CYB5D2 IN BREAST CANCER

CYB5D2 AS A POTENTIAL TUMOR SUPPRESSOR OF BREAST CANCER

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Abstract

Breast cancer (BC) the second leading cause of cancer death in Canadian women.[1] The disease is affected by numerous genes with more to be discovered. Our lab has recently published cytochrome b5 domain containing 2 (CYB5D2) as a candidate tumor suppressor in BC.[2] My research further supports this concept. In a tetracycline inducible CYB5D2 lines previously constructed in HER2+ HCC1954 BC cells, induction of CYB5D2 inhibited cell proliferation. The inhibition can be partially recovered once induction pressure was removed; recovery level was associated with the length of CYB5D2 expression with longer CYB5D2 induction accompanied with reduced recovery ability. This in vitro inhibition is supported by in vivo evidence; in xenografts formed by HCC1954 Tet-CYB5D2 cells, induction of CYB5D2 via administration of doxycycline in drinking water significantly inhibited tumor growth. To examine the potential mechanisms underpinning CYB5D2-derived inhibition, I was able to co-immunoprecipitate (Co-Ip) CYB5D2 and PTEN (phosphatase and tensin homolog) following ectopic expression of both in 293T cells, indicating the formation of the CYB5D2-PTEN complex. This possibility is further supported by co-localization of both ectopic proteins in 293T cells detected by immunofluorescence analysis. Furthermore, in HCC1954 Tet-CYB5D2 cells, a complex containing CYB5D2 and endogenous PTEN was co-immunoprecipitated following induction of CYB5D2 expression. Collectively, evidence supports an association of CYB5D2 with PTEN. I

further characterized this association. Binding of PTEN with CYB5D2 was determined in 293T cells using a set of CYB5D2 mutants, including ΔCYB5, ΔTM, D86G, R7P, R7G, and Q167K. Complexes containing PTEN and individual CYB5D2 mutants could be precipitated via either CYB5D2 or PTEN, but not both. While the situation was not ideal, evidence nonetheless indicates that these structural elements of CYB52 were not essential for its association with PTEN.

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The experience of graduate studies gives me a deeper understanding of researching and myself. I met tremendous challenges and finally figured them out. This experience no doubt improves me a lot with my personal abilities and way of thinking. I am looking forward to starting my career in this field with all my experience and gains in my graduate study and hope to be able to contribute to the scientific community.

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List of abbreviations

AD	Activation domain
AP-1	Activator protein-1
ATCC	American Type Culture Collection
ATM	Ataxia-telangiectasia mutated
BC	Breast cancer
BDNF	Brain-derived neurotrophic factor
BRCA 1	Breast cancer type 1
BRCA 2	Breast cancer type 2
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin D Kinase
CK2	Casein Kinase ||
Co-Ip	Co-immunoprecipitation
CYB5	Cytochrome b5 domain
CYB5D2	Cytochrome b5 domain containing 2
CYP	Cytochrome P450
CYPOR	Cytochrome P450 reductase
D	Aspartic acid
DAPI	4’,6- diamidino-2-phenylinodole

DDR	DNA damage response
DJBL	Duodenal-jejunal bypass liner
DMEM	Dulbecco’s modified eagle’s medium
DNA	Deoxyribonucleic acid
DOX	Doxycycline
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
EREs	Estrogen receptor elements
ERK	Extracellular signal-regulated kinase
ETOP	Etoposide
EV	Empty Vector
FBS	Fetal bovine serum
G	Glycine
G1	Gap 1 phase
G2	Gap 2 phase
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HER2	Human epidermal growth factor receptor 2
HER2+	Human epidermal growth factor receptor 2 positive type

HR	Homologous recombination
HR+	Hormone receptor positive type
IF	Immunofluorescence
IHC	Immunohistochemistry
INSIG1	Insulin-induced gene 1
IP	Immunoprecipitation
K	Lysine
M	Mitotic phase
MAPK	Mitogen-activated protein kinase
MAPR	Membrane associated progesterone receptor
mTOR	Mammalian target of rapamycin
NENF	Neuron-derived neurotrophic factor
NET	Progestogen norethisterone
OS	Overall survival
P	Proline
PDK1	Phosphoinositide-dependent kinase 1
PGRMC	Progesterone receptor membrane component
PI3K	Phosphoinositol 3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
POR	Cytochrome P450 oxidoreductase

PR	Progesterone receptor
PSB	Protein sample buffer
PTEN	Phosphatase and tensin homolog
Ptet	Tetracycline-responsive promoter
Q	Glutamine
R	Arginine
RKT	Receptor tyrosine kinase
S	Synthesis phase
SCAP	SREBP cleavage-activating protein
SOS	the guanine-nucleotide exchange factor son of sevenless
Sp1	Specificity factor 1
SREBP	Sterol-regulatory element-binding protein
Tc	Tetracycline
TetO	Tet operator
TM	Transmembrane domain
TMA	Tissue microarray
TNBC	Triple negative breast cancer
tTA	Tetracycline transactivator
TSC	Tuberous sclerosis proteins
TetR	Tet repressor protein
VP16	Herpes simplex virus

WNT1	Wnt Family Member 1

Declaration of academic achievement

While I was the primary contributor for the work presented in this thesis, others have contributed to this project.

