INVESTIGATION OF THE ACTION OF PHOSPHATASE OF REGENERATING LIVER ON PTEN USING MURINE MODELS

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INVESTIGATION OF THE ACTION OF PHOSPHATASE OF REGENERATING LIVER ON PTEN USING MURINE MODELS

The addition and removal of phosphate groups is a key regulatory mechanism for many cellular processes. The balance between phosphorylation and dephosphorylation is delicate and must be maintained in order for proper cell functions to be carried out. Protein kinases and phosphatases are the keepers of this balance with kinases adding phosphate groups and phosphatases removing them. As such, mutation and/or altered regulation of these proteins can be the driving factor in disease. Phosphatase of Regenerating Liver (PRL) is a family novel of three dual specificity phosphatases (DSPs) first discovered in the regenerating liver tissue of rats. PRLs have also been shown to act as oncogenes in cell culture and in animal models. However, the physiological substrate and mechanisms of the PRLs are not yet known. Recently, our lab has developed a PRL 2 knockout mouse and found several striking phenotypes all of which correspond to a significant increase in PTEN. We also found that PRL 2 is targetable by small molecular inhibitors that can potentially be used to disrupt tumor growth and spermatogenesis. Furthermore, a PTEN heterozygous mouse model crossed into our PRL 2 knockout line was generated to investigate the relevance of PRL interaction with PTEN in cancer.

Zhong-Yin Zhang, Ph.D.,Chair

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CHAPTER 1: Introduction

1.1 Phosphorylation

Phosphorylation is the addition and removal of phosphate groups to organic molecules in cells (9, 24, 62, 78). This evolutionarily conserved process is used as a regulatory mechanism for many cellular pathways (24, 62, 78). The reaction itself is covalent, reversible, and carried out on a variety of substrates by kinases and phosphatases (9, 24, 62, 78). Specifically, kinases add phosphate groups to substrates while phosphatases remove them (Figure 1.) (9, 24, 62, 78). These enzymes predominately phosphorylate/dephosphorylate amino acids on proteins (which this thesis will focus on) although examples of enzymes that phosphorylate/dephosphorylate lipids and nucleic acids exist (9, 24, 62, 78). The amino acids that can be modified are usually serine, threonine, and tyrosine due to their highly reactive hydroxyl groups (9, 24, 62, 78).

1.1.1 Kinases and Phosphatases

Kinases and phosphatases act as contrasting regulatory enzymes for many cellular pathways including metabolism, apoptosis, transcription, cell proliferation, and migration (9, 24, 62, 78). As such, kinases and phosphatases together constitute the largest families of genes in eukaryotic organisms (9, 24, 62, 78). Due to the diversity of the pathways they regulate, kinases and phosphatases are commonly found mutated or otherwise altered in many diseases, including cancer, diabetes, and neurodegenerative disorders, making them tempting targets for drug therapy. For many years, kinases have been the focus of drug discovery research due to the thought that phosphatases were too transient and

promiscuous to target with drugs (9, 24, 62, 78). However, recent studies have shown evidence that phosphatases do have substrate specificity and can, in fact, be targeted by small molecule inhibitors in a specific manner (9, 24, 62, 78). Drug discovery for phosphatases was greatly facilitated by studies that furthered our knowledge of phosphatase catalytic domains and the unique structural properties that allow for specific drug targeting (9, 24, 62, 78). However, catalytic domains tend to be highly conserved within families (9, 24, 62, 78). To increase drug specificity scientists broke new ground by designing novel drugs that target unique structural features near the catalytic domain and regulatory sites (9, 24, 62, 78).

1.1.2 Tyrosine Phosphatases

Protein phosphatases can generally be grouped into two superfamilies: enzymes that dephosphorylate serine/threonine residues (Serine/threonine phosphatases) and enzymes that are specific to tyrosine residues (Protein Tyrosine Phosphatases (PTPs)) (9, 24, 62, 78). A majority of phosphatase genes encode protein tyrosine phosphatases all of which contain a conserved HCX_5R active site motif (9, 24, 62, 78). Typical dephosphorylation by PTPs involves an invariant Asp residue brought down into the catalytic domain via the hinged WPD-loop (9, 24, 62, 78). The Asp then forms a hydrogen bond with the tyrosine residue substrate allowing the conserved active site cysteine to mount a nucleophilic attack (9, 24, 62, 78). PTPs can be further divided into 4 classes based on differences in the catalytic domain (Table 1.) (9, 24, 62, 78). Class I contains the largest and most diverse groups of PTPs including receptor-like,

non-transmembrane, and dual specificity phosphatases (DSPs) (9, 24, 62, 78). DSPs are named such due to their ability to dephosphorylate both serine/threonine and tyrosine residues (9, 24, 62, 78). My thesis focuses on the novel DSP, phosphatase of regenerating liver (PRL).

1.2 Phosphatase of Regenerating Liver(s)

PRL 1 was first discovered as a strongly upregulated, immediate-early gene in rat livers recovering from partial hepatectomy (45). Later, its family members, PRL 2 and PRL 3, were identified by searching the the Murine Expressed Sequence Tags database using the known sequence for PRL 1(75). The PRLs were found to be highly similar in both amino acid sequence and structure to each other with PRL 1 and PRL 2 exhibiting 87% identity while PRL 3 exhibited 76% and 79% identity to PRL 1 and 2 respectively (5, 75). Further structural and sequential analysis allowed the PRLs to be categorized within the PTP superfamily (Figure 2. Structural data collected by Sijiu Liu) (5, 58). PRL family members also share the CX_5R active site, P-loop, and WDP loop motifs typical of PTPs (5, 58). Furthermore, PRLs are hypothesized to be able to dephosphorylate both tyrosine and serine/threonine residues due to structural similarities to PTEN, Cdc14, MKP, and other dual specificity phosphatases (5, 58). One structural feature that makes the PRLs unique from all other phosphatases is the presence of a CAAX prenylation motif next to a polybasic region in the C-terminal domain (Figure 2. Structural data collected by Sijiu Liu.) (5, 57, 58). Prenylation is known to facilitate localization of proteins, such as oncogenes Ras and Rab, to the plasma membrane (8). PRL has indeed been

shown to localize to the plasma membrane and early endosome if the C-terminal CAAX motif is prenylated (76). The polybasic region preceding the CAAX motif is also thought to be required for nuclear localization in the absence of prenylation and for recruitment to the membrane (57, 76). Another interesting feature of the PRL family is the ability to form a trimer (Figure 2. Structural data collected by Sijiu Liu.) (23, 57, 58). This trait was discovered under crystallization conditions by Sun et al. and Jeong et al (23,57). It was later shown by Sun et al. that PRLs trimerize *in vivo* and that trimerization is required for proper cell migration (57).

In human tissue, PRL 1 and 2 mRNA was found to be expressed virtually ubiquitously throughout all organ systems, with PRL 1 expression levels being slightly more restricted and weaker than PRL 2 (14). PRL 3 however, has a much more restricted expression pattern, primarily in the heart, skeletal muscle, vasculature, and brain (38).

1.3 Phosphatase of Regenerating Livers as Oncogenes

1.3.1 PRL 3

PRL-3 was first discovered as a potential oncogene in a study by Vogelstein et al (55). Samples of metastatic liver lesions from colorectal cancer (CRC) patients were collected and applied serial analysis of gene expression (SAGE) technology to create a gene expression profile to compare to non-metastatic libraries (55). 144 genes were found to be misregulated in the metastatic lesions, and of those 144 genes, PRL 3 was the most consistently up-regulated (55). Interestingly, the expression level of PRL 3 in colorectal cancer primary tumors was much lower than in corresponding hepatic metastatic lesions, suggesting that PRL 3 may

play an important role in metastasis as opposed to carcinogenesis (55). Subsequent studies showed that, while PRL 3 was more consistently elevated in liver and lung metastasis, many other types of CRC metastasis exhibited high PRL 3 expression as well including brain, ovary, and lymph node lesions (25). Furthermore, it was shown that high levels of PRL-3 expression in the CRC primary tumor could predict the presence of distant metastasis (25). A Kaplan-Meier analysis for metastasis-free survival also revealed that patients with high levels of PRL 3 expression in resected CRC primary tumors were at a greater risk for liver/lung metastasis than those with low PRL 3 expression (25). This suggests that PRL 3 may be useful for determining patient prognosis, allowing doctors to prescribe aggressive CRC cancer treatment with greater accuracy (25).

The original studies linking PRL-3 expression to CRC patient prognosis resulted in the discovery of similar findings in other types of cancer including breast (20, 21, 52, 66), gastric (6, 10, 30, 43, 44, 47,), ovarian (50, 53), liver (71, 79), oral (22), cervical (36), esophageal (34), lung cancer (1, 73), multiple myeloma (7, 16), acute myeloid leukemia (AML) (72, 80), and nasopharyngeal cancer (81). As with CRC, PRL-3 overexpression is overwhelmingly correlated with poor prognosis and progression to metastasis in all of the aforementioned cancer types (Table 2).

1.3.2 PRL 1 and 2

Many studies have been conducted showing the significance of PRL 3 in cancer and later a similar correlation was discovered with PRL 1 and 2 (Table 2). Some of the earliest evidence of PRL 1 and 2 involvement in human cancer came from a study focusing on the generation of PRL 1 and 3 monoclonal antibodies to be used to diagnose cancer metastasis (28). Li et al. tested PRL 1 and PRL 3 specific antibodies on a human multiple cancer tissue array (28). The PRL 1 antibody reacted positively to 10 different cancer tissues including renal carcinoma and ovary lymphoma (28). Additionally, Wang et al. provided evidence that PRL 2 can serve as an oncogene in prostate cancer (67). In this study, PRL 2 was shown to be overexpressed in prostate cancer cell lines LNCaP, PC3, and DU145 (67). Concordantly, PRL 2 transcription was found to be elevated in three samples of advanced prostate cancer in comparison to the corresponding normal prostate tissue (67). Recently, a study by Dumaual et al. showed that both PRL 1 and 2 mRNAs are highly to moderately expressed in all but six tumor tissue samples examined in the study (15).

1.4 Cell function

The first insights to the molecular basis for PRL involvement in metastasis and cancer was from a study published in 2003 (74). Using a Chinese Hamster Ovary (CHO) cell line stably expressing Myc-tagged-PRL 1, 3, or β-Gal (control), the authors ascertained the effect of PRL 1 and 3 overexpression on cell mobility and cell invasiveness (74). PRL expressing cells were found to be significantly more mobile and invasive than the controls (74). Additionally, cells expressing a

catalytically inactive, mutant PRL showed significantly less activity and movement than WT controls, confirming that PRL 3 played an important role in cellular migration and that its ability to induce migration is dependent on its phosphatase activity (74). Several papers were published later with similar results in both mouse melanoma and HEK293 cells lines (3, 5, 11, 18, 51, 56, 69, 74). Studies by Sun et al. also revealed that PRL trimerization and the presence of the C-terminal polybasic regions and prenylation motif were also required to incite the oncogenic phenotype (57). Additionally, a study by Wang et al. revealed that PRL 2 can also affect cell migration and invasion (68).

Along with cell migration, invasion, adhesion, and proliferation PRLs have been shown to affect cell apoptosis and angiogenesis. Three papers revealed a novel connection between PRLs and p53 (Figure 3, Table 3). Basak et al. show that PRL 3 is upregulated in a p53-dependent manner after submitting control and Doxorubicin (DNA damaging agent)-treated murine embryonic fibroblast (MEF) cells to a microarray analysis (4). A scan of the PRL 3 locus revealed two p53 binding sites, one of which p53 was shown to bind to directly via chromatin immunoprecipitation (4). Additionally, two papers by Min et al. showed that p53 could also be regulated by PRL 1 and 3 in cancer cells (41, 42). Upregulation of PRL 1 and 3 can both inhibit p53 and p53-mediated apoptosis individually with the opposite being true when PRL 1 or 3 is knocked down (41, 42). Western blot analysis of PRL 1 or 3 overexpressing cells reveals that apoptosis inhibition can be caused by PRL-mediated activation of MDM2 via PI3K activation and PIRH2 (p53 ubiquitinase) transcription via EGR1 activation (Figure 3, Table 3) (41,42).

