Characterization of the Tet-On Grb7 and Tet-On 14-3-3σ Mouse Models

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ABSTRACT

ErbB2 is overexpressed and amplified in about 30% of all breast tumors and is correlated with poor patient prognosis. ErbB2 knock in mouse models mimic the amplification in chromosome 11 (human chromosome 17q21-25) comprising of *ErbB2* and *Grb7* and a deletion in chromosome 4 (human chromosome 1p35-36) including *14-3-3σ* seen in human breast cancers. Grb7 is an adaptor protein known to regulate cell migration and transcription by interacting with a cell migration protein FAK and a transcription factor protein FHL2. Grb7 overexpression has been linked to an increase in metastasis and poor patient outcome. 14-3-3σ is negative cell cycle protein that is upregulated by p53. Interestingly, 14-3-3σ has a role in down regulating ErbB2 expression by sequestering EGR2 from the nucleus to the cytoplasm. These results suggest that 14-3-3σ deletion and Grb7 overexpression in ErbB2 tumors represents an interesting target to study as its deletion may be a targeted event in the development of ErbB2 tumors.

To elucidate the role of Grb7 and 14-3-3σ I generated a mouse model utilizing the Tet-On mouse model system whereby these two proteins are overexpressed in the mouse mammary epithelium. Through this study we confirmed successful localized overexpression of both Grb7 and 14-3-3σ in the mouse mammary gland epithelium. Further, we were able to confirm that individual expression of both Grb7 and 14-3-3σ lead to a ductal outgrowth defect during mammary gland development. These observations confirm that Grb7 and 14-3-3σ both play a role in mouse mammary gland development.

RESUMÉ

ErbB2 est au-dessus d'exprimer et d'amplifier dans environ 30% de toutes les tumeurs de sein et est corrélé avec le pronostic patient pauvre. Le coup en ErbB2 dans des modèles de souris imitent l'amplification en chromosome 11 (chromosome humain 17q21-25) comportant *d'ErbB2* et de *Grb7* et une suppression en chromosome 4 (chromosome humain 1p35-36) comprenant le *14-3-3σ* vu dans les cancers du sein humains. Grb7 est une protéine d'adapteur connue pour régler la migration et la transcription de cellules par l'interaction avec une protéine FAK de migration de cellules et une protéine FHL2 de facteur de transcription. Grb7 au-dessus d'expression a été lié à une augmentation de métastase et de résultats patients pauvres. Le 14-3-3σ est une protéine négative de cycle de cellules qui upregulated par p53. Intéressant, 14-3-3σ a un rôle en réglant vers le bas l'expression ErbB2 en séquestrant EGR2 du noyau au cytoplasme. Ces résultats suggèrent que la suppression 14-3-3σ et le Grb7 au-dessus de l'expression dans les tumeurs ErbB2 représente une cible intéressante pour étudier pendant que sa suppression peut être un événement visé dans le développement des tumeurs ErbB2. Ces résultats suggèrent que la suppression 14-3-3σ et le Grb7 au-dessus de l'expression dans les tumeurs ErbB2 représente une cible intéressante pour étudier pendant que sa suppression peut être un événement visé dans le développement des tumeurs ErbB2.

Pour élucider le rôle de Grb7 et de 14-3-3σ j'ai produit d'une utilisation de modèle de souris Tet-Sur le système modèle de souris par lequel ces deux protéines soient plus de exprimées dans l'épithélium mammaire de souris. Par cette étude nous avons confirmé réussi localisé au-dessus de l'expression de Grb7 et de 14-3-3σ dans l'épithélium de glande mammaire de souris. De plus, nous pouvions confirmer que l'expression

individuelle des deux Grb7 et le 14-3-3σ mènent à un défaut ductal de conséquence pendant le développement de glande mammaire. Ces observations confirment que Grb7 et 14-3-3σ les deux jeu un rôle dans le développement de glande mammaire de souris.

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LIST OF ABBREVIATIONS

BAC- Bacterial Artificial Chromosome

Ca^{2+}- Calcium 2+ ion

CCAC- Canadian Council on Animal Care

CGH- Comparitive Genomic Hybridization

Cre- Cre Recombinase

EGFR- Epidermal Growth Factor Receptor

EGR2- Early Growth Response 2

ErbB2KI- ErbB2 Knock In

FAK- Focal Adhesion Kinase

FHL2- Four-and-a-Half LIM domain protein 2

FISH- Fluorescent In Situ Hybridization

GFP- Green Fluorescent Protein

Grb7- Growth Factor Receptor bound protein 7

HRP- Horseradish Peroxidase

IRES- Internal Ribosome Entry Site

LOH- Loss of Heterozygosity

MAPK- Mitogen Activated Protein Kinase

MMTV- Mouse Mammary Tumor Virus

MTB- MMTV Transactivator

NDL2-5- *Neu* Deletion 2-5 Mouse Model

Neu- Rat homologue of ErbB2

PI3K- Phosphoinositide 3 Kinase

PVDF- Polyvinylidene Fluoride Membrane

RTK- Receptor Tyrosine Kinase

SDS-PAGE- Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis

Sfn- Stratifin or 14-3-3σ

SH2- Src Homology 2 domain

TEB- Terminal End Bud

TR- Tetracycline Responsive Element

Tyr- Tyrosine

VP16- Viral Protein 16

1. INTRODUCTION

Breast cancer is the most prevalent cancer amongst women in Canada. The National Cancer Institute of Canada estimates that 1 in 9 women will develop breast cancer in their lifetime and 1 in 28 will die from the disease. Overexpression and amplification of the ErbB2 receptor tyrosine kinase (RTK) occurs in approximately 30% of all breast cancers and is associated with poor patient prognosis (Hodgson et al., 2005). ErbB2 is a member of the epidermal growth factor receptor protein (EGFR) family that regulates normal tissue development, cell proliferation, adhesion and migration (Figure 1) (Hynes and Stern, 1994).

1.1. ErbB2 Function and Misregulation

The EGFR family consists of four members including ErbB1, ErbB2, ErbB3 and ErbB4. These receptors function by forming homo and heterodimers with other family members upon extracellular ligand binding (Hynes and Stern, 1994; Yarden and Sliwkowski, 2001). ErbB2, however, does not bind any known ligand due to an open conformation within its extracellular domain that mimics ligand binding. Therefore ligand binding is not required for its activation and intracellular signaling. Activation through dimerization of the receptors leads to phosphorylation of the tyrosine residues on the intracellular portion of the receptor (Hynes and Stern, 1994; Yarden and Sliwkowski, 2001). The receptors (in all conformations) can activate the same intracellular pathways; however, not all combinations have the same signaling potential (Hynes and Stern, 1994; Yarden and Sliwkowski, 2001). For example, ErbB3 is kinase null and as a homodimer, ErbB3 is unable to confer a signal upon ligand binding. However, heterodimerization of ErbB3

with ErbB2 leads to the most potent signaling potential of all the combinations of the EGFR family (Hynes and Stern, 1994; Yarden and Sliwkowski, 2001).

The EGFR family normally functions during organogenesis (Hynes and Stern, 1994). Misregulation of the EGFR proteins by mutation or overexpression leads to transformation of normal cells to cancerous cells terming these proteins as oncogenic (Andrechek et al., 2000; Hynes and Stern, 1994; Montagna et al., 2002). Cells overexpressing ErbB2 have uncontrolled cell growth, decreased cell adhesion, and have the ability to metastasize to secondary sites within the body.

1.2. ErbB2 Mouse Models of Breast Cancer Reflects Human ErbB2 Positive Tumors

ErbB2 was first identified as a potent oncogene in breast cancer (Hynes and Stern, 1994). As mouse models were being developed, the effects of overexpressing or knocking out ErbB2 in the mammary epithelium allowed us to explore the role of ErbB2 during mammary gland development and tumorigenesis. Tumors that arise from the ErbB2KI and the NDL mouse models reflect the human disease genetically and molecularly (Hodgson et al., 2005; Montagna et al., 2002). These mouse models have proven to be a valuable resource for studying human breast cancer and allows for each event to be efficiently studied.

The NDL2-5 mouse model uses the mouse mammary tumor virus (MMTV) promoter to drive the expression of an activated *Neu* (rat isoform of ErbB2) containing an in frame mutation within the extracellular cysteine rich region of *Neu* (Figure 2A). Tumor latency occurs at approximately 6 months of age with 60% occurrence of lung metastases (Siegel et al., 1999). These tumors also display a frequent deletion on chromosome 4

(human chromosome 1p35-36) especially in ErbB2 positive human breast tumors (Horie-Inoue and Inoue, 2006; Lodygin and Hermeking, 2006; Ragnarsson et al., 1999).

The ErbB2KI mouse model is unique to other similar mouse models in that it uses the ErbB2 endogenous promoter to drive the expression of an oncogenic *Neu* in the mammary gland. *Neu* expression in this model is controlled by the placement of a Neo cassette flanked by two LoxP sites. Cre-recombinase, under the control of the MMTV promoter, mediating the excision and recombination of the LoxP sites and allows for the expression of oncogenic *Neu* (Figure 2B) (Andrechek et al., 2000). Tumors that arise from the ErbB2KI model have a latency of approximately 16 months with a low occurrence of lung metastasis (Andrechek et al., 2000). Approximately 85% of all the tumors contain an amplicon including ErbB2. Genes amplified on chromosome 11 are also amplified in human ErbB2 positive breast tumors (Table 1) (Hodgson et al., 2005; Montagna et al., 2002).

1.3. Mammary Gland Development

Mammary gland development requires a series of steps including cell proliferation, cell migration, cell adhesion and polarization (Smalley and Ashworth, 2003; Sternlicht, 2006). The mammary epithelium starts elongating from the nipple and forms ducts leading into the mammary fat pad (Figure 3) (Smalley and Ashworth, 2003; Sternlicht, 2006). At the end of the forming ducts lie the terminal end bud (TEB) structure, the leading end forming branches into the fat pad (Smalley and Ashworth, 2003; Sternlicht, 2006). The TEB is the site where there is increased cell proliferation and cell migration into the fat pad. Cells within the mammary ducts undergo apoptosis to form the hollow

lumen structure of the ducts (Smalley and Ashworth, 2003; Sternlicht, 2006). In female mice, mammary gland development starts from approximately 3 weeks of age in response to an increase in estrogen levels and is completed around 10-12 weeks of age when the mammary ducts completely fill the fat pad and the TEB's disappear (Sternlicht, 2006).

