we predict they will disrupt gene expression, when located in sense in the introns. Most importantly, we already hit 10274 different protein-coding genes carrying intragenic insertions, 6210 of which carry a single integration (Figure 15).

	# mapped clones	Multiple Insertions	Single Insertions	Intragenic
Tol2	14 376	7 248	7 128 (49%)	4 844 (68%)
Lenti	6 565	891	5 674 (86%)	3 924 (69%)
Retro	6 556	1 491	5 065 (77%)	2 458 (49%)
Σ	27 497	9 630	17 867 (65%)	11 226 (63%)

Number of genes associated to insertions	
All associated genes	21 922
associations up-/downstream	18 601
Genes with Intragenic insertions	10 274
In clones with single mapped insertion	6 210 (55% of # lines)
Tol2	3 712 (77% of # lines)
Lenti	2764 (70% of # lines)
Retro	1753 (71% of # lines)

Figure 15. Numbers of total clones mapped, single and multiple insertions in individual clones and the numbers and frequencies of intragenic insertions. The bottom panel shows total numbers of genes carrying intragenic insertions, clones with mapped single intragenic insertions (6210) and the vectors used to generate such single insertional clones.

Thus, within a few months we set-up mutagenesis systems that will allow us to perform near-genome saturated forward genetics and we have nearly mutated 1/3 of the expressed genome and generated ES cell clones that now can be used for reverse genetics and will be made available to all researchers.

RESEARCH ACCOMPLISHMENTS:

- **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**

REPORTABLE OUTCOMES:

We are currently setting up a homepage (**Haplobank**) where we will deposit all our mutant ES cell clones for the entire research community. All insertions will be mapped and recoded in a three-way traffic light system to report the status of confirmations (single vs multiple insertions, revertability, etc.), linked to genome browsers, protein sites, PubMed, etc. Clones will be provided for cost. It is anticipated that we become the key site for functional haploid ES cell genetics in the world that our technologies will strongly boost cancer research and allow all cancer researchers in the world to perform experiments that were previously not possible.

CONCLUSION:

Combining fly genetics and our unique haploid ES cell mutagenesis library 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 human candidate cancer genes. Using this approach we have already discovered and in mouse experiments functionally validated a novel tumor suppressor gene, the surface receptor TSPAN6.

We have also now moved forward to develop haploid ES cell systems that combine haploid whole genome scans with the potential of mammalian cell biology, with the grand vision and reachable goal to introduce genome-wide quantitative biology to perform for instance synthetic lethal screens and find genes that regulate resistance to cancer drugs. Moreover, we have started to generate a haploid murine ES cell library. The haploid ES cell library will be made available to all other breast cancer researchers and could markedly drive the field in the future because any new candidate gene/pathway could be immediately tested *in vivo*.

Further experimental elucidation of RANKL/RANK as critical molecules and cell types in breast cancer initiation, mammary stem cell biology, and metastasis, for instance in the context of BRCA1 mutations, combined with the development of already therapeutics directed to block RANKL could 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 on RANKL/RANK are not just “theoretic” but should have direct consequences for delaying metastases and, most importantly, might provide the experimental underpinning for breast cancer prevention.

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APPENDICES:

BRCA1 carriers - UK Familial Ovarian Cancer Screening Study (UKFOCSS) Cohort. The first group of subjects were participants in the UK Familial Ovarian Cancer Screening Study (UKFOCSS), a prospective single-arm screening study in a high-risk population (Trial registration - International Standard Randomized Controlled Trial Number (ISRCTN): 32794457). Eligible cases were premenopausal women who were confirmed *BRCA1* mutation carriers and who did not have a diagnosis of breast cancer 12 months after the last sample collection (114 samples from 42 *BRCA1* mutation carriers with median age 41.1 years; for 24 women serial samples were analyzed). Controls were premenopausal women who were initially recruited due to known *BRCA1* mutations in the family but who subsequently tested negative for a *BRCA1* mutation and did not have breast cancer before or after they provided serum samples (85 samples from 41 control women with a median age 41.9 years; for 23 women serial samples were analyzed). Samples were split into three groups in order to reconstruct the phases of the menstrual cycle: phase I (follicular phase) - days 1-11 (low progesterone [<2 ng/mL] and low luteinizing hormone (LH) [<10 mIU/mL]); phase II (mid cyclic phase) - days 12-17 (high LH [>10 mIU/mL] independent of progesterone); and phase III (luteal phase) - days 18-28 (low LH [<10 mIU/mL] and high progesterone [>2 ng/mL]). For intra-individual analysis we calculated correlation coefficients for each woman that were then averaged among controls and *BRCA1* mutation carriers.

MPA/DMBA-induced mammary carcinogenesis. MPA/DMBA treatment was performed as described¹⁶. Briefly, six-week old female mice were anesthetized with ketamine-xylazine and surgically implanted with slow-release Medroxyprogesterone Acetat (MPA) pellets (50mg, 90-day release; Innovative Research of America) subcutaneously on the right flank. 200 μ l DMBA (5mg/ml diluted in cottonseed oil) was administered by oral gavage 6 times throughout the following 8 weeks. Onset of mammary tumors was determined by palpation.

Statistics. For the Kaplan-Meier analysis of tumor onset a log rank test was performed. $P < 0.05$ was accepted as statistically significant.

SUPPORTING DATA:

All relevant data are shown in the main description of the tasks.