Many early PRL studies have noted PRL-3 to be strongly expressed in tumor vasculature and hypothesized that it may play a role in induction of angiogenesis (18, 19, 79). A recent paper by Xu et al. explored the molecular mechanism of PRL-driven HUVEC tube formation in detail (70). In this study, vascular endothelial growth factor (VEGF) is shown to induce the transcription of PRL-3 in HUVEC cells via MEF2C transcription factor (Figure 3, Table 3) (70). Furthermore, VEGF could not induce PRL-3 expression when MEF2C was knocked down by siRNA and PRL 3 knockdown in HUVEC resulted in compromised tube formation (70).

1.5 Cell Signaling

Since its discovery as an oncogene in colorectal cancer, PRL has been exhaustively researched in cell culture. However, no putative substrate has been discovered. Despite this, studies have shown evidence that PRLs can alter several major cell signaling pathways, including PTEN and Src (Figure 3, Table 3) (31,32,65). One of the first mechanistic pathways to be associated with PRL is Src and its downstream targets. A study by Liang et al. showed that when PRL 3 was overexpressed in HEK293 cells Src kinase activity was increased by 180% in comparison to control and PRL 3 C104S mutant cells (31). Downstream targets of Src, ERK1/2, STAT3, and p130Cas, also experienced increased phosphorylation (Figure 3, Table 3) (31). Purified PRL-3 was not able to phosphorylate Src directly; however, Csk protein (negative regulator of Src) expression was shown to be significantly reduced in cells overexpressing PRL 3 (Figure 3, Table 3) (31). When Csk was reconstituted to normal levels, via

tetracycline expression in cells overexpressing PRL 3, Src phosphorylation and the oncogenic phenotype of PRL 3 was negated (31). A follow up study on the activity of PRL 3 on Csk revealed that PRL 3 could inhibit Csk translation due to increased phosphorylation of Ser-51 of eIF2α (Figure 3, Table 3) (32). Subsequent studies indicated that PRL 1 can also activate the Src pathway (32, 35). However, unlike PRL 3, PRL 1 activation increased Tyrosine 416 as opposed to Tyr527 and increased FAK activation along with pre-established p130Cas, ERK1/2, and STAT3 (Figure 3, Table 3) (35). Additionally, active forms of matrix metalloproteinases 2 and 9 (MMP 2 and 9) were expressed at higher levels than in control cells (Figure 3, Table 3) (35). MMP2 and 9 are regulated by ERK1/2 and degrade collagen of the basement membrane (35). The two proteins are often found overexpressed in cancer, providing another pathway through which PRL can induce cell migration (35).

PRLs have also been shown to regulate Rho and Rac activity in SW480 cells (Figure 3, Table 3) (17). Rho A, C, and Rac are known facilitators of the actin polymerization associated with cell mobility (17). In cells that have been transfected with PRL 1 and 3 overexpression vectors, RhoA, and RhoC expression levels increased by 4- to 7-fold while Rac was greatly reduced (17). Further testing showed that in order to promote cell invasion and mobility in a Rho-dependent manor requires the presence of Rho effector ROCK and PRL phosphatase activity (17).

PRL is also known for interacting with integrins, E-cadherin, γ-catenin, vinculin and other adhesive proteins to modulate cell migration and invasiveness

(Figure 3, Table 3) (33, 46, 48, 61, 65). When upregulated, PRL can enhance integrin β activity directly and suppress integrin α via inhibition of c-fos (Figure 3, Table 3) (33). Epithelial markers such as E-cadherin are downregulated when PRL is over expressed while mesenchymal markers fibronectin and Snail are up-regulated (Figure 3, Table 3) (65). The adhesive proteins are upstream of PI3K/Akt, which has been shown to be up-regulated in DLD-1 cells overexpressing PRL 3 (65). The mechanism for this action may be the result of PTEN downregulation (Figure 3, Table 3) (65). PTEN is the negative regulator of the PI3K pathway and is down-regulated in DLD-1 cells over-expressing PRL 3 (65). PRL-mediated Src and PTEN/Akt activation can also be explained by upstream activity with receptor tyrosine kinases (RTKs) (Figure 3, Table 3). Indeed, PRL-3 has been shown to up-regulate PDGFR, Eph, and integrin receptor array in a proteomic analysis of HEK293 cells overexpressing PRL 3 (Figure 3, Table 3) (64). Another study also revealed evidence of PRL 3 induced activation of EGFR in A431 epidermoid carcinoma cells (2). PRL 3 overexpression induced a state of EGFR addiction in both cell lines and in patient tumor sample, causing hypersensitivity to EGFR inhibition (2). It was concluded that PRL 3 regulates EGFR by transcriptionally down-regulating PTP1B, causing EGFR hyperphosphorylation and activation (2).

Several independent studies also show that PRL effects some additional pathways including upregulation of KCNN4 potassium channels (27), activity toward phosphoinositides (40), induction of micro RNAs (miR) 21, 17, 19a (77), and interaction with miR-495 and miR551a (Figure 3, Table 3) (29). One study

proposes a novel role of PRL-3 downstream of an internal tandem duplication mutant of fms-like tyrosine kinase 3 (FLT3-ITD) present in approximately 25% of AML patients (Figure 3, Table 3) (80). While studying the mechanism behind FLT3-ITD-positive AML drug resistance Zhou et al. treated AML cell lines with both FLT3 inhibitor ABT-869 and a histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) and were successful in reducing AML colony formation and inducing apoptosis in tumor cells (80). Further studies revealed that PRL 3 was strongly downregulated in cells that received combined ABT-869 and SAHA treatment and showed evidence that PRL 3 may be responsible for FTL3 drug resistance through activation of the Stat-pathway, interaction with histone deacetylase 4, and upregulation of Mcl-1 (80).

1.6 Research Goals

Much of the pre-existing data on PRL was gathered from cell culture, thus there is a dearth of knowledge regarding the physiological relevance of PRL *in vivo*. The Zhang lab was amongst the first in the field to develop traditional PRL knock out (KO) mice. My project focuses mainly on the study of the physiological impact of PRL using the PRL KO and other murine models. Specifically, I investigate the impact of PRL 2 deletion on spermatogenesis and the potential for PRL as a target for male contraception using a small molecule inhibitor developed by our lab. I also created a PRL 2 KO mouse line crossed to a conditional, PTEN heterozygous knockout line to determine the relevancy of PRL 2 interaction with PTEN in tumorigenesis.

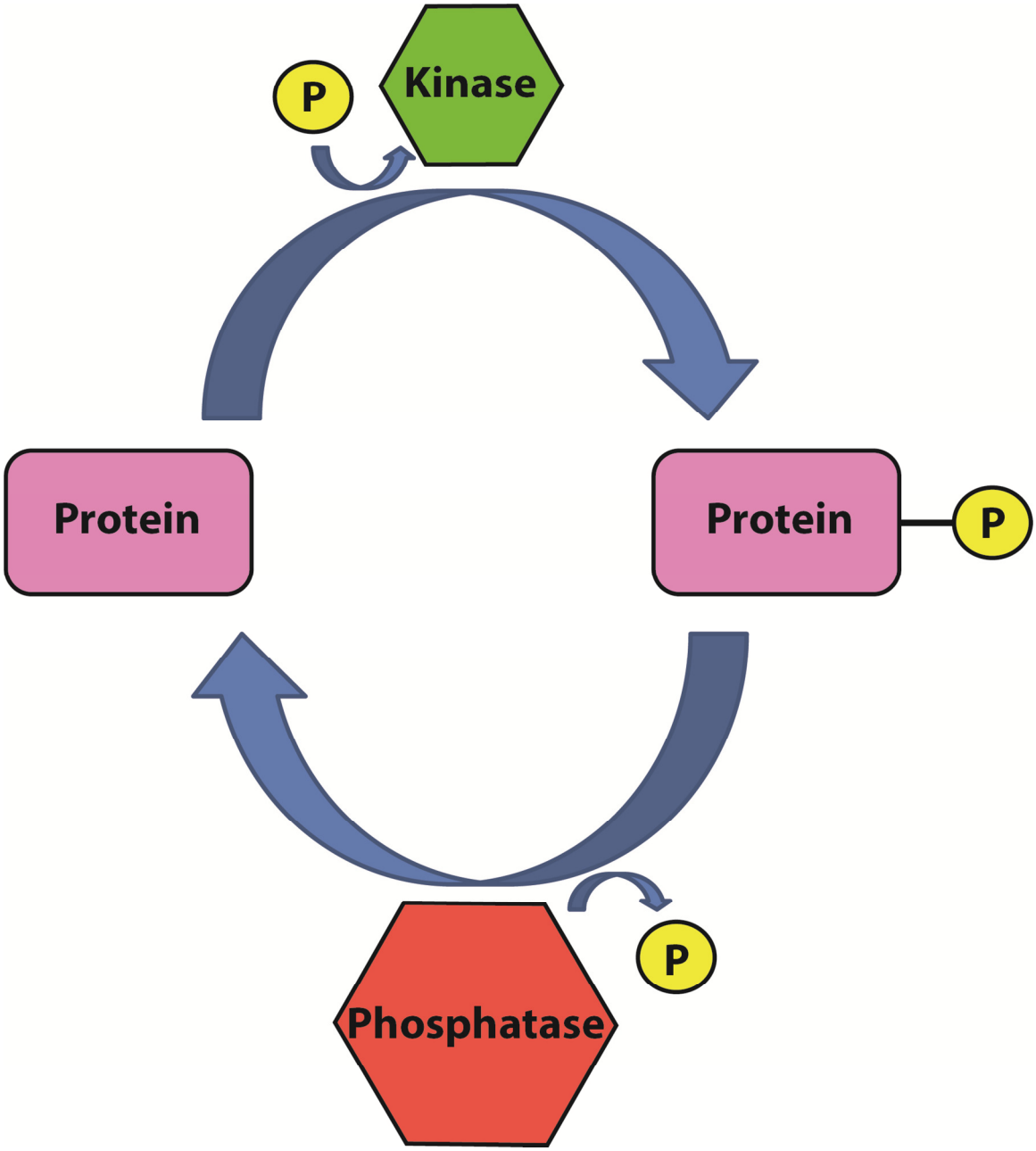


Figure 1. The basic mechanism of protein phosphorylation

Class I	Class II	Class III	Class IV
Receptor PTPs	LMW-PTP	CDC25	EYA
PTPRG			
PTPRZ			
PTPRD			
PTPRF			
PTPRS			
PTPRB			
DEP1			
SAP1			
PTPRO			
PTPRK			
PTPRM			
PTPRT			
PTPRA			
Non-Receptor PTPs			
PTPN13			
PTPN14			
PTPN21			
PTPN3			
PTPN5			
PTPN7			
PTP-PEST			
PTPN18			
PTPN6			
PTPN11			
PTPN1			
PTPN2			
DSPs			
PTENs			
SSHs			
MTMRs			
CDC14s			
PRLs			
MKPs			
Atypical DSPs			

Table 1. Tyrosine phosphatase classification (24).