Dr. Damu Tang and I designed this project. I have carried out research. Former members of Dr. Tang's lab provided the HCC1954 Tet-ON and HCC1954 Tet-CYB5D2. Plasmids of CYB5D2 mutants were constructed previously. David Rodriguez generated xenograft in NOD/SCID mice using HCC1954 cells. I extracted RNA from the frozen tumor tissues and quantified CYB5D2 expression by qRT-PCR. Dr. Xiaozeng Lin, Yan Gu, Mathilda Chow provided experimental training and assistance.

Chapter 1: Introduction

1.1. Breast Cancer

1.1.1. Subtypes and Treatments

Breast cancer (BC) is the most common cancer among women worldwide. In 2020, the incidence of breast cancer in Canada was estimated to be 27,400 and 5,100 patients died from the disease, representing 25% of all new cancer cases and 13% of cancer deaths respectively in Canadian women. Approximately, 1 in 8 Canadian women is affected by the disease and 1 in 33 Canadian women might die from BC. Although to a much lesser extent, men are also affected; the number of new BC cases in Canadian men were predicted to be 240 and the mortality was 55.[3] Breast cancer is a major health problem both in Canada and worldwide.[4]

Based on the histopathologic features, 15-30% of breast biopsies are carcinoma in situ, and the rest are invasive carcinoma.[5] Breast cancer is categorized into 3 main histological subtypes based on the expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor 2 (HER2): hormone receptor-positive type (HR+), HER2+ type, and triple-negative type (TN).[6] Estrogen receptors and progesterone receptors are expressed in over 70% BCs and associate with

favorable prognosis. ER and PR are members of the nuclear receptor family of evolutionarily related ligand-activated transcription factors. ER and PR are highly conserved, sharing the typical structural features of the nuclear receptor family.[7] ER stimulates transcription via binding to estrogens (a type of steroid hormone, formed from cholesterol), especially 17β-estradiol (E2); the binding results in ER conformation changes, dimerization and the interaction with estrogen receptor elements (EREs), leading to increasing its transcription activity and the regulation of target gene expression.[8] (Fig. 1) Another typical function of estrogen is its nongenomic effects acting via a process independent of RNA metabolism. This nongenomic function might mediate gene expression via modulating signaling pathways that are relevant to transcription factors, like activator protein-1 (AP-1).[8] This effect is associated with mobilisation of intracellular calcium, activation of adenylyl cyclase activity and cyclic adenosine monophosphate (cAMP), activation of the mitogen-activated protein kinase (MAPK) signaling pathway, and activation of the phosphoinositol 3-kinase (PI3K) signaling pathway.[8] (Fig 1) Some reports indicate that ERα and ERβ located at the plasma membrane also show the nongenomic effect.[9] ERα and ERβ are encoded by different genes. ERα contributes to the development of mammary gland and proliferation, while ERβ often associates with differentiation, anti-proliferation and pro-apoptosis and can counteract the proliferation effect induced by ERα.[7] Besides, ERβ plays a more critical role on central nervous and immune systems.[8]