By studying how ErbB2 behaves during mammary gland development allows us to determine its role during tumorigenesis. A knock-in mouse model containing a loxP flanked *Neu* (rat isoform of ErbB2) in place of the first exon of the endogenous ErbB2 followed by a selection marker showed that excision of ErbB2 in the mammary epithelium delays ductal outgrowth in the early stages of development (Figure 2B) (Andrechek et al., 2005). In 3 week old homozygous females ($ErbB2^{Flox/Flox}$ MMTV-Cre) ductal outgrowth was severely delayed compared to littermate controls ($ErbB2^{Flox/Flox}$) and also displayed more than a 3 fold decrease in the number of terminal end buds (Andrechek et al., 2005). This defect is seen in mice up to 12 weeks of age, however, in later adulthood the null mice ($ErbB2^{Flox/Flox}$ MMTV-Cre) eventually catches up to the littermate controls (Andrechek et al., 2005). These results demonstrated that ErbB2 has a role in mammary gland development although no functional defect results as mothers are able to lactate and suckle their young normally through multiple rounds of pregnancy and lactation periods.

In contrast to ablating ErbB2 expression in the mammary gland, the ErbB2KI mouse model and the NDL2-5 mouse model demonstrate that overexpression of oncogenic *Neu* in a developing mammary gland leads to altered ductal branching with the formation of lobular alveolar budding (Figure 2A,C). (Andrechek et al., 2000; Andrechek et al., 2005; Siegel et al., 1999). Additionally, the mammary ducts in these

mice branch beyond the confides of the mammary fat pad displaying an alteration in migration of the mammary epithelium (Andrechek et al., 2000; Siegel et al., 1999). NDL2-5 female mice are able to lactate and sustain their young normal but they form mammary tumors in response to the upregulation of pregnancy and lactation hormones which drive expression of *Neu* from the MMTV promoter (Siegel et al., 1999). ErbB2KI females are also able to lactate normally and sustain their young and are able to undergo multiple rounds of pregnancy and lactation before mammary tumors appear (Andrechek et al., 2000). The upregulation of pregnancy and lactation hormones does decrease tumor onset.

By investigating the role that ErbB2 plays during mammary gland development, insight is given into the role of this protein during tumorigenesis. Overexpression of ErbB2 in a developing mammary gland leads to an increase of ductal outgrowth into the mammary fat pad which reflects ErbB2's role during tumorigenesis where the cells are highly proliferative and often migrate and metastasize to distal sites in the body. In the reverse experiment ablation of ErbB2 in the mammary gland leads to a ductal outgrowth defect meaning that the cells are less proliferative and do not migrate into the fat pad as efficiently as wild type cells.

1.4. Genetic analysis of ErbB2 tumors and the role of Grb7

Using human data and mouse models of breast cancer it became evident that ErbB2 overexpression in a large subset of cases was due to genetic amplification on the ErbB2 locus (Andrechek et al., 2003; Hodgson et al., 2005; Stein et al., 1994). Genetic amplification is a process whereby a portion of a chromosome is copied multiple times.

Not all genes in the ErbB2 amplicon are overexpressed in tumors, and may only be amplified due to their proximity to ErbB2 (Table 1) (Hodgson et al., 2005; Montagna et al., 2002; Stein et al., 1994). However, upon closer investigation of the ErbB2 amplicon, 10 genes were found to be consistently amplified along with ErbB2 (Hodgson et al., 2005). Comparative Genomic Hybridization (CGH) and Bacterial Artificial Chromosome (BAC) analyses of ErbB2KI tumors have shown a recurring amplicon on the ErbB2 locus or chromosome 11 in the mouse (Hodgson et al., 2005). The core of the amplified region in the ErbB2KI tumors included a minimal 280kb section found also in human breast cancer (Kauraniemi et al., 2003). Of those 10 genes Grb7 was singled out as potentially important during ErbB2 tumorigenesis as it is amplified and overexpressed in these tumors (Table 1) (Andrechek et al., 2003; Hodgson et al., 2005). Grb7 is an adaptor protein known to interact with ErbB2, ErbB3 and the focal adhesion kinase (FAK), four-and-a-half LIM domain protein 2 (FHL2), and calmodulin.

1.5. Chromosomal Deletion in ErbB2 Tumors and the Role of 14-3-3σ

Various studies on human breast cancer have shown that there is a recurring loss of heterozygosity (LOH) on chromosome 1p35-36 (Hodgson et al., 2005; Montagna et al., 2002; Ragnarsson et al., 1999). LOH at this region in human breast cancer in associated with poor patient prognosis and therefore it became vital to identify genes located in this region (Table 2) (Ragnarsson et al., 1999). Chromosome 4 was found to be partially or completely deleted in ErbB2KI tumors. Using CGH, fluorescent in situ hybridization (FISH) and BAC analysis the minimal region of chromosome 4 loss was identified (Hodgson et al., 2005).

Frequent chromosomal deletions were also identified in the same data set used for the ErbB2 amplicon. Recurring deleted portions of the genome in cancer cells elucidate pathways that may be disrupted upon deletion of certain proteins that control cell growth checkpoints and cell death initiation. Using FISH and BAC analysis, a list of 30 genes located on mouse chromosome 4 (human chromosome 1p35-36) were found to be the frequent minimal deletion site (Montagna et al., 2002). 16 of the 30 genes have unknown functions and the remaining genes are involved in processes such as cell cycle control and transcriptional regulation (Table 2). The negative cell cycle protein 14-3-3σ was the most interesting target for investigation as it is frequently down-regulated in various cancers such as gastric cancer, esophageal cancer and breast cancer (Hermeking, 2003; Horie-Inoue and Inoue, 2006; Lodygin and Hermeking, 2006). 14-3-3σ 's expression is limited to epithelial tissue and is upregulated by the well known tumor suppressor protein p53 (Hermeking and Benzinger, 2006; Yang et al., 2006). Previous work from the Muller lab has shown that 14-3-3σ down-regulates ErbB2 transcription by sequestering the transcription factor Early Growth Response 2 protein (EGR2) from the nucleus to the cytoplasm (Dillon et al., 2007).

1.6. Summary of ErbB2 tumors

The dominating characteristic of ErbB2 tumors is the overexpression of ErbB2, however, genetic events such as amplification and deletions at the chromosomal level represent major events that occur before and during tumorigenesis. In ErbB2 tumors a portion of the ErbB2 locus is frequently amplified in both human breast cancers and ErbB2 mouse models of breast cancers (Hodgson et al., 2005; Stein et al., 1994). Several genes

included in the ErbB2 amplicon and are seen in both the human and mouse and, one of the genes (Grb7) has been shown to be amplified and overexpressed in the same capacity as ErbB2 (Andrechek et al., 2003; Hodgson et al., 2005; Stein et al., 1994). Besides amplification occurring in ErbB2 tumors, there are also frequent deletions on chromosome 4 (human chromosome 1p35-36) that helped identify an important role for 14-3-3σ in downregulating ErbB2 expression and its role as a negative cell cycle regulator (Ragnarsson et al., 1999; Stein et al., 1994). Both Grb7 and 14-3-3σ will now become the focus of the next sections.

1.7. Grb7 Structure and Function

Grb7 is a member of the Grb7 family of adaptor proteins including Grb7, Grb10 and Grb14 (Daly, 1998). Each member has a highly conserved molecular architecture consisting of an N-terminal conserved proline rich motif, a central region exhibiting homology to Mig10 which contains a pleckstrin homology domain and finally a C-terminal SH2 binding domain (Daly, 1998; Han et al., 2001) (Figure 4A). There is a 70% amino acid identity between each family member however they bind different SH2 domains with high specificity. The BPS region upstream from the SH2 domain is the least conserved region between all the family members and is believed to mediate specific interactions through the SH2 domain (Daly, 1998). Only Grb7's interaction through its SH2 domain has been studied. However, the central region that has high homology to Mig10 has been hypothesized to be involved in cell migration as the Mig10 protein in *C.elegans* is involved in neural cell migration during development (Daly, 1998; Han et al., 2001; Shen and Guan, 2004). Grb7 expression is tissue specific. In humans

Grb7 is normally expressed in the pancreas, kidney, placenta, prostate, and small intestine (Daly, 1998). Importantly, Grb7 expression is not detected in normal mammary epithelium.

Moreover the SH2 domain of Grb7 was shown to be sufficient for interaction with ErbB2 (Figure 4B) (Daly, 1998). It was also shown that Grb7 associated through its SH2 domain with the adaptor protein ShcA after growth factor treatment (Figure 4B) (Daly, 1998). Phospho-Shc's interaction with the adaptor protein Grb2 is a well defined pathway to Ras activation suggesting that their SH2 specificity may overlap. Activation of the Ras pathway through ErbB2 and Shc increases cell proliferation (Daly, 1998).

Grb7 also interacts with ErbB3 at 2 tyrosine residues (Y1180 and Y1243) (Fiddes et al., 1998) (Figure 4B). These residues are able to target Grb7 but not Grb2 suggesting that Grb7 and Grb2 binding is not always overlapping (Fiddes et al., 1998; Shen and Guan, 2004). Variable residues between Grb7 and Grb2 may alter binding affinities for each RTK and determine the competition between both proteins. Grb7 interaction with FAK is known to mediate cell migration and cell survival signals (Han et al., 2000; Han et al., 2001; Shen and Guan, 2001). FAK cooperation with growth factor receptors such as ErbB2 is often increased in cancer leading to altered cell growth and cell migration (Figure 4C) (Ivancic et al., 2003; Ivancic et al., 2005; Janes et al., 1997).

FAK is a cytoplasmic tyrosine kinase that mediates integrin signal transduction which is involved in cell adhesion, cell survival, proliferation, spreading, and cell migration (Han et al., 1999; Han et al., 2000; Shen et al., 2001). FAK is activated, subsequently autophosphorylated, and co-localizes with integrins when cells become adherent to an extracellular matrix (Han et al., 1999; Han et al., 2000; Shen et al., 2001).

Phosphorylation on Tyr-397 is necessary for FAK-mediated cell spreading, survival, and cell cycle progression. The Tyr-397 site is known to associate with multiple SH2 domains including the Src family kinases, phosphatidylinositol 3-kinase (PI3K), phospholipase C-γ, and Grb7 (Shen et al., 2001). Moreover, the SH2 domain in Grb7 is vital for FAK-mediated cell migration and in turn Grb7 is phosphorylated by FAK during this process (Han et al., 1999; Han et al., 2000). Interestingly, Grb7 phosphorylated by FAK in a cell adhesion dependent manner, and this phosphorylation and interaction with FAK is necessary for normal cell migration mediated through FAK (Han et al., 1999; Han et al., 2000; Shen et al., 2001).