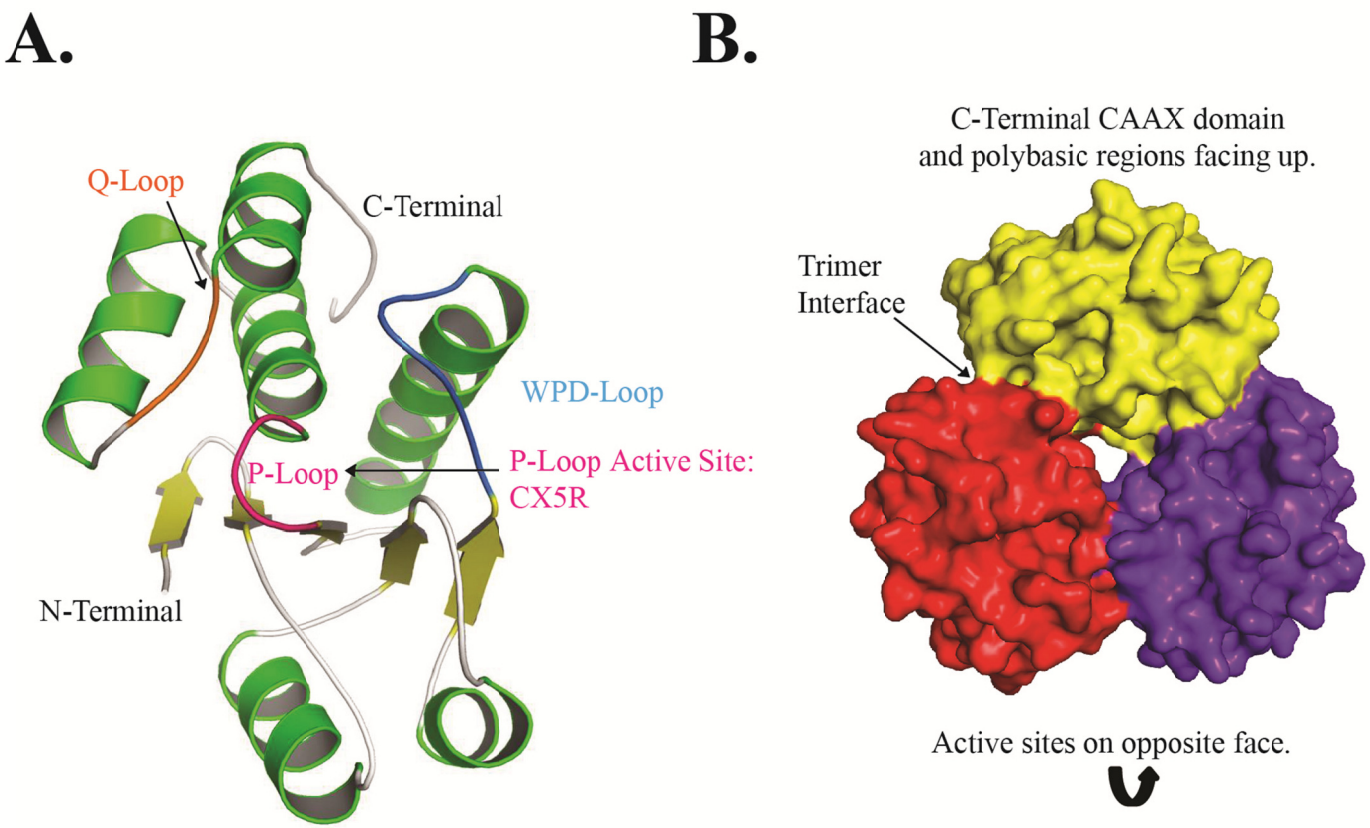


Figure 2. PRL 1 can form a trimer under crystallization conditions. .A. Ribbon diagram of the structure of PRL-1. B. PRL Trimer: PRL crystalizes in a trimer that fixes the C-Terminal ends of each monomer such that they all face towards the plasma membrane. This trimer is oriented with the C-terminal ends facing out (into the "membrane") with the active sites on the opposite face (in the "cytoplasm"). This is how the PRL trimer is hypothesized to dock onto the membrane. Structural data collected by Sijiu Liu.

Cancer Type	Primary Tumors with High PRL Expression	Metastasis with High Levels of PRL Expression?	High expression of PRL in Primary tumor predictive of Metastasis?	Clinical Features Affected by PRL Overexpression	Clinical Features NOT Affected by PRL Overexpression	Reference
Colorectal	Yes	Yes: Especially in lung and liver	Yes: Distant Metastasis	Chance of Distant metastasis Significant reduction in Disease-free survival (DFS)	Lymph-node metastasis. Tumor Size. Tumor stage.	(25, 49, 55, 60)
Breast	Yes: 116 out of 135 (85.9%) samples of DCIS and 190 out of 246 (77.2%) samples of invasive carcinoma	Yes	Yes: Distant Metastasis	Decrease in disease-free survival, overall trend Significant decrease in DFS in node-positive samples (but not node-negative)	Lymph-node metastasis. Tumor stage.	(52)
Breast	Yes: 133 out of 382 (34.8%) samples	Yes	No Data	Significant decrease in DFS in node-negative patients (but not node-positive)	Tumor stage. Age	(66)
Breast	Yes	Yes	Yes: Increased lymph-node and distant metastasis	Significantly correlated with lymph-node metastasis Decrease in DFS	Tumor stage.	(20)
Gastric	Yes	Yes	Yes: Increased lymph-node and distant metastasis	Significant decrease in DFS and distant metastasis	Tumor stage. Tumor size.	(6, 10, 30, 37, 43, 44, 47)
Ovarian	Yes	Yes	Yes: Increased metastasis	Significant decrease in DFS and distant metastasis	No data.	(50, 53)
Liver	Yes	Yes	Yes: Increased metastasis	Significant decrease in DFS and distant metastasis	---	(71, 79)
Oral	Yes	Yes	Yes: Increased metastasis	Significant decrease in DFS and distant metastasis	No data	(22)
Cervical	Yes	Yes	Yes: Increased metastasis	Significant decrease in DFS and distant metastasis	No data	(36)
Esophageal	Yes	Yes	Yes: Increased metastasis	Significant decrease in DFS and distant metastasis	No data	(34)
Lung	Yes	Yes	Yes: Increased metastasis	Significant decrease in DFS and distant metastasis	---	(1, 73)
Multiple Myeloma	Yes	No Data	No Data	Significant decrease in DFS	No data	(7, 16)
Acute Myeloid Leukemia	Yes	No Data	No Data	Significant decrease in DFS and possible driver for drug-resistance	No data	(72, 80)
Nasopharyngeal	Yes	Yes	Yes: Increased metastasis	Significant decrease in DFS and distant metastasis	No data	(81)
Prostate	Yes	No Data	No Data	No Data	No data	(67)

Table 2. A summary of studies of PRL in cancer.

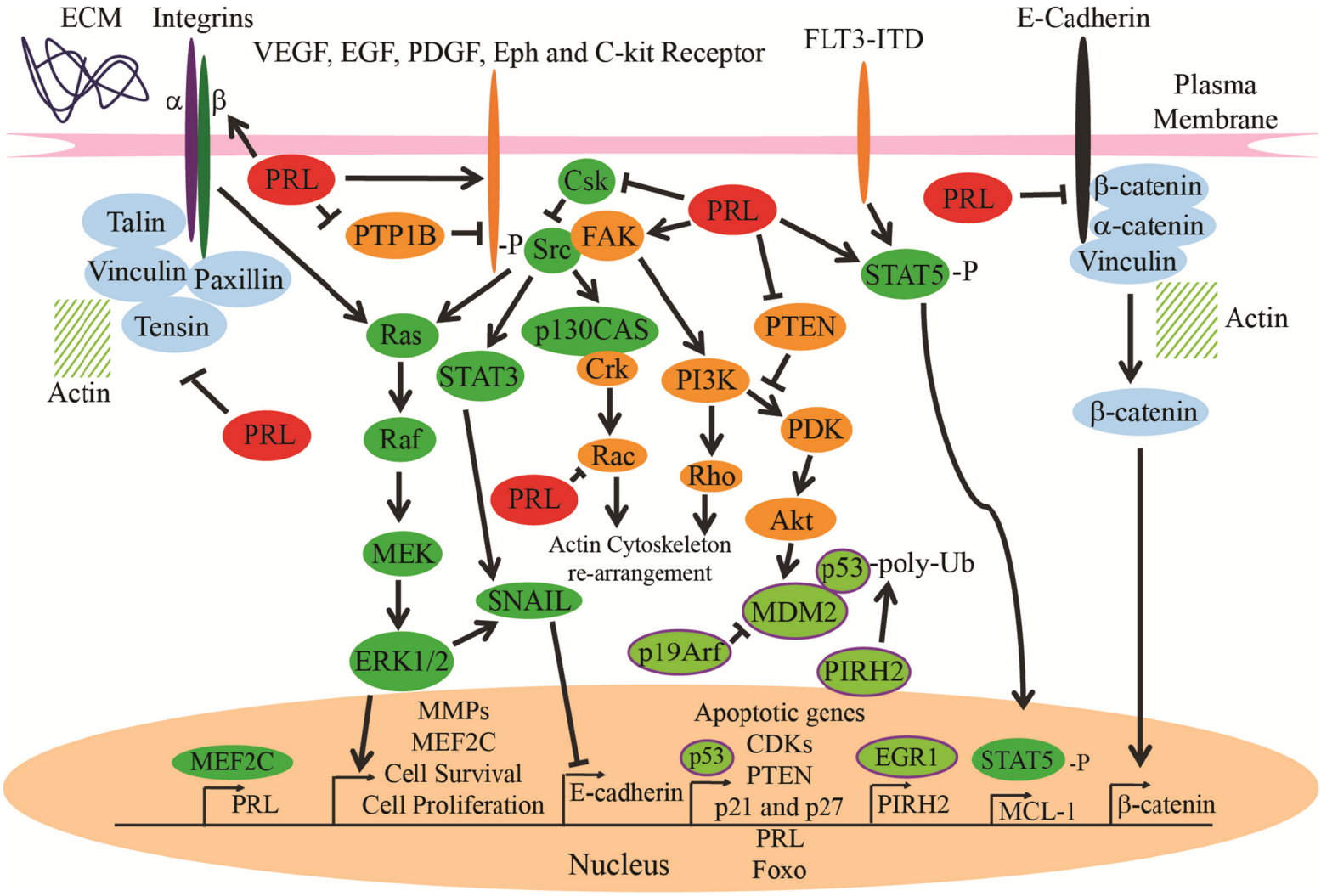


Figure 3. Summary of purposed PRL signaling pathways.

Binding Partner	Effect of PRL Overexpression	Reference
p53	Inhibition	(4,41,42)
Src	Activation	(31,32,25)
ERK1/2	Activation	(31,32,25)
STAT3	Activation	(31,32,25)
p130Cas	Activation	(31,32,25)
Csk	Inhibition	(31,32)
MMPs	Activation	(35)
FAK	Activation (PRL-1)	(35)
Rho A and C	Activation	(17)
Rac	Inhibition	(17)
Integrin α	Inhibition	(33, 48)
Integrin β	Enhance Src interaction	(61)
c-fos	Inhibition	(33)
Actin Cytoskeleton	Uncoupling from adhesive proteins	(46)
E-Cadherin	Inhibition	(65)
γ-catenin	Inhibition	(65)
Vinculin	Inhibition	(65)
Fibronectin	Protein level Up-regulation	(65)
Snail	Protein Level Up-regulation	(65)
PTEN	Inhibition	(12,13,65)
Akt	Activation	(12,13,65)
PDGFR	Activation	(64)
Eph	Activation	(64)
EGFR	Activation	(2)
PTP1B	Inhibition	(2)
KCNN4	Activation	(27)
Phosphoinositides	Dephosphorylation	(40)
miR 21,17,19a	Induction	(77)
miR 495, 551a	Induction	(29)
STAT5	Activation	(80)
Histone Deacetylase 4	Activation	(80)
Mcl-1	Induction	(80)

Table 3. Summary of potential PRL binding partners

CHAPTER 2: Materials and Methods

2.1 Mouse Model Lines

The PRL 2 Genetic Knockout line used in this study was originally generated by Yuanshu Dong and Yunpeng Bai from the lab of Zhong-Yin Zhang. The mice were developed using a commercially available gene trap embryonic stem cell (AQ0673; 129P2/OlaHsd) acquired from the Sanger Institute that contained a pGT01 cassette inserted within the first intron of the Prl2 gene (13). The embryonic stem cell (ES) was then injected into C57BL/6 blastocysts by the Indiana University School of Medicine Transgenic Core Facility (13). Wildtype, heterozygous, and knockout animals were used (Figure 4. Figure made by Yuanshu Dong).

The conditional PTEN knockout line used was originally developed by Antonio D. Cristofano from the Pandolfi Lab and was generously donated to the Zhang Lab by Yan Liu of Indiana University's School of Medicine (Figure 5. Figure made by Lloyd C. Trotman) (63)).

The pure C57BL/6 mouse line and EIIA-Cre mouse line were both purchased from Jackson Lab.

2.2 Genotyping

All DNA used for genotyping our experimental mice were gathered from toe tissue clipped from 7-10 day old pups. The tissue was then lysed in Tissue Lysis Buffer (25mL 2M NaCl, 10mL 1M Tris pH 8.0, 10 mL 0.5M EDTA, 1% SDS) with freshly added proteinase K overnight at 65℃. The DNA was then separated

from solution with isopropanol and re-suspended in TE buffer (10mM Tris, pH 8.0, 1 mM EDTA) for long-term storage. All PCR was performed using the Go Taq DNA Polymerase kit from Promega.