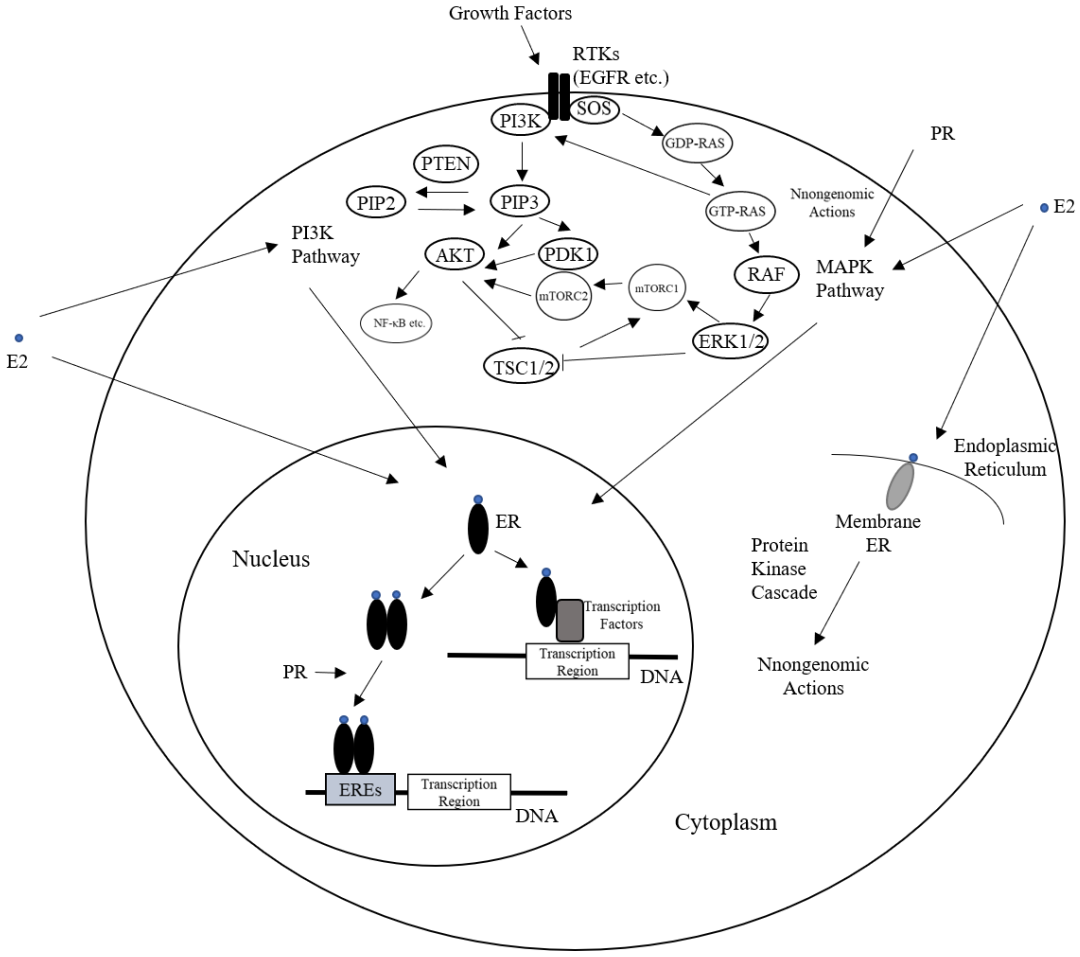


Figure 1. A schematic outline of the PI3K, MAPK and ER signaling pathways with respect to ER signaling. Interactions of among the indicated pathways are indicated. E2: estrogens.

As an ER-target, progesterone receptors can associate with ER and contribute to gene expression with good clinical outcome. Some observations suggest that the activation of PR exerts anti-tumorigenic effect in estrogen-driven ER+ BCs.[10] In ER signaling pathway, the activated PR binds to ERα and mediates the interaction between the complex PR/ERα/p300 (a co-activator) and DNA.[10] PR has two forms, PRA and PRB, with tissue-specific functions: mice without PRA have defects in uterine

development and reproductive function; mice lack of PRB are associated with abnormal mammary gland development.[7] The ratio of PRA/PRB is relevant to the tamoxifen resistance and BC prognosis.[11] Phosphorylation of PRB correlates with response of estrogen and transcription activities regulated by CDK2. Phosphorylated PR contributes to tamoxifen resistance and poor prognosis in BCs. The complex containing phosphorylated PR, select specificity factor 1 (Sp1), and cyclin D1 is required for cell cycle progression at the G2/M phase.[10] As for the nongenomic effects, PR contributes to rapid activation of the MAPK pathway and facilitates progesterone treatment in BCs via regulating WNT1; WNT1 signaling stimulates the action of epidermal growth factor receptor (EGFR, a tyrosine kinase receptor involved in the activation of oncogenic signaling pathways), and thus prolong the activity of the MAPK signaling pathway.[12]

Another molecule target in breast cancer is the human epidermal growth factor receptor 2, encoded by the HER2 oncogene, a member of the human epidermal growth factor receptor family.[13] HER2 is a membrane tyrosine kinase that promotes cell proliferation and survival. Around 15%-20% of breast cancers are HER2+ and the rate of HER2+ is higher in metastatic BCs.[14] HER2 dimerizes and stimulates tumorigenesis via activating several intracellular signaling pathways, including PI3K/Akt pathway, Ras/mitogen-activated protein kinase pathway etc. by transphosphorylation.[15] Compared with its homodimers, HER2 heterodimers has the strongest signal transduction capacity with higher kinase activity.[14] The partnership between HER2 and