Besides regulating cell migration, Grb7 has also been shown to bind to the transcriptional regulator FLH2 (four-and-a-half LIM domain protein 2) (Siamakpour-Reihani et al., 2008). FLH2 contains four LIM domains (double zinc finger motifs) and one single zinc finger domain and is known to alter gene expression by interacting with various transcription factors (Kleiber et al., 2007; Siamakpour-Reihani et al., 2008). As previously mentioned, Grb7 interacts with FAK and is vital for normal cell migration in a cell adhesion dependent manner and autophosphorylation dependent manner. FHL2 has also been shown to colocalize with FAK and Grb7 in cells (Siamakpour-Reihani et al., 2008). A model proposed by Reihani et al suggests that Grb7 interacts with phosphorylated FAK, and FAK in turn phosphorylates Grb7 on its tyrosine residue (Siamakpour-Reihani et al., 2008). Phosphorylated Grb7 then is better able to interact with FHL2, thereby preventing FHL2 from interacting with downstream transcription factors.

Calmodulin is a calcium-dependent protein that inhibits tyrosine kinase activity within the cell by acting on EGFR and ErbB2 directly (Li et al., 2004). As tyrosine kinase activity increases within the cell, Ca^{2+} ions increases in the cytoplasm and then binds to calmodulin forming a Ca^{2+}-Calmodulin complex that directly binds and inhibits EGFR and ErbB2 signaling (Li et al., 2004). Moreover, Grb7 has been shown to bind to calmodulin in a Ca^{2+}-dependent manner (Li et al., 2004; Li et al., 2005). This interaction has yet to be fully elucidated, however, it has been shown that Grb7's interaction with calmodulin induces angiogenesis (Li et al., 2005).

1.8. 14-3-3σ Structure and Function

In humans, the 14-3-3 family consists of seven isotypes (14-3-3β, γ, ε, η, σ, τ and ζ). They all function as homo or heterodimers that interact in signal transduction pathways controlling cell cycle regulation. Most of the 14-3-3 isotypes are expressed in various tissues in the body and function as a cell cycle checkpoint, however, one isotype (14-3-3σ) is a negative cell cycle regulator and is exclusively expressed in epithelial tissue and only functions as a homodimer. P53 up-regulates *14-3-3*σ after the cell undergoes DNA damage or stress through binding to a p53 response element in the *14-3-3*σ promoter (Figure 5) (Hermeking and Benzinger, 2006; Lee and Lozano, 2006). Up-regulation of *14-3-3*σ leads to the sequestering of Cdc2/cyclin complexes and ultimately initiates G2-M cell cycle arrest which suggests that 14-3-3σ is a tumor suppressor protein (Hermeking, 2003).

14-3-3σ has been shown to be downregulated in breast and gastric cancers as well as other by various methods such as CpG methylation, targeted ubiquination and

chrosomal deletion. Besides being frequently downregulated in various cancers, published data from the Muller lab has demonstrated that 14-3-3σ has a role in down regulating ErbB2 expression by sequestering the early growth response-2 (EGR2) transcription factor from the nucleus to the cytoplasm. EGR2 binding to the ErbB2 promoter leads to up-regulation of ErbB2 (Dillon et al., 2007). These results suggest that 14-3-3σ deletion in ErbB2 positive breast tumors is a valid target for study as it is now known to upregulate ErbB2.

Recently published data from the Muller lab has further elucidated the role of 14-3-3σ on cell migration and polarity (Chen et al, 2010). Re-expression of 14-3-3σ in a cell line derived from an ErbB2KI tumor (TM15 cells) showed that 14-3-3σ had little impact on cell proliferation but has a role in the migratory behavior of the cells leading to a decrease in metastasis of the tumor cells. Further, 14-3-3σ also restored adherent and tight junction proteins, E-cadherin and ZO-1, resulting in a decrease of metastasis or cell migration. Additionally, a conditional 14-3-3σ knockout mouse model displayed a loss of epithelial polarity due to a decrease in ZO-1 (tight cell junction) and E-Cadherin (adherent cell junction) expression in the mammary epithelium (Chen et al, 2010).

1.9. Tetracycline Inducible Mouse Models (Grb7 and 14-3-3σ)

Conditional mouse models are an important tool in studying breast cancer. They involve controlling the expression of particular genes by various methods such as the cre/loxp system (knock-in), complete knockout, or the tetracycline inducible system. The Tetracycline inducible system has two variants, the Tet-On system and the Tet-Off system (Albanese et al., 2002; Zhu et al., 2002). In the Tet-On system the inducible gene

is only expressed when doxycycline (a derivative of tetracycline) is administered to the mouse, and in the Tet-Off system the inducible gene is turned off when doxycycline is administered (Albanese et al., 2002; Zhu et al., 2002).

There are two gene components involved in the Tet-on mouse model (Figure 6). One component is the tetracycline dependent transactivator (MTB) which is a fusion protein composed of the viral transactivation protein (VP16) linked to a mutated tetracycline repressor (Albanese et al., 2002; Gunther et al., 2002; Zhu et al., 2002). This mutated Tet-repressor is unable to bind to a Tet-operator in the absence of doxycycline. Once doxycycline is present, the Tet-repressor is able to bind to a Tet-operator where then the transactivator is then in close proximity to the promoter sequence and transcription occurs (Zhu et al., 2002). The second construct uses two Tet-operators upstream of the minimal pCMV promoter followed by the gene of interest (Grb7 or 14-3-3σ) and is followed by an internal ribosomal entry site (IRES) sequence and a green fluorescent protein (GFP) reporter gene. The MMTV promoter used to control the expression of the tetracycline dependent transactivator restricts the expression of Grb7 or 14-3-3σ to the mammary gland (Gunther et al., 2002). This system also allows us to study any developmental effects of overexpressing either protein in the mammary gland.

The MMTV promoter is active around 3 weeks of age in female mice and the focus of my initial research involved developing mice carrying the Grb7 or 14-3-3σ gene in the Tet-responsive constructs and inducing expression of each gene in a developing mammary gland up until 8 weeks of age. Through the addition of doxycycline to the mice drinking water Grb7 or 14-3-3σ expression is upregulated in the mammary epithelium.

My research clearly showed that over-expressing Grb7 or 14-3-3σ in the mammary gland leads to a ductal outgrowth defect.

EXPERIMENTAL RATIONALE

ErbB2 positive breast tumors frequently manifest one of two significant genetic changes; (a) gain of function mutations involving cell growth division and cell cycle, i.e Grb7 may function as a positive regulator of cell growth through its interaction with ErbB2, (b) loss of function mutations in tumor suppression genes involved in preventing unrestrained cellular growth, i.e the negative cell cycle regulation 14-3-3σ.

The use of transgenic mouse models that take advantage of the tightly controlled Tet inducible system allows the expression of genes of interest directly in the mammary epithelium allowing the elucidation of their function within a variety of aspects of mammary gland development and more specifically ErbB2 tumorigenesis.

In order to determine the roles of Grb7 and 14-3-3σ in mammary gland development we developed and characterized a tetracycline inducible mouse model to discern the role of both Grb7 and 14-3-3σ in mammary gland development. This model will provide the basic understanding of the roles of these genes in an individual sense during mammary gland development, thereby allowing the study of their role in the interactive signaling aspects of ErbB2 mediated tumorigenesis.

Through this approach we expect to confirm successful overexpression of each gene in the mouse mammary epithelium. We anticipate, based on current knowledge of protein function that Grb7 will increase ductal outgrowth during mammary gland development, whereas 14-3-3σ, being a negative cell cycle regulator will have an opposing effect and lead to decreased ductal outgrowth.

2. MATERIALS AND METHODS

2.1 Generation of TR-Grb7 and TR-Sfn Mice

Plasmids were linearized for microinjection into FVB embryos by restriction enzyme digest. Linearization removes as much of the bacterial DNA sequence as possible without cutting at the immediate start of the promoter or the end of the SV40. The TR-Grb7 construct was digested with PvuI restriction enzyme and the TR-Sfn construct was digested with PvuII restriction enzyme. After digestion, the fragment was electrophoresed on a 0.8% agarose gel containing ethidium bromide. The band containing the fragment was excised from the gel and gel purified using the Qiagen Gel Pur kit according to manufacturer's protocol. The DNA was not precipitated with Ethanol or purified with Phenol:Chloroform as these chemicals are lethal to the embryos. Exposure to UV light was kept to a minimum so as to prevent DNA damage. Purified sample was resuspended in 0.22um filter sterilized 1XTE buffer pH 8. 20ug of each linearized plasmid was submitted to the McGill Transgenic Mouse Facility for pronuclear injection into fertilized mouse eggs derived from FVB strain. Founder animals were determined by genotyping for the EGFP reporter gene and subsequently crossed with the MTB line. 8 week old females carrying both transgenes were induced with doxycycline and expressing lines were determined by GFP detection in the mammary gland and western blot analysis. The mice with the highest measured expression of Grb7 and Sfn were maintained and used for all experiments.

2.2 Mouse Colony Maintenance

For simplicity, males carrying both transgenes (MTB/TR-Grb7-3 and MTB/TR-Sfn-3) were mated with FVB females. Pups were tagged and tailed at 2 weeks of age and female positive carriers of both transgenes were determined through genotyping from tail DNA. At 3 weeks of age the pups were weaned and positive females were induced by doxycycline (2mg/ml) and control mice were either induced or given untreated water. At the end of the induction period female mice were euthanized and necropsied.

2.3 Genomic DNA Extractions from Mouse Tails

A tail size 0.5-1cm was digested in 500uL of tail buffer (10mM Tris pH8.0, 100mM NaCl, 10mM EDTA pH8.0, 0.5% SDS) containing 10uL of 20mg/mL Proteinase K and incubated at 55˚C overnight. After incubation the tubes were vortexed and 500uL of 50:50 Phenol:Chloroform was added and agitated to extract the DNA from the organic layer. Samples were centrifuged for 10 minutes at 13.2 rpm to separate the organic and DNA layer. The top layer was carefully removed and transferred to a clean eppendorf tube where 1 mL of 100% ethanol was added to precipitate genomic DNA. The sample was gently mixed by hand and the tube was centrifuged at 4˚C for 15 minutes at 13.2 rpm. The ethanol was carefully aspirated off and the pellet was air dried for 10-15 minutes before the DNA was resuspended in 200uL of sterile 1X TE buffer (100mM Tris pH8.0, 10mM EDTA pH8.0).

2.4. Doxycycline Administration

All preparations of doxycycline were performed in light protecting bottles to prevent UV degradation of the drug. To make the 100X doxycycline stock doxycycline powder (Sigma) was dissolved in ddH_2O to a final concentration of 20mg/ml and then sterilized through a 0.22um filter. 1mL aliquots of the 100x stock were kept at -20˚ C in a light protecting box. To prepare drinkable water for the mice, 100mL of cold tap water was sterilized through a 0.22um filter and 1mL of 100X doxycycline stock was added in a light protecting bottle. The water was thoroughly mixed for 1 minute and then added to a sterile red drinking bottle. Dox water was changed every 3-4 days or when needed to ensure that the potency of the drug is not altered by the room temperature conditions in the mouse room. Induced mice are only provided with dox water and were not attached to the automatic watering system to ensure that the Tet-on system was constantly induced. Water levels were checked every day for leakage and to ensure sufficient water was available to the mice.