2.2.1 PRL 2

To determine the genotype of PRL 2 mice the primer set (donated by Qi Zeng): Forward 5'GGCTGTAACAGGGTGGAAGA3' and Reverse 5'GCCACCAACATCTGGGTACT3' was used along with the following PCR cycle program:

Initial Denaturation: 94℃ for 5 mins

Denaturation: 94℃ for 30 seconds

Annealing: 55℃ for 30 seconds

Elongation: 72℃ for 1 min

35 cycles

Final Elongation: 72℃ for 1 min

2.2.2 PTEN flox

To detect the PTEN floxed allele the primer set (donated by Yan Liu): Forward 5'TGTTTTTGACCAATTAAAGTAGGCTGTG-3' and Reverse 5'AAAAGTTCCCCTGCTGATGATTTGT3' were used.

To detect the PTEN deleted allele the primer set Forward 5'CCCCCAAGTCAATTGTTAGGTCTGT3' and Reverse 5'AAAAGTTCCCCTGCTGATGATTTGT3' were used along with the following PCR cycle program:

Initial Denaturation: 95℃ for 3 mins

Denaturation: 95℃ for 30 seconds

Annealing: 57℃ for 30 seconds

Elongation: 72℃ for 1 min

35 cycles

Final Elongation: 72℃ for 10 min

2.3 Western Blot

Tissues were snap frozen in liquid nitrogen on the day of the mouse dissection and later lysed using buffer containing 25mL 1M Tris HCl (pH 7.4), 18.75mL 4M NaCl, 2.5mL Triton X, 50mL Glycerol, and H2O to 500mL along with freshly prepared protease and phosphatase inhibitors (cOmplete Tablets EDTA-free, EASYpack, and PhosSTOP EASYpack from Roche). 3x SDS-PAGE buffer was added to the lysate to a final concentration of 1x and equal amounts of lysate were resolved on SDS-PAGE gels. The proteins were then transferred to nitrocellulose membranes and treated with primary and secondary antibodies according to the official product recommendations. The proteins on the membrane were visualized on CL-XPosure film (Thermo Scientific) after treatment with SuperSignal West Femto Chemiluminescent and/or Maximum Sensitivity Substrate from Thermo Scientific. Visible bands were quantified using ImageJ software.

2.4 Histology

Tissues were fixed in 4% paraformaldehyde for 48 hours at room temperature, embedded in paraffin, serially sectioned (7 micron thickness), and stained with hematoxylin and eosin according to standard procedures.

2.5 Sperm Count

The epididymis from treated and untreated mice were collected and minced in separate petri dishes containing 10mL of 1x PBS. The plates were incubated for 1 hour at room temperature to allow sperm to migrate from the minced epididymis out into the PBS. The sperm was then collected in conical tubes, vortexed, and counted using a hemocytometer.

2.6 Treatment with Compound 43 and DMSO

Compound 43 injections were prepared by first dissolving Compound 43 in DMSO then adding 1x PBS to 10mL. This leaves a final concentration of 38.4 mM Compound 43 in 37% DMSO. 37% DMSO in 1x PBS was prepared as the control treatment. The mice then received intraperitoneal injections of Compound 43 or DMSO in PBS at a concentration of 50mg/mL once a day for three weeks.

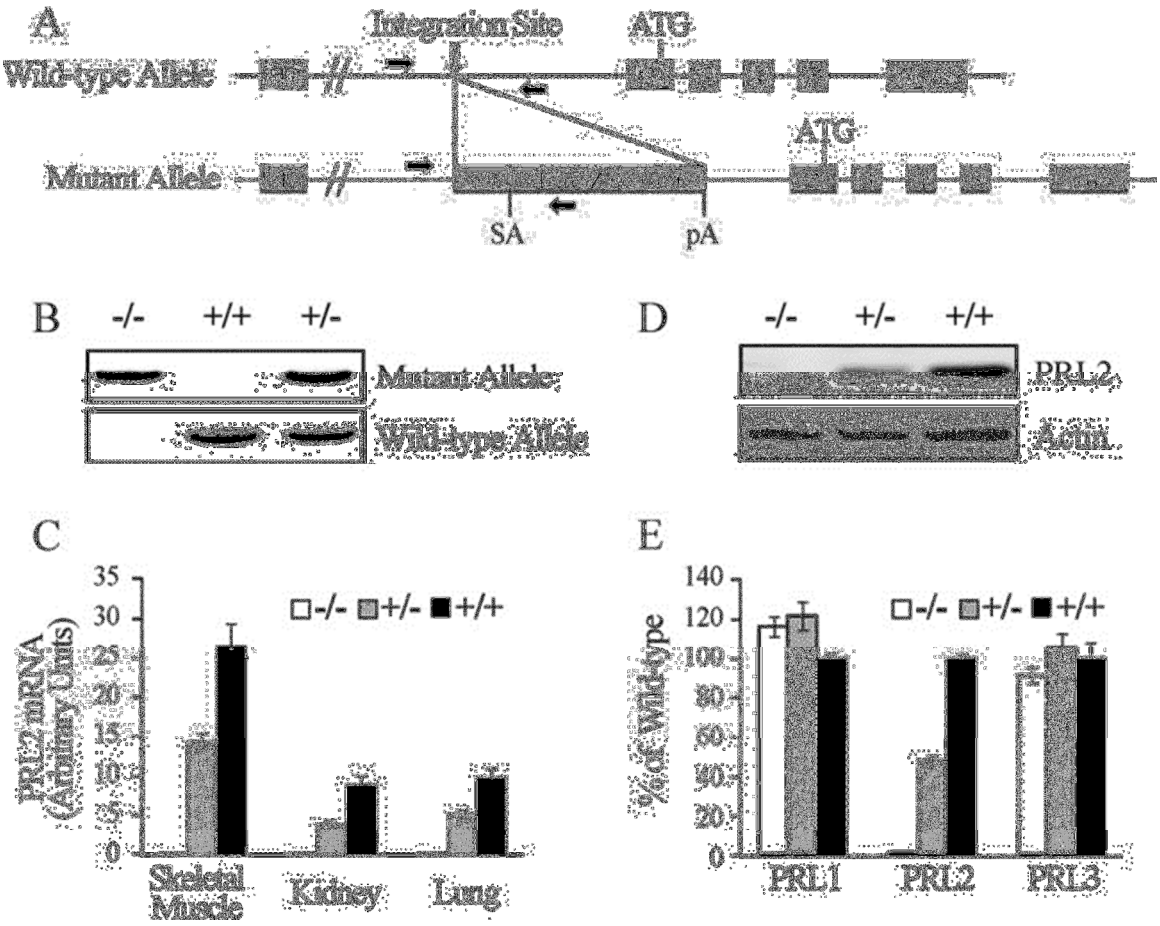


Figure 4. Generation of PRL 2 Knockout mice. A) Representation of the PRL 2 locus and the mutant allele containing the pGT01 gene trap cassette. Numbered exons (ATG) of PRL2 are shown (13). The boxes labeled Lac Z and neo indicate β-galactosidase and neomycin resistance genes respectively (13). pA and SA indicate the polyadenylation signal and splice acceptor respectively (13). The black arrows show primer sites for PCR genotyping (13). B) PCR amplification bands for PRL 2 genotyping (13). C) Quantitative RT-PCR analysis of PRL2 mRNA expression in tissues from PRL2 KO, PRL2 HET, and PRL2WT mice in skeletal muscle, kidney and lung tissue. n= 3 mice per group(13). D) Western blot analyses of PRL2 protein levels in skeletal muscle from PRL2 KO, PRL2 HET, and PRL2 WT mice (13). E) Quantitative RT-PCR analysis of PRL1, PRL2, and PRL3 mRNA expression as a percentage of WT(13). Figure made by Yuanshu Dong.

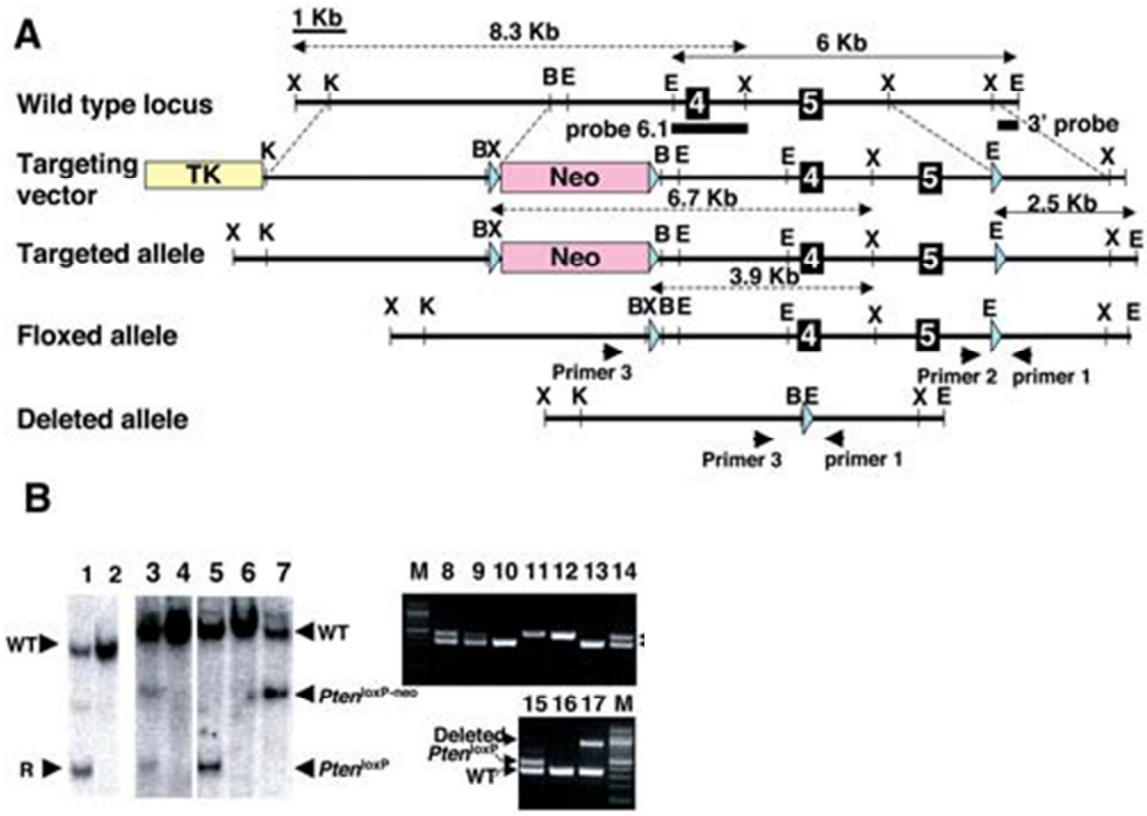


Figure 5. Generation of conditional PTEN knockout mice. A) The wild type and LoxP mutant loci are represented here (63). Endonuclease restriction sites are labeled along with the neomycin resistance gene, thymidine kinase gene, and sections targeting homologous recombination (blue dotted line) (63). LoxP sites are indicated by light blue triangles flanking exons 4, 5, and the neomycin gene (63). The thick, black arrows indicate primer sites used for PCR genotyping (63). B) Bottom right: Genotyping bands for PTEN WT allele, floxed allele, and deleted allele (63). Figure created by Lloyd C. Trotman, Pandolfi

CHAPTER 3: PRL 2 Deletion Causes Progressive Degradation of Spermatogenesis and Loss of Germ Cells in Murine Testis

3.1 Introduction

3.1.1 Generation of PRL 2 Genetic Knockout Mouse Line

PRL 2 KO mice were generated by Dong et al. using a commercially available gene trap embryonic stem cell (Sanger Institute) that contained a pGT01 cassette inserted within the first intron of the Prl2 gene (13). The embryonic stem cell (ES) was then injected into C57BL/6 blastocysts by the Indiana University School of Medicine Transgenic Core Facility (13). The resulting chimeric mice were crossed to pure C57BL/6 wildtype (WT) mice (Jackson Lab) and the first PRL -/+ animals were successfully obtained (13). The heterozygous animals produced all three genotypes (PRL 2 -/-, -/+, and +/+) when interbred (15). Deletion of PRL 2 was confirmed using PCR and Western Blot (Figure 4. Figure made by Yuanshu Dong) (13).

3.1.2 Deletion of PRL 2 Causes Reduced Testis Weight and Infertility in Male Knockout Mice

After knockout PRL 2 mice were obtained, several abnormal phenotypes were observed. The most noticeable result of PRL 2 deletion in mice was growth retardation in KO mice in comparison to wildtype siblings (13). Newly born knockout mice were, on average, 20% smaller than WT mice and remained smaller into adulthood and beyond (13). Most organ systems in the KO mice were proportionately small and revealed no abnormalities under histological

scrutiny (13). Two exceptions to this finding were the testis and placenta of the KO animal (12, 13). Both organs were significantly smaller than the testis and placenta of WT control mice (12,13). My work focuses on the testis phenotype.

Since the KO mice were naturally smaller that their WT siblings, Dong et al. normalized the organ to body weight and found that the testis were still significantly smaller than WT (12). In order to investigate the impact of the reduced testis size on fertility cohorts of PRL 2 KO and WT males at three and six months old were mated to two, age-matched C57BL/6 WT females every day for 6 days (12). Vaginal plugs were observed and the plug efficiency was found to be similar between PRL 2 KO and WT males, indicating no reduction in sex drive in the KO males (12). However, sperm counts for KO males indicated significantly less active spermatozoa than at both 3 and 6 months old (12). Furthermore, the number of females plugged by PRL KO males that became pregnant was 20% as opposed to 70% for PRL 2 WT (12). This reduction in fertility was observed in PRL 2 KO males at 3 months and 6 months with 6 month old males being significantly less fertile that 3 month old mice (12).

Since testosterone can drastically affect fertility, serum testosterone levels were measured for PRL 2 KO and WT males. No significant difference was discovered(12).

3.2 PRL 2 Knockout Males Suffer Progressive Degradation of Spermatogenesis and Loss of Germ Cells

To investigate the cause of the progressive infertility in PRL 2 KO male mice, I gathered samples of 3 month old and 6 month old PRL 2 KO and WT testis and

preserved them in paraffin wax (Figure 6). I then analyzed histological testis cross-sections for abnormalities. In the testicular cross-sections of 3 month old PRL 2 KO, I found that many of the seminiferous tubules had stared to shed some of the germ cells into the lumen (Figure 6B). This was evidenced by a smaller seminiferous tubule diameter and the presence of large, multi-nuclear germ cells in the lumen (Figure 6B). Otherwise, most cross-sections had well-structured seminiferous tubules with mature spermatids being ejected into the lumen. However, when I examined 6 month old PRL 2 KO testis, I found that the shedding of the germ cells had worsened significantly (Figure 6B). Large sections of seminiferous tubules appeared to have completely shed the germ cell lining into the lumen. Furthermore, 86% of seminiferous tubules were at end stage spermatogenesis (mature spermatids in the lumen) in 6 month old PRL 2 WT testis while only 29% of tubules were at end stage spermatogenesis in PRL 2 KO mice. Additionally, shed germ cell were found in the epididymis of PRL 2 KO mice at both 3 and 6 months old along with a visibly reduced amount of stored, mature sperm (Figure 7B).

3.3 Discussion

The study was continued by Dong et al. to determine the mechanism responsible for the progressive infertility and germ cell loss in the testis of PRL 2 KO male mice. The seminiferous tubules of PRL 2 KO mice were found to be hypocellular which is consistent with the shorter diameter of seminiferous tubules as well as the smaller testis size (12). Seminiferous tubules cells consist of three cell types, Sertoli cells, germ cells, and peritubular myoid cells (12). Sertoli cells provide

nourishment and structural support for the germ cells (12). Thus, the loss of Sertoli cells could result in loss of germ cells (12). However, visualizing the Sertoli cells using and anti-vimentin marker showed that the average number of Sertoli cells in each seminiferous tubules was similar between the two genotypes (12). Thus, it can be concluded that the progressive infertility of the PRL 2 KO males is due to the loss of germ cells due to PRL 2 deletion (12). Our mutant KO allele of PRL 2 contains lacZ under the expression control of the native PRL 2 promoter, and can be used to monitor PRL 2 expression patterns via X-gal staining (12). In PRL 2 KO mice, the germ cells stained positive for lacZ indicating that PRL 2 is expressed in the testis and particularly in spermatogonia and spermatocytes (12).

To determine whether the testicular hypocellularity was due to decreased proliferation, increased apoptosis, or a combination of both, immunohistological staining for PCNA (Proliferating Cell Nuclear Antigen, a proliferation marker) and cleaved PARP (Poly ADP-Ribose Polymerase, an apoptosis marker) was performed on testis sections of PRL2 KO and WT mice at 2 weeks of age (12). There was no significant decrease in the amount of PCNA present between genotypes, but Cleaved-PARP was greatly increased in KO testis in comparison to WT (12). The shedding of the germ cells from the lining of the seminiferous tubules of PRL 2 KO mice was ultimately shown to be due to an increase in apoptosis (12).

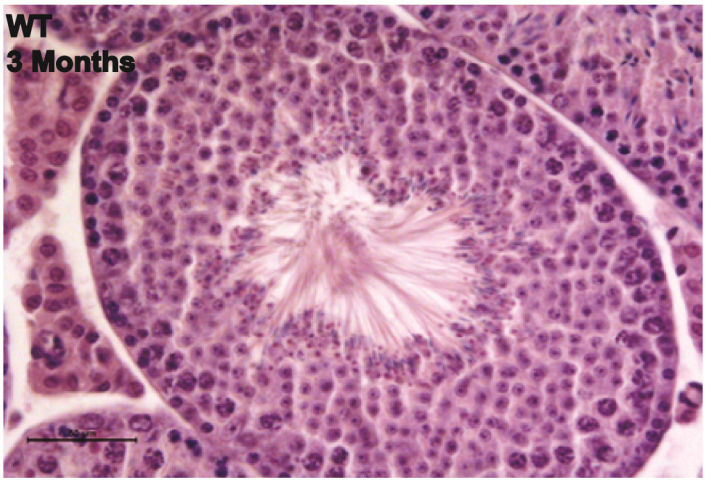
Additionally, PTEN was found to be upregulated in KO testis along with the concordant attenuation of the c-kit-PI3K-Akt pathway (12). PI3K-Akt

signaling is critically important for cell survival, thus it's attenuation in PRL 2 KO germ cells could explain the dramatic increase in apoptosis in comparison to wild type (12).

This study was the first to reveal that PRL 2 plays an important role in spermatogenesis. PRL2 KO males display testis hypotrophy, impaired reproductivity, and decreased sperm production all as a result of compromised spermatogenesis (12). As in the PRL 2 KO placenta, hypocellularity of the knockout testis can be explained by an increase in PTEN and attenuation of the PI3K-Akt pathway (12, 13). However, unlike the KO placenta, this effect is reflected in an increase in apoptosis as opposed to a decrease in cell proliferation as seen in the placenta (12,13).

The ability of PRL 2 to affect PTEN *in vivo* presents exciting possibilities as a potential target for cancer therapy. PTEN is the negative regulator of the PI3K-Akt pathway and one of the most commonly mutated tumor suppressor genes found in cancer. Further studies targeting PRL 2 with small molecule inhibitors can establish whether or not PRL 2 can serve as a novel target for cancer therapy as well as determine the value as a potential drug for male contraception.

A.



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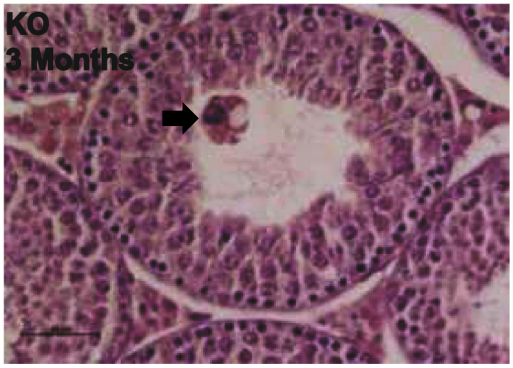


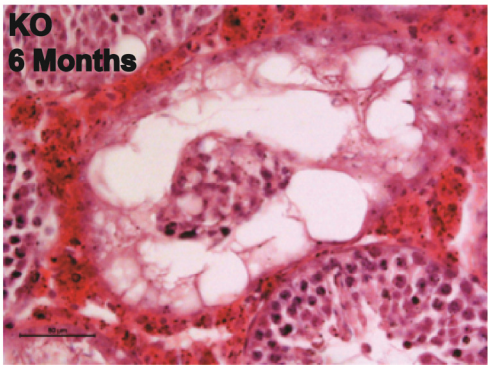


Figure 6. Seminiferous tubules of PRL 2 KO male mice display progressive degradation of spermatogenesis and shedding of germ cells into the lumen. A) This PRL 2 wildtype seminiferous tubule is healthy and well-structured with spermatogonia lining the outside, spermatocytes in the middle, and mature spermatids about to be released into the lumen tail first. B) At 3 months old, PRL 2 KO seminiferous tubules are still well structured but have a significantly reduced thickness in comparison to WT and have large, apoptotic cells being shed into the lumen (black arrow). At six months large sections of PRL 2 KO seminiferous tubules have completely shed their germ cell lining into the lumen.

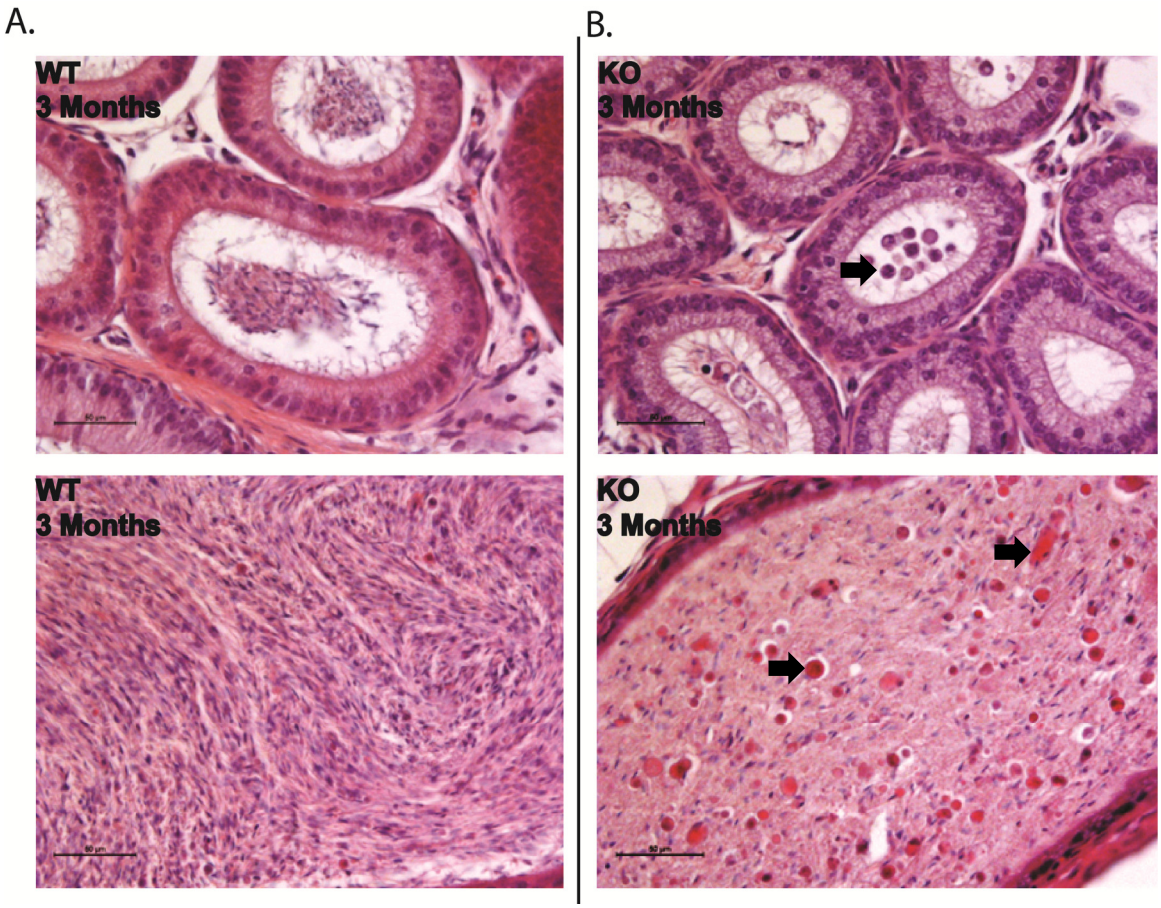


Figure 7. Germ Cells are shed into the epididymis of PRL 2 KO mice. A) At three months of age, PRL 2 WT epididymis is healthy and full of mature sperm (stained purple). B) The epididymis of PRL 2 KO male have apoptotic germ cell debris shed in the lumen (black arrows) and show significantly less healthy sperm stored in comparison to WT.