2.5. Mammary Gland Wholemounts

Transgenic mice were euthanized prior to necropsy under the guidelines of the Canadian Council for Animal Care (CCAC). The number 4 mammary glands of the virgin females were excised and mounted onto a glass slide (Figure 7). The mammary gland was placed in acetone for 2 days or more to remove the fat from the gland. The mammary gland was then dried and placed in Harris Modified Hematoxylin with acetic acid (Fisher Scientific) overnight. A 70% ethanol and 1% HCl solution was used to de-stain the mammary gland until the ducts were visible in the fat pad. Slides were transferred to 70% ethanol for 30

minutes and then 100% ethanol for 30 minutes to dehydrate the mammary gland. Samples were then placed in xylenes for no more than 2 days. Slides were dried and mounted under a coverslip using Permount (Fisher Scientific) and left to dry for 2 days.

2.6. Protein Extraction from Mouse Tissue

The number 3 mammary glands were excised out from the animal, flash frozen in liquid nitrogen and placed in a cryovial to be stored in liquid nitrogen until extraction (Figure 7). Frozen tissues were ground into a powder in liquid nitrogen using a chilled mortar and pestle and lysed for 15 minutes on ice using modified TNE lysis buffer containing protease inhibitors (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40 [NP-40], 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1mM Na_3VO_4, 10mg/mL aprotinin, 10ug/mL leupeptin). Lysates were cleared by centrifugation for 10 minutes at 4˚C and the protein concentration was determined by Bradford assay (Bio-Rad) using manufacturer's protocols.

2.7. Western Blotting

Samples were resuspended in 6X SDS-PAGE loading buffer and denatured by boiling for 10 minutes. Samples were loaded and separated by 12% polyacrylamide gels at 100 volts. The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane using a wet transfer (Bio-Rad) with transfer buffer (25mM Tris, 190mM Glycine, 20% Methanol) for 90 minutes at 60 volts. Membranes were blocked in 3% milk in TBS-T (20mM Tris-HCl [pH 8.0], 0.1% Tween) at 4˚C with gentle agitation overnight. After blocking, the membranes were incubated with primary antibody prepared in 3%

milk for 2 hours at room temperature and then washed 5 times with TBS-T. The appropriate horseradish perioxidase (HRP) linked secondary antibody was added to the membrane for 1 hour at room temperature with agitation and then washed 5 times with TBS-T. The membrane was then incubated in enhanced chemiluminescence (ECL, Amersham) reagent and exposed onto a film (Kodak Biomax Light), following manufacturer's protocols.

3. RESULTS

3.1 Tet-On constructs are functional in 293T cells

A tissue culture expression system was used to test the DNA constructs prior to making a mouse model to ensure functionality. To do this, the Tet-responsive plasmid carrying either the TR-Grb7 or TR-14-3-3σ gene (both are mouse sequences) and the tetracycline dependent transactivator plasmid (MTB) were transfected using FuGene into 293T cells. Control cells held either one gene or transfected with an empty pcDNA construct. Doxycycline was added to the cell culture dishes 24 hours after transfection, and induction lasted for 24 hours. Cells were lysed using modified TNE lysis buffer and run on a SDS-PAGE gel and blotted for Grb7 or 14-3-3σ. As expected, Grb7 expression was upregulated in the cells containing both the Tet-responsive DNA and the transactivator DNA, and Grb7 was not detected in the control cells (Figure8B). 14-3-3σ, however, was undetectable in the 293T cells and the cells were not growing as fast as the Grb7 transfected cells. This is likely due to 14-3-3σ role in negative cell cycle control and the cells overexpressing 14-3-3σ were dying. In order to determine successful expression of the 14-3-3σ a fluorescent approach was undertaken to measure the level of GFP fluorescence, as a GFP reporter gene is simultaneously expressed with 14-3-3σ. GFP expression was clearly detected in cells transfected with the 14-3-3σ gene (Figure 8A)

These results demonstrate the Tet-responsive constructs holding either Grb7 or 14-3-3σ are successfully expressed in mammalian tissue. These constructs were sequenced to ensure that no mutations are present in either gene and are suitable for injection into FVB mouse embryos.

3.2 Determining Positive Founder Lines

Linearized Tet-responsive (TR) constructs (TR-Grb7 or TR-Sfn) were introduced into FVB mouse embryos and tagging and tailing of pups born from this procedure was carried out to determine the presence of the transgene. In total, 22 mice successfully carried the Tet-responsive 14-3-3σ (TR-Sfn) gene and 12 mice carried the Tet-responsive Grb7 (TR-Grb7) gene. After this was determined, 4 mice from each set were crossed with the MTB mice and resulting females carrying both transgenes were induced with doxycycline for expression of each gene for 3 days at 6 weeks of age.

To determine if the transgene was functional in the mouse strain, fluorescent microscopy was used to detect any GFP expression in the mammary gland. Control mice either non-induced or carrying one trangene were also used. For each strain, 3 mouse strains were confirmed as expressors and used for further experiments (Figure 9). Of the 3 lines maintained it was necessary to determine which line had the highest expression of either transgene. To do this a western blot procedure was employed. It was determined that the TR-Grb7-3 line maintained the highest expression levels of the transgene of the 3 lines. For the 14-3-3σ mouse lines where we could see GFP expression by fluorescent microscope we were unable to see 14-3-3σ expression by western blot. To see if this event was temporary or can be overcome by extending the time that 14-3-3σ is induced I repeated the experiment and induced these mice for 1 week. I was able to detect GFP expression in the mammary gland as shown previously and successfully detected 14-3-3σ expression by western blot, albeit at a lower levels than seen with Grb7 expression using this system. Interestingly the TR-Sfn-3 mouse line has the highest level of 14-3-3σ

expression and it is important to note that all experiments from this point use this mouse strain (Figure 9).

3.3 Characterization of Mammary Gland Development

As mentioned before, after creating a new mouse strain it is important to characterize any developmental effects that may occur in these specific strains. This will allow a baseline phenotype for each mouse strain when the construct is induced at an early age. All mice are induced at 3 weeks of age and were assessed at weekly time points starting at 4 weeks of age and the final set at 8 weeks of age. Mammary gland development is complete between 8 weeks of age to 12 weeks and determined by the absence of terminal end buds and by the length of the mammary ducts filling the mammary fat pad past the lymph node (Figure 10B). Ductal outgrowth in the mammary gland is highly variable within individual mice and also between littermates. To control for this variation both number 4 mammary glands were excised out of each mouse and an average was taken between the two glands. Then this average was added to the other experimental mice and an average was taken between those 3 mice. The same procedure was performed for the littermate controls.

3.3.1 TR-Grb7-3 Characterization

Overexpression of Grb7 in a developing mammary gland leads to a ductal outgrowth defect up until 8 weeks of age. Doxycycline induced overexpression starts at 3 weeks of age in female mice containing the MTB and TR-Grb7 transgenes as well as littermate controls. Figure 10A clearly shows that female mice that contain both transgenes (MTB

and TR-Grb7-3) have a ductal out growth defect up until 8 weeks of age. At 8 weeks of age the p-value for this defect is statistically significant and the mammary ducts in the induced glands are about 6mm shorter compared to the control mammary glands. Weeks leading up to the 8 week time point clearly follow the same trend and are significantly shorter than the control mice. Figure 10C clearly shows that Grb7 is overexpressed in female mice containing both MTB and TR-Grb7-3 trangenes and the littermate controls do not express Grb7. E-Cadherin, an epithelial marker, was used as the loading control in the western blot as the mammary gland extract contains a lot of stromal tissue and the levels of Grb7 might not be representative of how much Grb7 is actually expressed in the mammary epithelium.

3.3.2 TR-Sfn-3 Characterization

Overexpression of 14-3-3σ in a developing mammary gland leads to a ductal outgrowth defect up until 8 weeks of age. Doxycycline induced overexpression starts at 3 weeks of age in female mice containing the MTB and TR-Sfn-3 transgenes as well as littermate controls. Figure 11A clearly shows that female mice that contain both transgenes (MTB and TR-Sfn) have a ductal out growth defect up until 8 weeks of age. It appears that at 8 weeks of age the experimental set of mice are possibly overcoming the ductal outgrowth defect seen in the earlier weekly time points as displayed with the error bars overlapping at the 8 week time point. Weeks leading up to the 8 week time point clearly follow the same trend and are significantly shorter than the control mice. Western Blot analysis on mammary gland extracts show that 14-3-3σ is overexpressed in female mice containing both MTB and TR-Sfn trangenes and the littermate controls do not express Sfn Figure

11B. The positive control, MCF10A cell line, shows exactly where 14-3-3σ runs on a gels and the antibody is much cleaner with the sample is pure epithelial tissue. E-Cadherin, an epithelial marker, was used as the loading control in the western blot and it shows that there is a clear upregulation of 14-3-3σ in the mammary gland.

4. DISCUSSION

The aim of this project was to determine the role of Grb7 and 14-3-3σ during ErbB2 tumorigenesis. To do this I developed 2 transgenic Tet-On Grb7 and 14-3-3σ mouse models that overexpress these proteins when given doxycycline. Characterization of mammary gland development in these mouse models has shown that overexpression of Grb7 or 14-3-3σ in the mammary leads to a ductal outgrowth defect up to 8 weeks of age. Grb7 is often amplified and overexpressed in ErbB2 tumors and with a functional Tet-On Grb7 mouse model we can determine if Grb7 overexpression in a mouse mammary gland can accelerate ErbB2 tumorigenesis. 14-3-3σ is often downregulated or deleted in ErbB2 tumors and with a functional Tet-On 14-3-3σ mouse model we can overexpress 14-3-3σ in an established ErbB2 tumor.

4.1. TR-Grb7-3 Future Directions

ErbB2 and Grb7 are amplified and overexpressed in approximately 85% of ErbB2 tumors (Andrechek et al., 2003; Hodgson et al., 2005; Stein et al., 1994). Grb7 is an adaptor protein shown to have a role in cell polarity and possibly could downregulate 14-3-3σ expression. Grb7's interaction with FAK has also shown its role in regulating normal cell migration (Han et al., 2000; Han et al., 2001; Shen and Guan, 2001). Normally Grb7 is not expressed in the mammary epithelium making its overexpression in ErbB2 mammary tumors an interesting and important target to study.