CHAPTER 4: EVALUATION OF PRL AS A POTENTIAL TARGET FOR MALE CONTRACEPTION USING PRL INHIBITOR, COMPOUND 43

4.1 Treatment of Wildtype C57BL/6 mice with Compound 43

Since the ability of PRL to disrupt spermatogenesis was shown by Dong et al. the potential for use as a male contraceptive became a tempting cause for research. Recently, our lab has developed a novel, small molecular inhibitor of PRLs (Figure 8. Structure made by Yunpeng Bai.). The drug targets the trimerization domain of PRL and prevents trimerization which was previously shown to be important for PRL-driven cell migration and invasiveness through PTEN-Akt and ERK signaling (57). Since, the drug inhibits PTEN signaling through PRL and it is known that the ability of PRL 2 to disrupt spermatogenesis is correlated to PTEN, I decided to conduct a mouse study exploring the effects of Compound 43 on male WT C57BL/6 mice. I hypothesized that treating the WT mice with compound 43 would result in a PRL 2 KO phenotype of reduced spermatogenesis and infertility. I treated one cohort of 4 male mice with 50 mg/kg of Compound 43 dissolved in dimethyl sulfoxide (DMSO), and a control cohort of 5 male mice with an equal concentration of pure DMSO once a day for three weeks. The cohorts were subjected to a breeding efficiency experiment and at the end of the three week period were sacrificed and dissected for analysis.

4.1.1 Treatment of Mice Cohorts for Three Weeks Did not Induce Growth Retardation

At the end of the three week treatment period, mice were weighed to determine if inhibiting PRL with Compound 43 would reproduce the growth retardation phenotype present in the PRL 2 genetic knockout. The weights were not significantly different between the treatment groups (Figure 9C).

4.1.2 Three Week Treatment with Compound 43 Was Not Sufficient to Induce Infertility

PRL 2 KO males have difficulty producing pups. To assess the ability of PRL 2 inhibitor to induce infertility, each male mouse was mated to two virgin, C57BL/6 WT females during the last two and a half days of the three week treatment with Compound 43 and DMSO. Treatment was continued during breeding. Treatment did not affect plugging efficiency. Over the two and a half days 8 out of 8 of the females paired to Compound 43 treated males were plugged and 8/10 females paired to the DMSO controls were found to be plugged. Of the females plugged, however, 5 out of 8 females paired to Compound 43 treated males became pregnant while only 4 of the 10 females paired to control mice gave birth. Additionally, sperm collected from the minced epididymis of the mice showed no difference between treatment groups when counted with a hemocytometer (Figure 9B). Thus, we can conclude that treatment with Compound 43 for three weeks will not reproduce the infertility phenotype of the genetic PRL 2 KO mice.

4.1.3 Three Week Compound 43 Treatment Did Not Reproduce PRL 2 Knockout Phenotypes in the Testis

In the PRL 2 KO male, the testis were significantly smaller than WT and hypocellular (14). Shedding of germ cells into the lumen of the seminiferous tubules was also observed(14). To test if treatment with compound 43 could reproduce this phenotype the testis of treated and untreated mice were collected and weighed. The size of the testis where PRL 2 was inhibited were not smaller than untreated testis (Figure 9A,D). In fact, the testis treated by Compound 43 were slightly larger than DMSO controls (Figure 9A,D). This may be due to inflammation caused by off-site effects of the drug. Furthermore, no germ cells were observed in the lumen of the seminiferous tubules or epididymis of either treatment group when the testis were analyzed histologically (Figure 10 and Figure 11).

4.1.4 Weak PTEN Pathway Activation Detected after Three Week Treatment

To determine if Compound 43 could upregulate PTEN and downregulate PI3K-Akt signaling in the testis, I used whole testis lysate for Western Blot analysis. PTEN was found to be weakly upregulated in Compound 43 treated testis, as expected based on data from the PRL 2 KO mouse (Figure 12A,B). C-kit was also downregulated and apoptosis slightly increased (based on Cleaved PARP levels) in Compound 43 testis (Figure 9A). However, p-Akt was not downregulated in PRL 2 inhibited mice (Figure 9A). I would expect p-Akt to be decreased in Compound 43 mice in comparison to control mice, however, Akt phosphorylation seems to be upregulated in PRL 2 inhibited mice (14).

4.2 Discussion

This study shows that treatment with Compound 43 for three weeks is insufficient to reproduce the infertility phenotype present in the PRL 2 KO model although weak activation of PTEN was detected. The expected results were that treatment with Compound 43 would induce infertility, testis hypocellularity, shedding of c-kit positive germ cells into the lumen, and activation of PTEN as was observed in the genetic knockout of PRL 2. In fact, since the drug targets all three members of the PRL family equally, I expected greater degradation of spermatogenesis as is seen in PRL 2 KO: PRL 1 HT mice. However, the shedding of germ cells is a prolonged process in our genetic models, even with the combined partial deletion of PRL 1 (hypothesized to compensate for deleted PRL 2 in the testis). Thus, in future studies I would propose to treat the mice for 12 to 16 weeks. Furthermore, I would improve the statistical data by using larger cohorts of male mice in each treatment group. I would like at least 8-10 animals per group. Also, the discrepancy in the birth rates between PRL inhibited and DMSO treated groups may be due to the fact that the females used for breeding were not age matched and only two females per male was used. In future studies, I would like to have at least six females per male to assess pregnancy efficiency, and would like females to be no younger than 3 months old and no older than 5 months old. This will ensure accurate statistics and reduce the skewing of pregnancy rates due to the females being too old or too young.

Before this study, Compound 43 had not been assessed for toxicity. This is important for assessing the usability of Compound 43 as a male contraceptive.

One way to check for off-site effects is to measure testosterone, luteinizing hormone, and follicle stimulating hormone levels in the serum using an ELIZA assay (39). If the protein levels of these hormones differ from the control group, there is an increased chance that the PRL drug can disrupt organ systems and pathways outside of the testis in a toxic manner(39). The kidneys can also be assessed for histological evidence of kidney damage (39). Detection of blood in the urine is also indicative of drug toxicity(39). During long term treatments, the mice may suffer a general degradation in health and well-being.

Furthermore, if treatment of WT mice with Compound 43 for a longer period does result in infertility I would be interested in testing if the effect is reversible. Reversibility is particularly important if the drug is to be used as a contraceptive. In order to do this, I would treat cohorts of 8-10 male mice with Compound 43 or DMSO for a sufficient time to induce infertility (will be determined based of previous study and confirmed with a pregnancy efficiency test) then cease treatment in increasing and decreasing increments of time (3, 6, 9, 12, and 16 weeks). Then, the breeding and plugging efficiency tests will be repeated to determine if fertility is regained. Testis will also be collected and compared to the testis of mice treated with Compound 43 or DMSO to an equivalent point of infertility. If infertility caused by treatment with Compound 43 is reversible, I would expect a complete return of fertility and no difference in the histology and PTEN signaling of the testis between DMSO and Compound 43 treated groups.

Before this study, Compound 43 has never been used on mice as a tool to study PRL. If we find that Compound 43 does cause significant off-site effects in organ systems besides the testis it can still serve as a powerful tool to study the role of PRL in normal physiology. Our lab has created knockout lines of all three PRL family members, but only PRL 2 has shown an observable phenotype. There is evidence that the other PRLs can partially compensate for deleted PRL family members due to their high sequence and structural similarity. Thus, in order to truly dissect the role of PRLs *in vivo* a triple knockout must be developed. However, this had been confounded in our mouse models due to the embryonic lethality of PRL 1 and 2 double knock out mice. Compound 43, on the other hand, knocks out all PRL family member with equal affinity and can be delivered during adulthood to circumvent any lethal developmental abnormalities present in the genetic model. This has the potential to bring out new phenotypes in organ systems that express more than one PRL family member. I would like to treat WT C57BL/6 mice with Compound 43 and observe different PRL-rich organ systems such as the brain and nervous system, colon, bones, spleen, regenerating liver, and thyroid gland for new abnormalities. This can help close the knowledge gap in the role of PRL in normal physiology and may reveal additional potential binding partners for research. We can also check for increased severity in other phenotypes present in PRL 2 KO animals. PRL 2 KO animals have hypocellular placentas and a more recently discovered deficiency in hematopoiesis (12, 13, 26). PRL-2 null hematopoietic stem and progenitor cells (HSPCs) are more quiescent and show reduced activation of the Akt and

ERK1/2 signaling as a result of an increase in PTEN level (26). Furthermore, PRL-2 is found to be important for SCF-mediated HSPC proliferation and loss of PRL-2 decreased the ability of oncogenic KIT/D814V mutant in promoting hematopoietic progenitor cell proliferation (26). Thus, PRL-2 plays critical roles in regulating HSC self-renewal and mediating SCF/Kit signaling(26).

PRL was brought into the spotlight when it was discovered as an oncogene. Compound 43 can potentially be used for cancer therapy. In the future, we can treat mice injected with cancer cells with Compound 43 to determine if inhibition of PRL trimerization is sufficient to delay tumorigenesis. Compound 43 has great potential as a tool to study the role of PRL in cancer and in normal physiology in future studies.

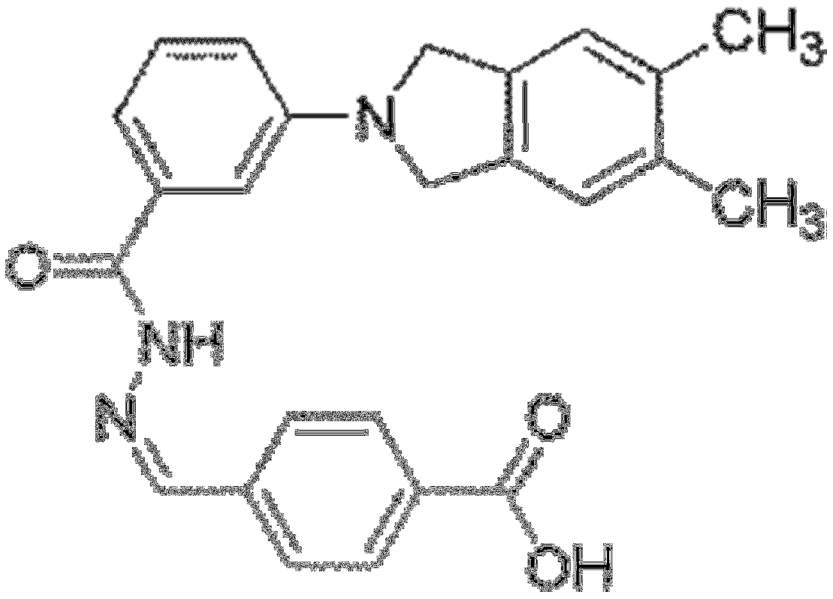


Figure 8. The chemical structure of Compound 43. Structure made by Yunpeng Bai.