My research has shown that when Grb7 is overexpressed in a developing mouse mammary gland this leads to a ductal outgrowth defect. My original hypothesis was that overexpression of Grb7 in these mice would lead to an increase or no change in ductal

outgrowth in the mammary gland. These results, however unexpected may be due to the fact that there was not sufficient growth factor stimulation proportional to the amount of Grb7 present in the mammary epithelium cells. It is possible that for Grb7 to have its full effect on the growth and migration of epithelial cell it may require the presence of an equal or higher copy number of its interacting partners ErbB2 and ErbB3. Adaptor proteins like Grb7 on their own have no intrinsic enzymatic activity and only function as mediators that link other proteins to their final target. This could explain why overexpression of Grb7 in the mammary gland does not lead to an increase of ductal outgrowth, but rather leads to a ductal outgrowth defect. It is possible that overexpression of an adaptor protein simply disrupts normal signal transduction events that occur during development.

4.1.1. Ductal Outgrowth Defect

My work has looked at the developmental effect of overexpressing Grb7 in the mammary gland up until 8 weeks of age. To see if this ductal outgrowth defect can be overcome the experiment will be continued up to 12 weeks of age. Sections of the mammary glands leading up to 8 weeks of age may display morphological variations in the Grb7 overexpressed glands. Sections of 7 week old Grb7 induced glands and littermate control cells have preliminarily suggested that in the Grb7 overexpressed mammary glands, the terminal end buds are abnormally formed compared to the littermate control mice. The terminal end buds appear enlarged and contain multiple layers (data not shown). This preliminary result has not been quantified and more sections from the all weekly time points need to be investigated before this can be confirmed. If this result is true, it may

suggest that prolonged overexpression of Grb7 may lead to an abnormal terminal end bud formation and possibly leading to a hyperplasia in these glands.

Mammary gland epithelial cells isolated from 8 week old non-induced MTB/TR-Grb7-3 female mice will be used to do some migration and invasion assays. By inducing one set of cells containing both transgenes and using a set of non-induced cells as the control we will be able to see if overexpressing Grb7 in the mammary epithelium alters the ability of these cells to migrate or invade like normal epithelial cells.

4.1.2. Lactation Studies

The results from the developmental effects of overexpressing Grb7 the mammary gland leads to a ductal outgrowth defect in these mice. To further investigate the effect this has on the mammary gland it will be important to do a lactation study on these mice to see if overexpression of Grb7 impairs the females from sustaining their young after birth. To do this a female containing both transgenes MTB and TR-Grb7-3 will be given doxycycline water starting from 3 weeks of age and continued throughout her pregnancy and after birth. Doxycycline has been administered to pregnant female mice before and does not alter mammary gland development, lactation or involution after weaning (Gunther et al., 2002). Time points during pregnancy can give us insight as to whether or not the overexpression of Grb7 inhibits ductal branching that occurs before birth so that the gland is prepared for lactation postpartum. My hypothesis is that overexpression of Grb7 will not affect normal lactation or involution after weaning in these mice.

4.1.3. Effects of Overexpressing Grb7 in the ErbB2KI Mouse Model

The most important experiment using the TR-Grb7-3 mice is the cross between these mice with the ErbB2KI mouse model. Normally in the ErbB2KI mouse model tumors arise at 16 months of age and these resulting tumors have similar genetic and molecular markers like the human ErbB2 positive breast tumors (Andrechek et al., 2000; Andrechek et al., 2003; Hodgson et al., 2005). I propose that we overexpress Grb7 in these mice starting at 3 weeks of age and continue doxycycline induction until a palpable tumor is detected. My hypothesis is that the early overexpression of Grb7 will decrease tumorigenesis in this model. It will also be important to see if the amplicon is reduced by the induced overexpression of Grb7 in the mammary epithelium.

4.2. TR-Sfn-3 Future Directions

14-3-3σ on the other hand is deleted from a large percent of ErbB2 tumors and has been shown to be downregulated in other various cancers. Upregulation of 14-3-3σ occurs after cell damage by the well known tumor suppressor protein p53. 14-3-3σ deletion and downregulation in ErbB2 tumors is of high interest due to its role in negative cell cycle regulation and its role in negatively controlling ErbB2 expression at the transcription level.

As expected overexpression of 14-3-3σ in a developing mammary gland leads to a ductal outgrowth defect. The reason 14-3-3σ may not be detected by western blot but GFP expression can be seen in the same cells may suggest that when 14-3-3σ is upregulated in cells, especially using a system such as the Tet-on system where the

protein is grossly overexpressed, the cells respond by downregulating the protein as it is toxic to the cells.

As mentioned before when the Tet-responsive construct was tested in 293T cells the cells expressed 14-3-3σ but we could not detect expression by western blot probably due to a downregulation event. This result further suggests that 14-3-3σ is being downregulated when overexpressed in the mammary epithelium; however this can be overcome by prolonged overexpression.

4.2.1. Ductal Outgrowth Defect

My work has looked at the developmental effect of overexpressing 14-3-3σ in the mammary gland up until 8 weeks of age. To see if this ductal outgrowth defect can be overcome the experiment will be continued up to 12 weeks of age. Mammary gland development can take up to 12 weeks to be completed and I hypothesize that in the TR-Sfn-3 mice this ductal outgrowth defect will be overcome by that age.

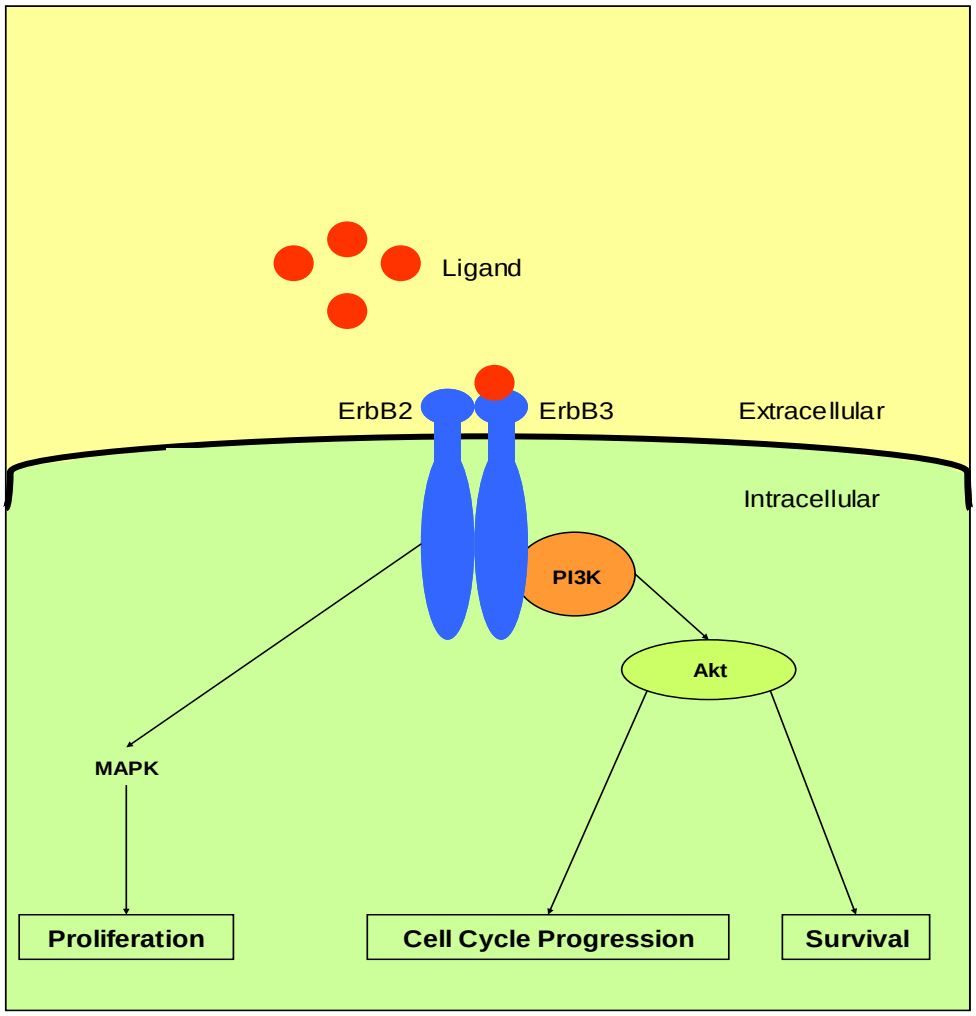
Sections of the mammary glands leading up to 8 weeks of age may display morphological variations in the 14-3-3σ overexpressed glands. Since 14-3-3σ is a negative cell cycle regulator and has been show in various cell lines to increase cell polarity and decrease cell migration it would make sense that the same event is occurring in these mice (data not shown). I would hypothesize that the ductal structures in these mice appear normal but just have a delay in growth and migration into the fat pad.

4.2.3. 14-3-3σ Overexpression in the NDL2-5 Mouse Model

The most important experiment using the TR-Sfn-3 mice is the cross between these mice with the NDL2-5 mouse model. Normally in the NDL2-5 mouse model tumors arise at 6 months of age and these resulting tumors have similar genetic and molecular markers like the human ErbB2 positive breast tumors (Hodgson et al., 2005; Montagna et al., 2002; Siegel et al., 1999). I propose that we overexpress 14-3-3σ in these mice starting at 3 weeks of age and continue doxycycline induction up to 6 months of age. Another important experiment will be to turn on the expression of 14-3-3σ upon detection of a tumor and at various time points during tumorigenesis. My hypothesis is that the early overexpression of 14-3-3σ will delay tumorigenesis in the NDL2-5 model and lung metastasis will also decrease.

5. FIGURES AND TABLES

Figure 1



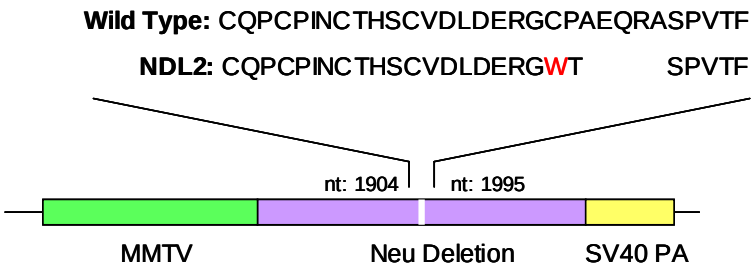
Adapted from: Hynes et al. *Nature Reviews: Cancer* 2005;5:341-54

Figure 1: ErbB2 Signal Transduction Pathways

Ligands such as epidermal growth factor and neuregulins bind to the extracellular region of ErbB1, ErbB3, and ErbB4 but not ErbB2. ErbB2 and ErbB3 dimerization confers the strongest signal transduction potential of any combination. Extracellular ligand binding to ErbB3 leads to activation of the MAPK pathway and the PI3K/AKT pathway.

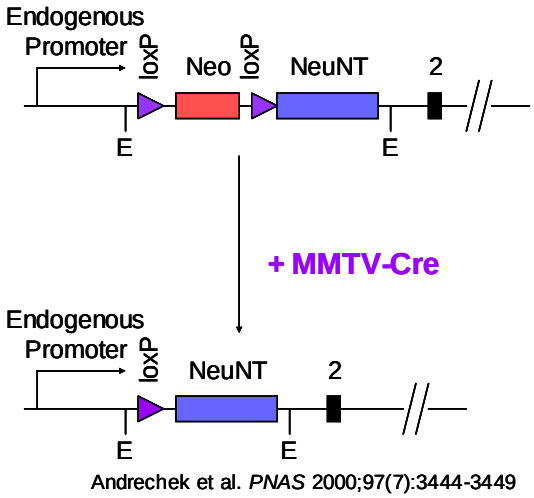
Figure 2

A



Siegel et al. *EMBO J* 1999;18(8):2149-2164

B



Andrechek et al. *PNAS* 2000;97(7):3444-3449

C

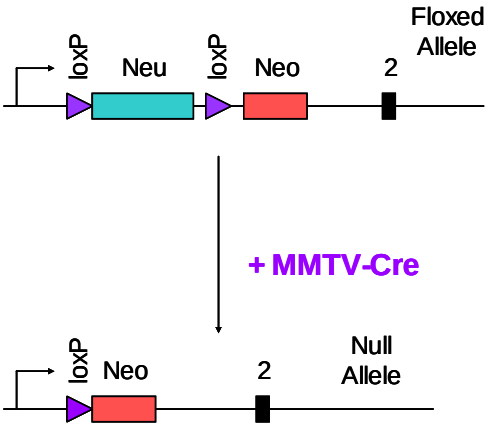
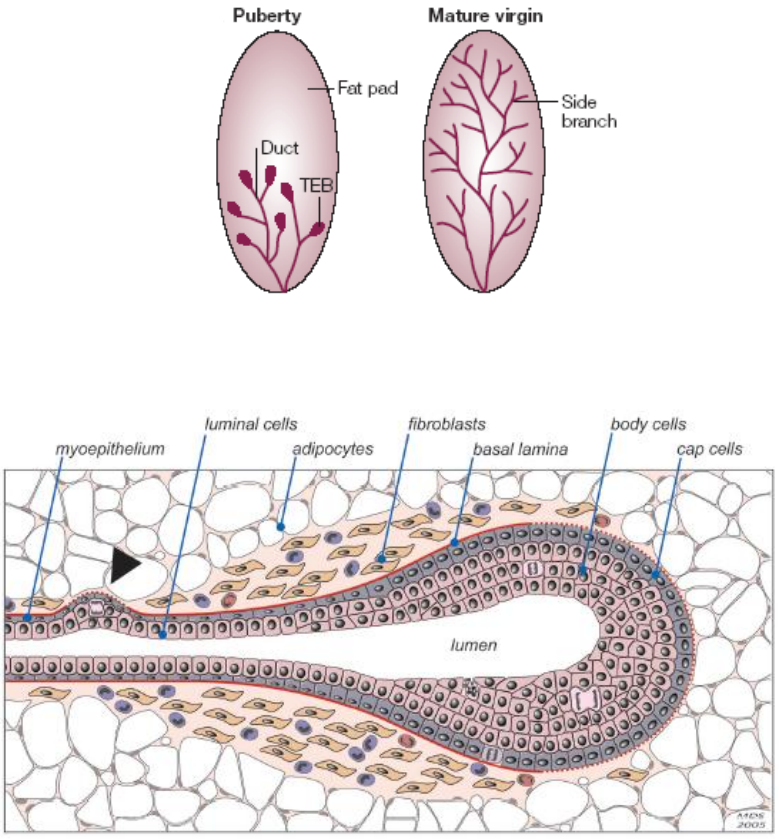


Figure 2: ErbB2 Conditional Mouse Models

A) ErbB2 knock-in conditional mouse model. LoxP sites flank a neomysin stop cassette which is downstream of the ErbB2 endogenous promoter. Expression of Cre-recombinases under the control of the mouse mammary tumor virus (MMTV) promoter leads to the excision of the Neo cassette whereby the ErbB2 endogenous promoter and activated Neu is expressed in the mammary epithelium. **B)** ErbB2 knock-out conditional mouse model. Exon 1 of ErbB2 was replaced by a loxP flanked *Neu* sequence followed by a Neomycin stop cassette. Expression of Cre-recombinases under the control of the MMTV promoter leads to the excision of the *Neu* sequence and places the Neo stop cassette by the endogenous ErbB2 promoter. **C)** *Neu* deletion 2-5 (NDL2-5) mouse model uses the MMTV promoter to drive the expression of the full length *Neu* sequence that has a deletion site… A frame shift mutation in the extracellular cysteine rich region of *Neu* which confers a conformational change and allows for constitutive dimerization. The expression of this *Neu* deletion gene is controlled by the MMTV promoter which allows for the expression of *Neu* in the mammary gland.

Figure 3



Adapted from : Sternlicht *Breast Cancer Research* 2006;8(1): 201-11

Figure 3: Mouse Mammary Gland Development

A) Ductal outgrowth and branching starts around 3 weeks of age and is characteristic of the terminal end buds (TEB) formed at the leading edge of the ducts. **A)** In a mature virgin mammary gland once the TEB's reach the edge of the mammary gland the TEB's disappear. **B)** TEB structure consists of body cells at the leading structure and the hollow lumen is formed by the body cells undergoing apoptosis.

Figure 4

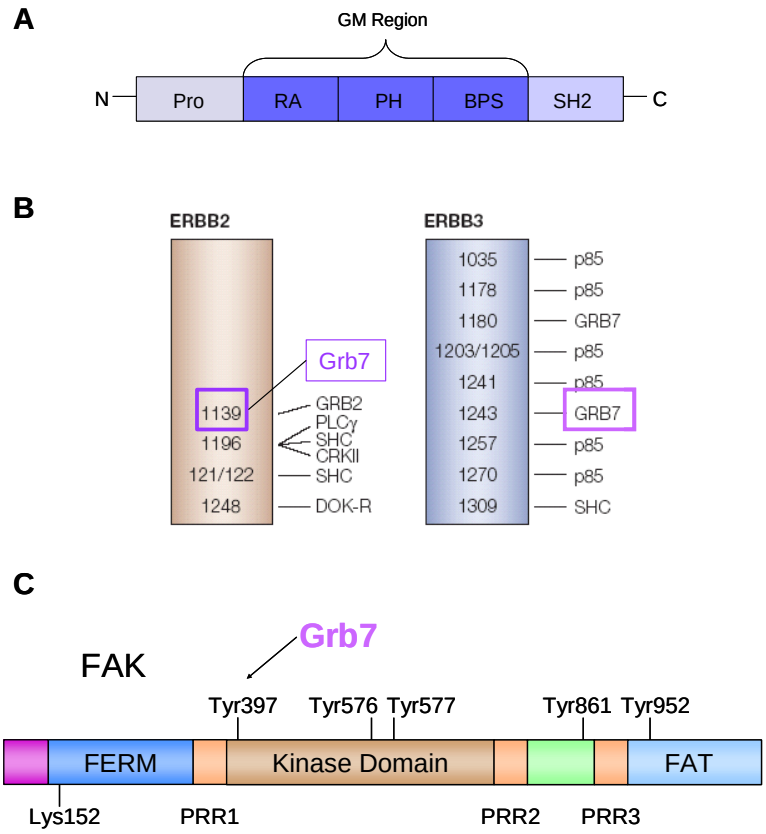
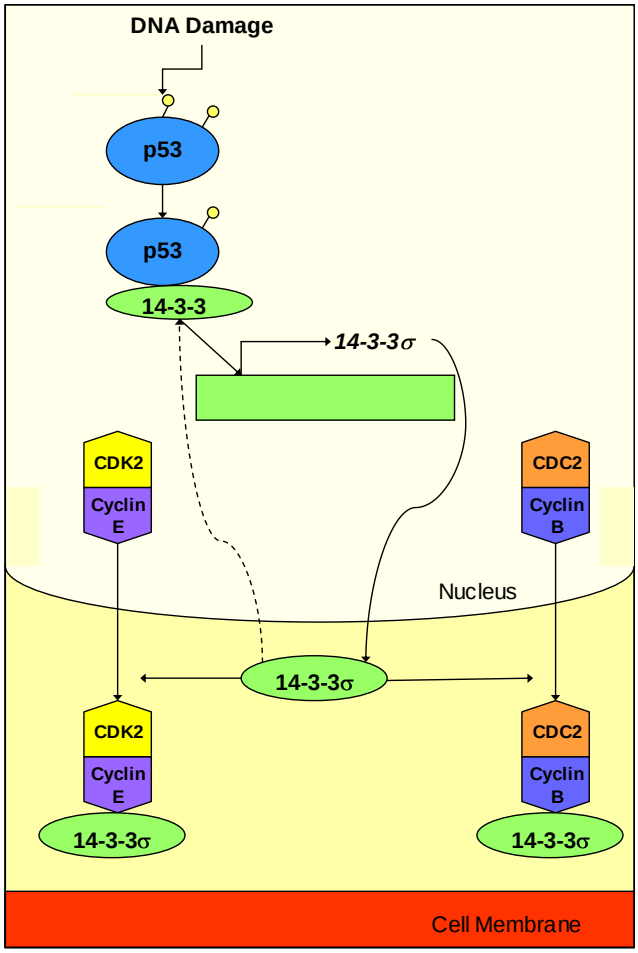


Figure 4: Grb7 structure and binding sites on ErbB2, ErbB3 and FAK

A) Grb7 consists of a proline rich region (Pro) followed by a central Grb and Mig section (GM) and ending with an SH2 domain. The central GM region has a high homology to the C. elegans protein Mig10 and contains a Ras associating domain (RA), a pleckstrin homology domain (PH) and a between the pleckstrin and SH2 domain region (BPS). **B)** Grb7's SH2 domain binds with specificity to ErbB2 at AA site 1139 which overlaps with the Grb2 binding site, and Grb7 binds to ErbB3 at AA sites 1180 and 1243. **C)** Grb7's binding site on FAK (focal adhesion kinase) at tyrosine 397.

Figure 5



Adapted from: Hermeking, Nature Reviews 2003

Figure 5: The negative cell cycle regulator protein 14-3-3s is up regulated by p53

Upon DNA damage, p53 dephosphorylates once and is then able to bind 14-3-3 isoforms (γ, ε, τ, σ). This construct then binds to a p53 binding element located in the promoter region of 14-3-3σ and upregulates its expression. 14-3-3σ then translocates to the cytoplasm and sequesters CDK2/Cyclin E to cause G1 cell cycle arrest or CDC2/Cyclin B to cause G2 cell cycle arrest.