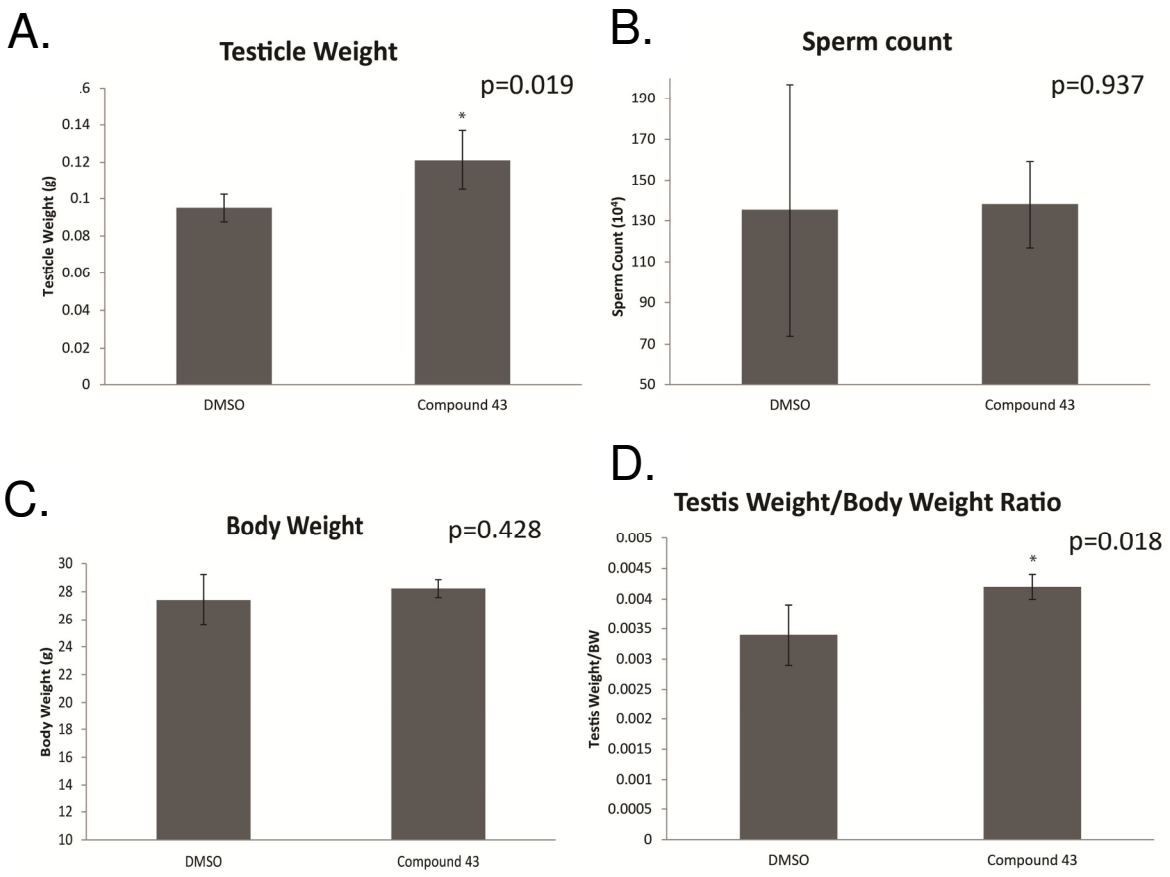


Figure 9. Treatment of C57BL/6 WT males with Compound 43 for three weeks did not replicate PRL 2 KO phenotypes. Expected results were that mice treated with Compound 43 would have reduced testis weight and body weight to testis weight ratio and have reduced sperm count. However, results

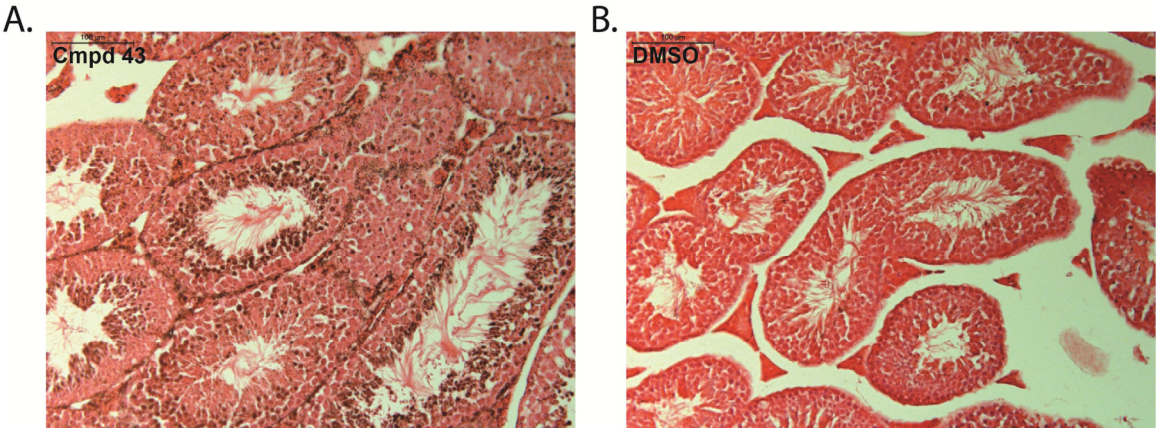


Figure 10. The seminiferous tubules of Compound 43 treated mice and DMSO treated mice are both healthy and have no shed germ cells in the lumen.

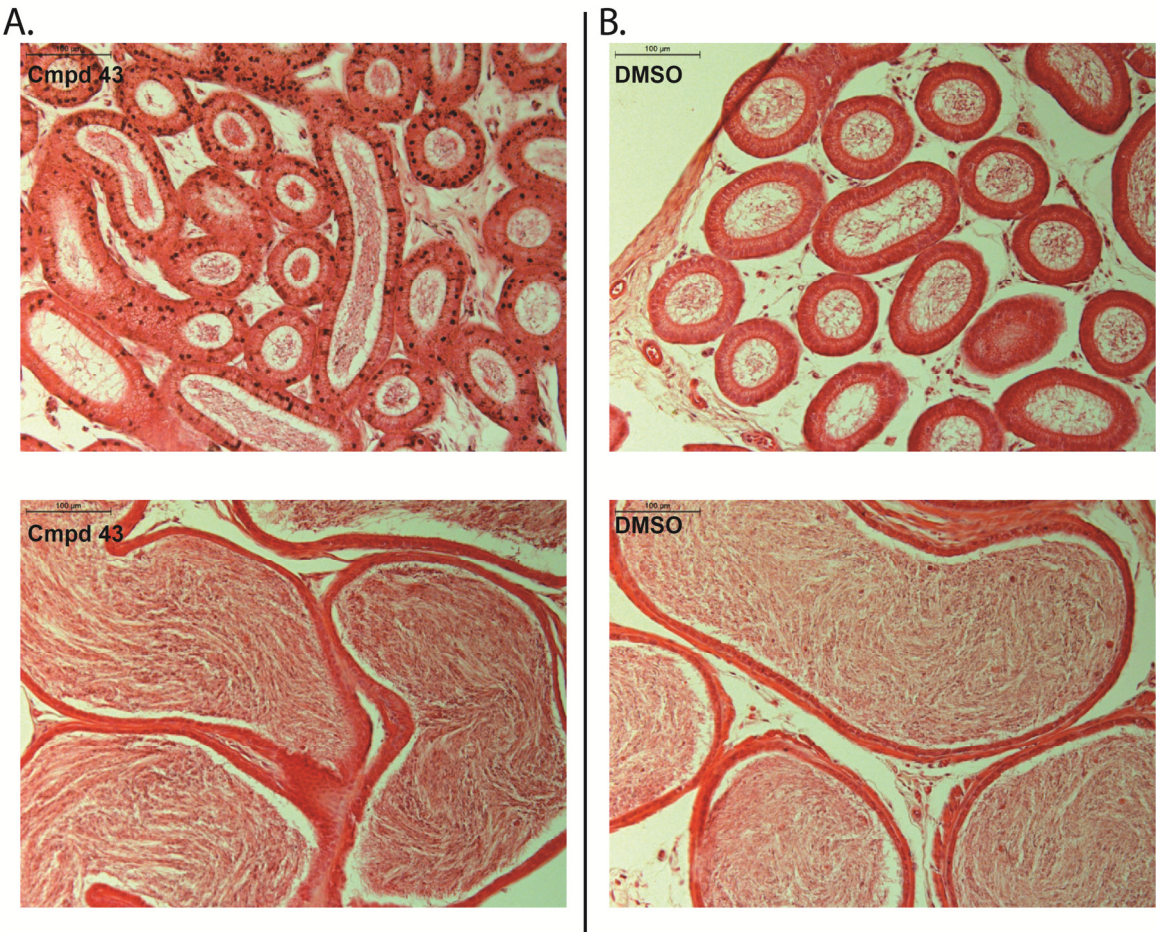


Figure 11. The epididymides of Compound 43 and DMSO treated mice. Healthy, contain no she germ cells and are full of mature sperm.

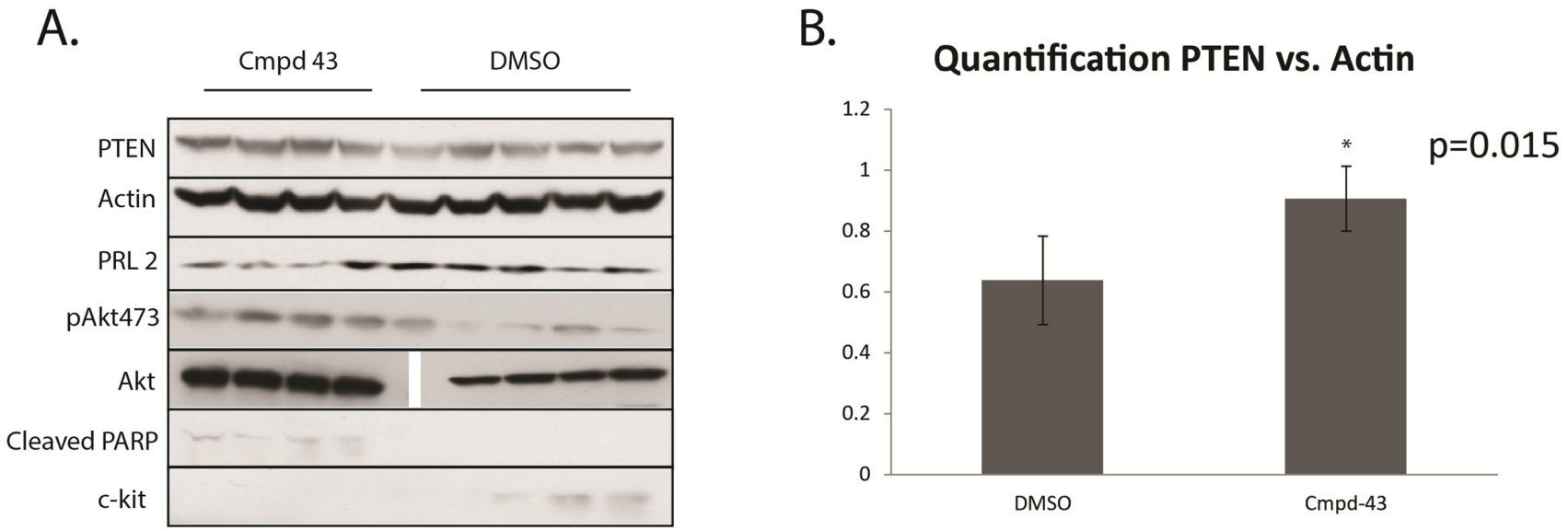


Figure 12. PTEN is upregulated in Compound 43 treated mice. A) As expected PTEN is upregulated in the whole testis lysate of PRL 2 inhibited mice in comparison to DMSO controls. C-kit is also downregulated and apoptosis is slightly elevated as indicated by Cleaved-PARP. Akt activation is strongly upregulated in Compound 43 treated mice, which is the opposite if what is expected. Might possibly be due to higher total Akt in compound treated mice in comparison to DMSO controls. B) Quantification of PTEN levels normalized to actin by ImageJ software. PTEN is significantly higher in Compound 43 treated testis.

CHAPTER 5: Creation of a Heterozygous PTEN Murine Cancer Model Crossed on PRL 2 Knockout Background

5.1 Mouse Model Generation

Using small molecular inhibitors is one way to investigate the role of PRL in cancer. Genetic models can also be used in conjunction with chemical models to provide more physiologically relevant data. Previous studies from our lab shows that PRL 2 can act as a negative regulator of PTEN in spermatogenesis (12). Since PTEN is one of the most commonly mutated tumor suppressors found in cancer, I generated a Cre-driven PTEN heterozygous knockout line crossed with our PRL 2 KO line to investigate whether PRL interaction with PTEN is relevant during tumorigenesis. Akira Suzuki et al. created a mouse line with traditional knockout deletion of PTEN on exons 3-5 and demonstrated that PTEN deletion can increase cancer risk in a dose-dependent manor (59). The PTEN Null mice were found to be embryonic lethal (59). However, heterozygous null mice were viable and exhibited an increased incidence of breast, thyroid, prostate, intestinal, brain, and liver cancer as well as T-cell lymphoma/leukemia by the time they reached six months of age (59). I hypothesize that PRL 2 deletion in a PTEN heterozygous murine model will delay the PTEN tumorigenesis phenotype.

5.1.1 Generation of PTEN flox/flox : PRL -/+ Mice

To create my PTEN/PRL 2 cross, I acquired a conditional knockout PTEN mouse line (C57BL/6 background) from Yan Liu (Indiana University School of Medicine, Department of Pediatrics) originally developed by Trotman et al. This PTEN

mouse model uses LoxP/Cre method of deletion with exons 4 and 5 flanked by LoxP sites. Upon introduction of Cre recombinase, PTEN will become truncated and inactive.

My first round of breeding involved two male PTEN flox/flox mice acquired from the Liu lab and four PRL 2 knockout female mice backcrossed onto C57BL/6 for seven generations arranged into two trios (Figure 13A). The resulting pups were all PTEN flox/+ and PRL 2 -/+. Next, I backcrossed the PTEN/PRL 2 double heterozygous offspring to their PTEN flox/flox father to generate PTEN flox/flox : PRL 2 -/+ mice (Figure 13A,B).

5.1.2 Generation of Cre-induced PTEN heterozygous Knockout Mice on PRL 2 KO background

To create a full body PTEN heterozygous knockout mouse on a PRL 2 knockout background I choose to purchase two C57BL/6 females homozygous for EIIA-Cre from Jackson Lab. EIIA-Cre puts Cre recombinase under the control of the strong, viral promoter, EIIA. I then create a single trio of two EIIA-Cre +/+ female mice crossed to one PTEN flox/flox : PRL 2 -/+ male (Figure 14A). The resulting litter should produce pups with one PTEN flox^{deleted} and one wildtype PTEN allele as well as either PRL 2 +/+ or PRL 2 -/+ (Figure 14A). From the first litter, I obtained my first PTEN flox^{deleted}/+ mouse as determined by PCR genotyping (Figure 14B). Unfortunately, she inherited two wildtype PRL2 alleles so she will have to be bred back into the PRL 2 KO line.

5.2 Discussion

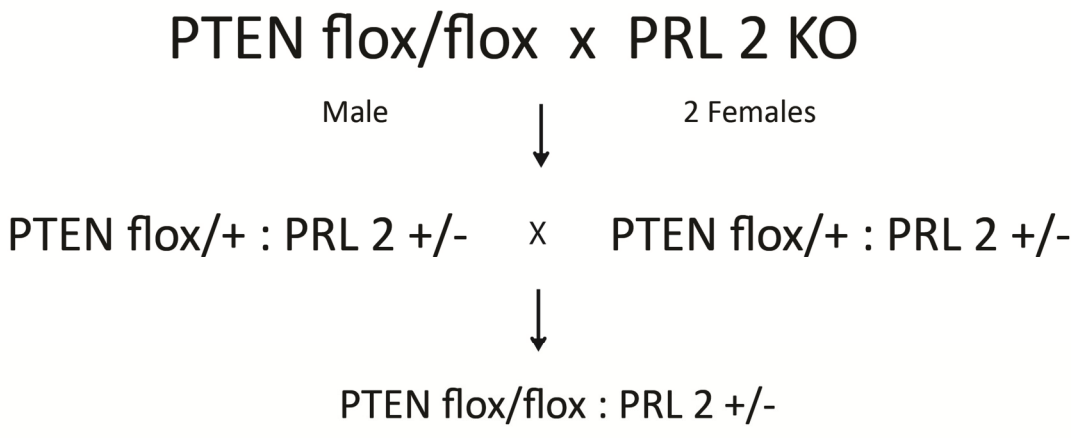
In this project I successfully obtained my first PTEN flox^{deleted}/+ mouse. Once I breed the female back into the PRL 2 knockout line I will obtain pups with PTEN flox^{deleted}/+ : PRL 2 +/- genotypes. My original cross of PTEN flox/flox : PRL 2 -/+ with the two EIIA-Cre females will also provide me with pups of this genotype. At this point I can dissect the pups to confirm ubiquitous deletion of PTEN via PCR and Western blot. I will then breed PTEN flox^{deleted}/+ : PRL 2 +/- mice together to obtain PTEN flox^{deleted}/+ : PRL 2 -/-, PTEN flox^{deleted}/+ : PRL 2 +/+, PTEN +/+ : PRL 2 -/-, and PTEN +/+: PRL 2 +/+ pups (Figure 15). These four genotypes will serve as the experimental and control cohorts for my future study on the effect of PRL 2 deletion on PTEN-driven tumorigenesis. I plan to generate 8-10 mice per genotype and gender, with PTEN flox^{deleted}/+ : PRL 2 -/-, PTEN flox^{deleted}/+ : PRL 2 +/+ as the experimental cohorts and PTEN +/+ : PRL 2 -/-, and PTEN +/+: PRL 2 +/+ serving as controls. I hypothesize that PTEN flox^{deleted}/+ : PRL 2 -/- mice will have a delayed tumorigenesis phenotype in comparison to PTEN flox^{deleted}/+ : PRL 2 +/+. I do not expect my control cohorts to develop PTEN-driven cancer. After establishing my cohorts, I will observe my mice throughout their lifespan. Mice will be monitored for cancer and end point determination will be based on signs of sickness and distress. The signs include: hunched posture, reduced food/water intake, lack of grooming, palpable tumors, and general health deterioration. At the end point, tumors from the mice will be counted and weighed. Tumors from the different cohorts will also be saved and examined for differences in tumor structure, cell death, cell proliferation, and development

through histology and immunohistochemistry. If PRL works up stream of PTEN as a negative regulator we should see a delayed tumorigenesis phenotype. On a western blot we should see PTEN levels higher than 50% and decreased Akt levels. If PRL does not affect the PTEN pathway there will be no difference between PTEN flox^{deleted}/+ : PRL 2 +/+ and PTEN flox^{deleted}/+: PRL -/-. Furthermore, PTEN level reduction in PTEN may also rescue the PRL 2 KO fertility and growth phenotypes.

While crossing PRL 2 with PTEN heterozygous cancer model is one way to interrogate PRL 2 interaction with PTEN, the model will take at least nine months to produce any noticeable difference in tumor load between the experimental and control cohorts. PRL 2 was originally hypothesized to be an oncogene. Thus, crossing PTEN flox mice to PRL 2 transgenic mice would quickly provide complementary confirmation to results gathered from the PTEN/PRL 2KO cross. I hypothesize that PRL 2 transgenic mice would act as a second-hit mutation for the PTEN heterozygous mice and expedite tumorigenesis in the PTEN flox^{deleted}/+:PRL2 Transgenic mice in comparison to PTEN flox^{deleted}/+:PRL2 +/+ controls. Cohorts will include 8-10 mice from the following genotypes: PTEN flox^{deleted}/+:PRL2 Transgenic, PTEN flox^{deleted}/+:PRL2 +/+, PTEN +/+:PRL2 Transgenic, and PTEN +/+: PRL 2 +/+. Mice with PTEN flox^{deleted}/+ will already be established as a line from the preceding study, simplifying the breeding strategy. PRL 2 transgenic mice have yet to be developed in our lab, however, once a transgenic line has been established I will cross PTEN flox^{deleted}/+ to PRL 2 transgenic mice to produce my cohorts. Again,

I will observe the mice until palpable tumors are present. The mice will then be sacrificed and the tumors collected and counted. Along with a heavier tumor load, I expect decreased PTEN protein levels if PRL works upstream of PTEN and no effect on PTEN if it works on a parallel pathway. As in the above study, Akt, p-Akt, ERK, p53, Src and their downstream targets will also be examined via western blot. Performing this study will allow us to evaluate PTEN as a potential target of PRL.

A.



B.

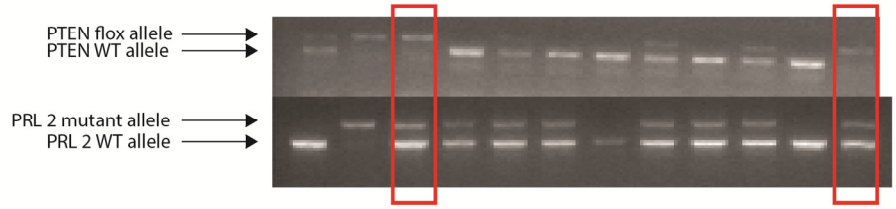


Figure 13. Breeding strategy to obtain PTEN flox/flox mice on PRL 2 background. A) Breeding strategy. B) The red boxes are examples of mice that have the desired genotype.

A.

PTEN F/F : PRL 2 +/- x EIIA-Cre +/+

↓

PTEN flox^{deleted}/+ : PRL 2 +/-

B.

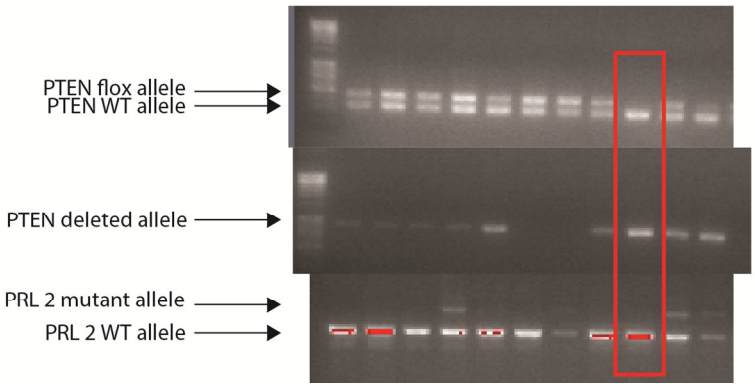


Figure 14. Strategy to obtain PTEN heterozygous Cre mice on PRL 2 background. A) Breeding strategy. B) The red box is mouse #48. She is the first PTEN heterozygous Cre mouse generated.

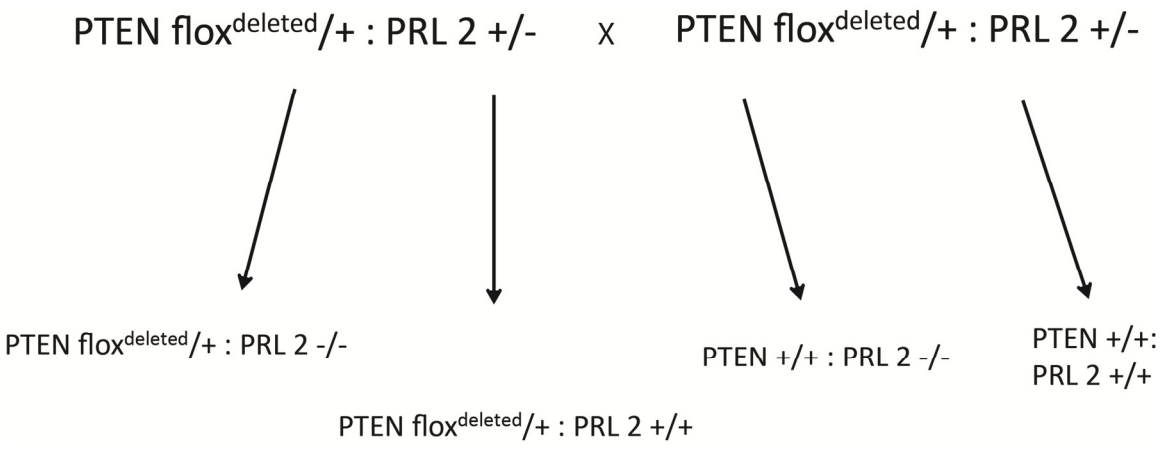


Figure 15. Breeding strategy for four experimental cohorts.

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