Figure 6

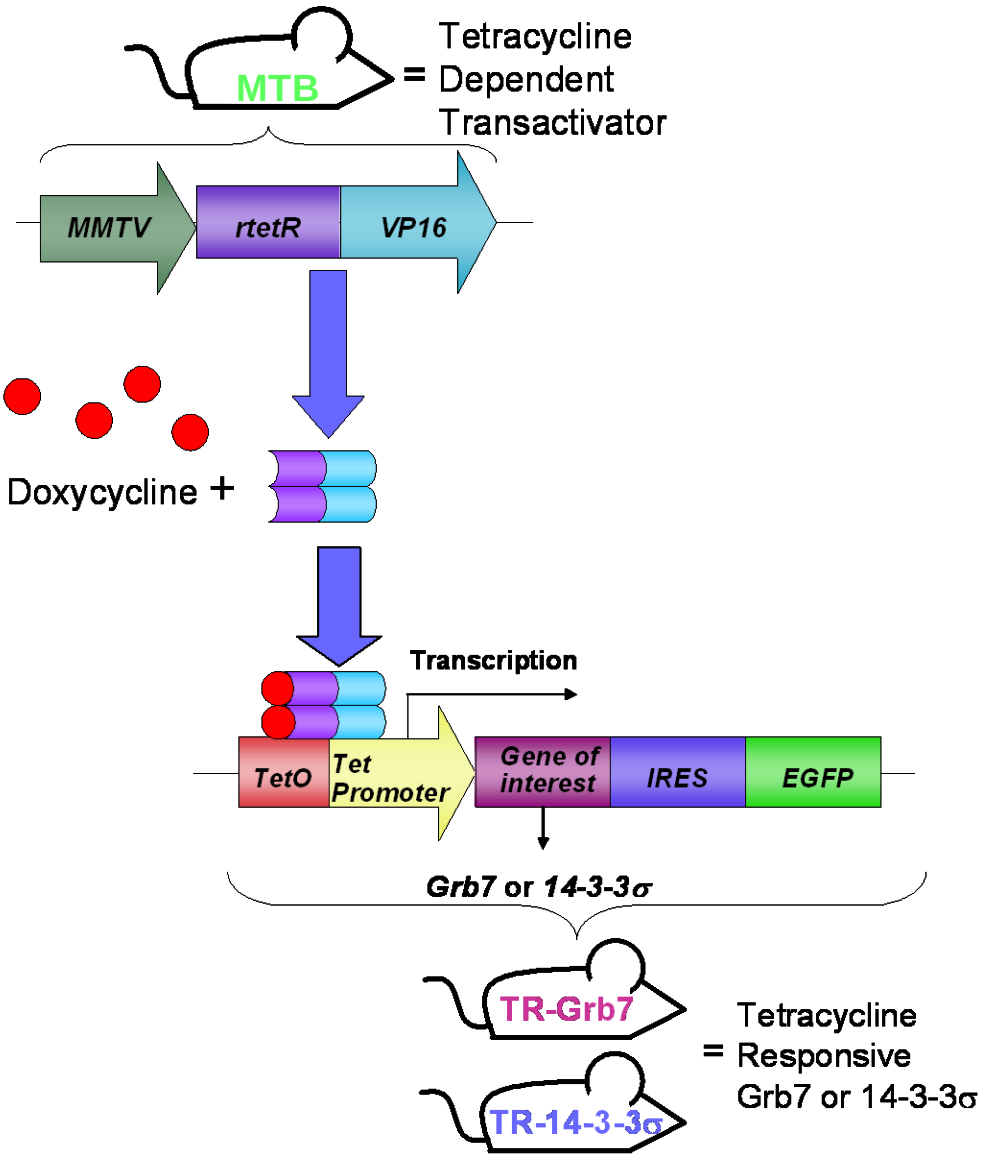

MTB
= Tetracycline Dependent Transactivator
MMTV
rtetR
VP16
Doxycycline +
Transcription
TetO
Tet Promoter
Gene of interest
IRES
EGFP
Grb7 or 14-3-3σ
TR-Grb7
TR-14-3-3σ
= Tetracycline Responsive Grb7 or 14-3-3σ

Figure 6: Tet-On mouse models

The tetracycline dependent transactivator transgene is located downstream from the mouse mammary tumor virus promoter (MMTV) which is only expressed in the mammary gland. The MTB mouse expresses a fusion protein which consists of a mutated Tet-repressor that is unable to bind to a Tet-operator in the absence of doxycycline or tetracycline. Linked to this Tet-repressor is a viral protein (VP16) that is a transactivator. When doxycycline or tetracycline is administered to the mouse this transactivator is able to bind to a Tet-operator. The second transgene consists of 2 tet-operators located upstream of the minimal pCMV promoter. Once the transactivator is bound to doxycycline or tetracycline, Grb7 or 14-3-3σ is expressed and the reporter gene EGFP located after an internal ribosomal entry sequence (IRES) is also expressed in the mammary gland.

Figure 7

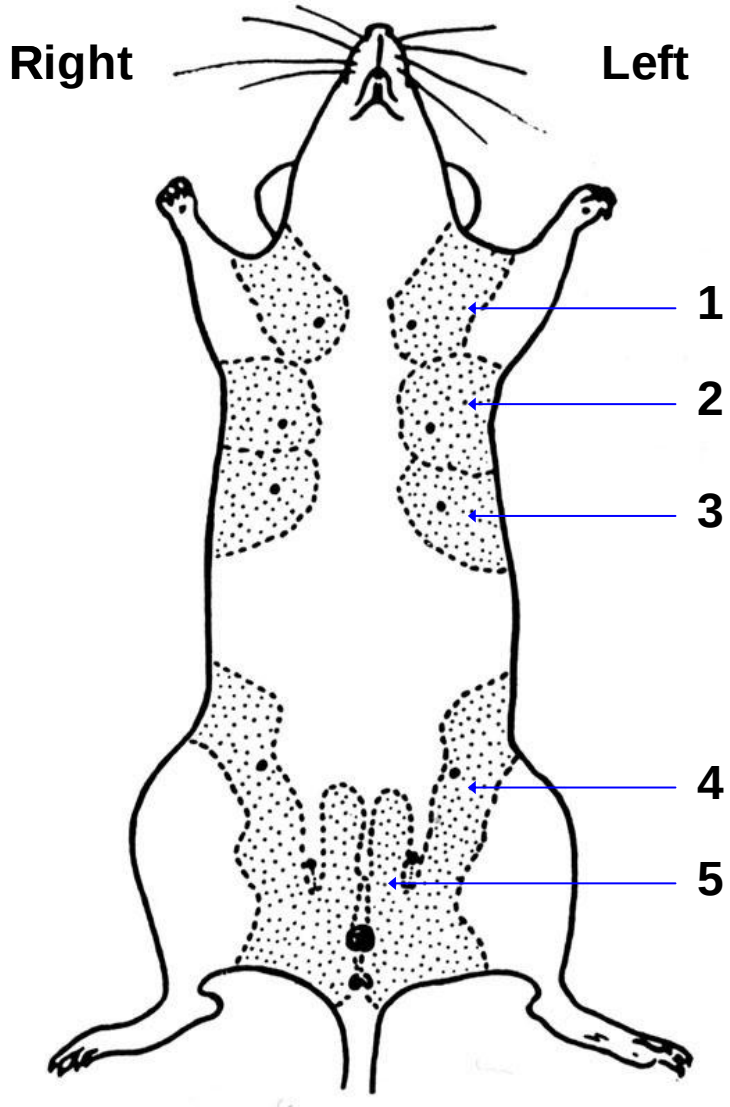
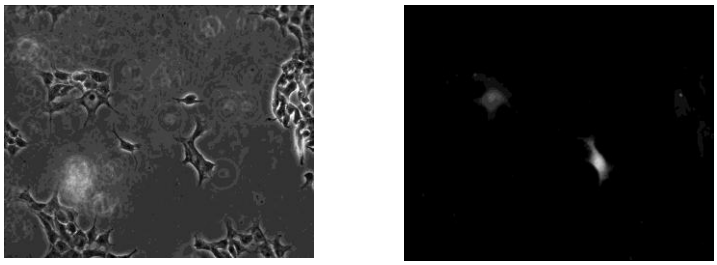


Figure 7: Mouse Mammary Gland Positions

Diagrammatic representation of mouse mammary gland positions.

Figure 8

A



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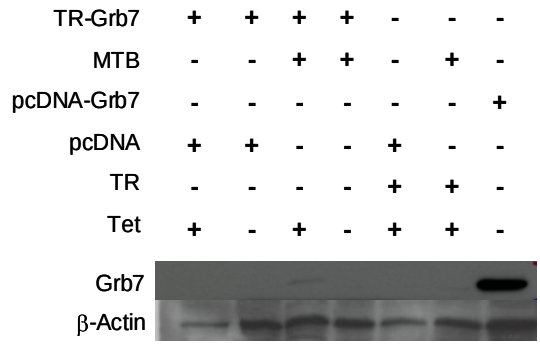

Figure 8: Tet-On Grb7 and Tet-On Sfn constructs are functional in 293T cells

The tetracycline dependent transactivator construct (MTB) and the TR-Grb7 (tetracycline responsive Grb7) or TR-Sfn (tetracycline responsive 14-3-3σ) construct was transfected into 293T cells. Tetracycline was administered 24 hours after transfection and cells were collected 24 hours later. **A)** GFP was detected in 293T cells transfected with the TR-Sfn construct. **B)** Western blot analysis on cell lysates show upregulation of Grb7 in tetracycline induced 293T cells containing the MTB and TR-Grb7 constructs. pcDNA-Grb7 transfected 293T cells were the positive control and β-actin was used as the loading control.

Figure 9

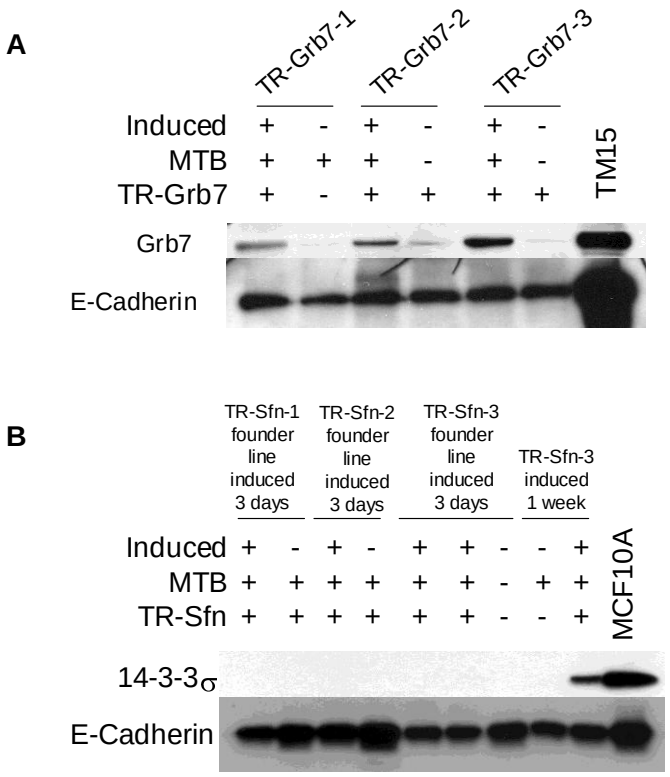

Figure 9: Determining positive founder lines

A) 8 week old female MTB/TR-Grb7 positive mice and littermate controls were induced with dox for 3 days. Western blot analysis on the number 3 mammary glands shows upregulation of Grb7 in the mammary epithelium and the TR-Grb7-3 line having the highest level of expression compared to the littermate controls. TM15 lysate is from an ErbB2KI tumor cell line known to overexpress Grb7 and used as a positive control. **B)** 8 week old female MTB/TR-Sfn positive mice and littermate controls were induced with dox for 3 days and one set was induced for 1 week. Western blot analysis on the number

3 mammary glands shows upregulation of 14-3-3σ for the 1 week induction but not for the 3 day induction. MCF10A cell line was used as a positive control for 14-3-3σ.

Figure 10

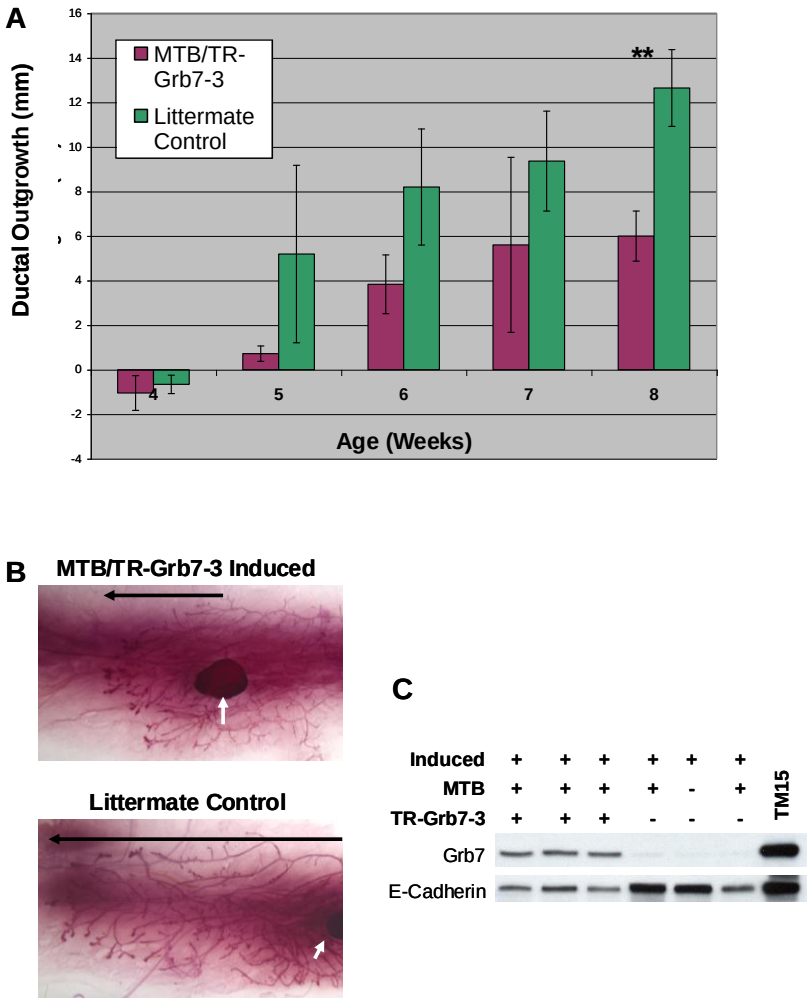

Figure 10: MTB/TR-Grb7-3 characterization

All mice are induced with 2mg/mL Doxycycline administered in drinking water starting at 3 weeks of age and ductal outgrowth was measured between 4-8 weeks of age. Each bar consists of measurements collected from 3 female mice. **(A).** Ductal outgrowth measured from midpoint of the Lymph Node (white arrow) into the fat pad (black arrow) **(B).** Western Blot of mammary gland lysates showing Grb7 expression in 8 week old dox

induced females and their littermate controls. TM15 cells were used as a Grb7 positive control and E-Cadherin shows the level of epithelial cells in each tissue lysate sample.

Figure 11

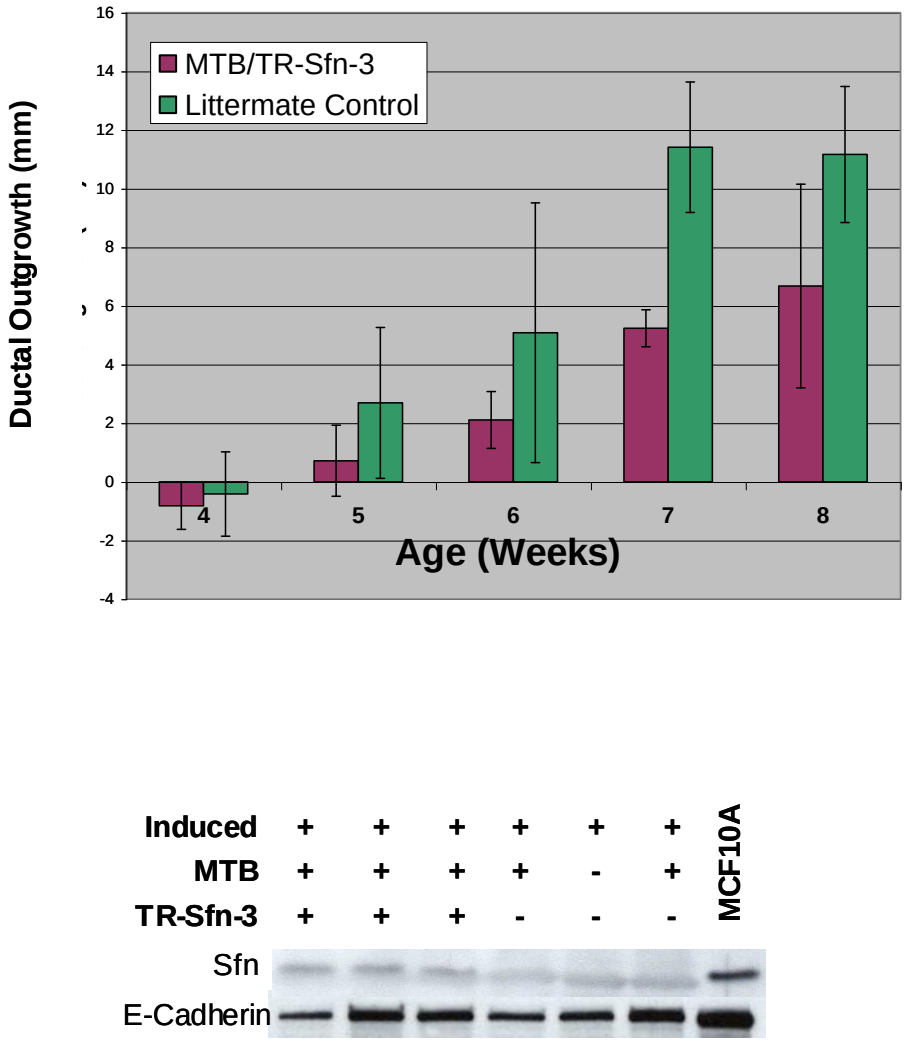

Figure 11: MTB/TR-Sfn-3 characterization

A) All mice are induced with 2mg/mL Doxycycline administered in drinking water starting at 3 weeks of age and ductal outgrowth was measured between 4-8 weeks of age. Each bar consists of measurements collected from 3 female mice. **B)** Western Blot of mammary gland lysates showing 14-3-3σ expression in 8 week old do induced females

and their littermate controls. MCF10A cells were used as the 14-3-3σ positive control and E-Cadherin shows the level of epithelial cells in each tissue lysate sample.

Table 1: ErbB2 Amplicon

Mouse gene	Human gene	Description
Neurod2	*NEUROD2*	Transcription factor; neuronal differentiation
Ppp1r1b	*PPP1R1B*	Protein phosphatase
Stard3	*STARD3*	STAR-related lipid transfer; sterol transport
Tcap	*TCAP* (*MLN64*)	Telethonin; sarcomere assembly
Pnmt	*PNMT*	Phenylethanolamine *N*-methyltransferase
BC030368	*PERLD1* (*MGC9753*)	Perl-like domain containing protein
Erbb2	*ERBB2*	Oncogenic RTK
1810046J19Rik	*C17orf37* (*MGC14832*)	Open reading frame of unknown function
Grb7	*GRB7*	Growth factor receptor bound protein 7
AF001293	*ZNFN1A3*	Zinc finger transcription factor
1700017D11Rik	*ZPBP2*	Zona pellucida binding protein
Ormdl3	*ORMDL3*	Orosmucoid (ORM1) like; plasma drug transport

Genes in bold are also amplified in human ErbB2 positive breast tumors

Table 2: Genes contained in the minimal chromosomal 4 deletion site

Gene	Function
Ythdf2	Unknown
LOC664903	Unknown
Gmeb1	Inhibition of caspase-induced apoptosis
Rnu11	Unknown
Taf12	General RNA polymerase II transcription factor activity
9530096D07Rik	Unknown
Rcc1	Detecting unreplicated DNA and inhibiting mitotic activation
Rnu17d	Unknown
Phactr4	Unknown
Med18	Stimulating basal RNA polymerase II transcription and enables transcriptional regulation
Sesn2	Involved in regulation of cell viability in response to different stress conditions
Atpif1	Inhibition of the mitochondrial ATPase
Eya3	Unknown
Ppp1r8	Type 1 serine/threonine specific protein phosphatase inhibitor activity
Stx12	Involvement in endocytic trafficking and neurite outgrowth
BC008163	Unknown
LOC433762	Unknown
Gpr3	Maintenance of meiotic arrest in mouse oocytes
Cd164l2	Unknown
Map3k6	Weak JNK pathway activation
Wdtc1	Unknown
4732473B16Rik	Unknown
2300002D11Rik	Unknown
Gpatc3	Unknown
Sfn	Negative regulator of cell cycle; Stabilizing p53 and p21; Anit-apoptosis; Involved in skin development
Zdhhc18	Unknown
LOC545683	Unknown
Arid1a	Nucleosome remodeling activity associated with transcriptional regulation
Rps6ka1	Inhibiting neuronal nitric oxide synthase
Lin28	Involved in early developmental timing

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