HER3 is crucial for various developmental processes.[15] HER2 overexpression increases its dimerization with EGFR and HER3. EGFR-HER2 heterodimers reduces endocytic degradation as degradation of EGFR by this process favors EGFR homodimerization. By sustaining its presence, HER2 enhances its promotion of BC via forming heterodimer with EGFR. HER3 contributes to the activation of PI3K/AKT pathway via heterodimerizing with HER2.[15] Besides, it is reported that Src kinases facilitate HER2 functions at multi-levels from upstream to downstream through protein-protein interaction involving SH2 domain.[15]

Hormone receptor-positive BCs include ER+ and/or PR+ but HER2- tumors; these cancers rely on hormone signaling and can be treated through endocrine therapy using tamoxifen (a selective estrogen receptor modulator competitively inhibiting the binding between estrogen and ER) and aromatase inhibitors (reducing circulating estrogen level and only effective in postmenopausal women).[7,16] For patients treated with chemotherapy, ovarian suppression plus exemestane is superior to tamoxifen, while tamoxifen is more tolerable than ovarian suppression with exemestane during the first 2 years.[17] It is recommended that patients should receive endocrine therapy for at least 5 years.[17] Resistance remains a major issue. Some studies have shown that extending the usage of both tamoxifen and aromatase inhibitors can only have small benefits with additional toxicity[6].

HER2+ subtype BC is classified based on HER2 expression regardless of the presence of ER or PR; these BCs are treated with a targeted therapy. The most common treatments include using trastuzumab (a monoclonal antibody targeting the extracellular domain of HER2) which should be used in addition to chemotherapy for one year[17-18] and newer drugs such as small molecule tyrosine kinase inhibitor lapatinib, as well as others such as pertuzumab (another monoclonal antibody targeting a different area on the HER2 receptor than trastuzumab[17]) and ertumaxomab.[19]

Triple-negative BCs do not express all three receptors; this subtype accounts for 15% of breast cancer cases[20]. Compared with the other two types of BCs, TN BCs are associated with higher rates of recurrence and 5-year mortality.[21] Besides, triple-negative breast cancer occurs more frequently in young women and is associated with alterations in BRCA1.[22] Therapeutic options for triple-negative BCs are mainly chemotherapy as TNBC is not sensitive to endocrine therapy or molecular targeted therapy.[8] However, due to the limited therapy option, TNBC is likely to relapse and metastasize with a poor prognosis.[23] Therefore, optimization of chemotherapy regimens is important for treatment outcome and prognosis. Currently, the combination of adriamycin, cyclophosphamide and other drugs like taxel/docetaxel or fluorouracil is preferred.[23] The monoclonal antibody bevacizumab is another way to treat TN breast tumors in combination with paclitaxel, aiming at angiogenesis.[24] Although discovering new targets for treatment is difficult in TNBC, there are several potential targets in

researching, including EGFR, PARP inhibitors, androgen receptor and estrogen receptor ER-α36. Of note, the application of immunotherapy like PD-LI, CTLA-4 in TNBC also seems promising.[23]

1.1.2. BC Genetics

A variety of genes are associated with BC pathogenesis, including BRCA1, BRCA2, TP53, PTEN, STK11, CDF1, ATM, CHEK2, etc.[25]

Breast cancer susceptibility gene 1 (BRCA1) and gene 2 (BRCA2) are two major genes related to breast cancer. The lifelong risk of breast cancer is around 60%-85% if mutants occur in either of these two genes. BRCA1 is important in the development of the mammary gland and plays important roles in DNA damage repair, transcriptional regulation, chromatin remodeling and cell cycle. BRCA2 is also involved in DNA recombination and repair processes.[26] In response to DNA damage, BRCA1 is likely to be phosphorylated at different residues by different kinases. The homologous recombination (HR) plays a central role in the repair of double-strand DNA breaks to maintain the genomic integrity. HR is highly dependent on ataxia-telangiectasia mutated (ATM), BRCA1 and BRCA2.[27-28] In this process, ATM phosphorylates BRCA1, which plays a major role in HR along with BRCA2 and RAD51.[29] BRCA1 acts more as a signal integrator in DNA damage response, while BRCA2 mainly regulates RAD51 activity by recruiting RAD51 to DNA damage site and facilitating a transition of

inactive RAD51 to its active form.[30] Although BRCA1 might not bind to RAD51 directly, it regulates the length of single strand DNA generation at DNA damage sites by binding to DNA directly and regulating the complex of MRE11/RAD50/NBS1 (MRN).[31] At transcriptional level, BRCA1 regulates transcription through forming a complex with RNA polymerase II via RNA helicase A. In addition, BRCA1 also binds to several transcriptional factors, like ZBRK1 and STAT1. While the effect of BRCA2 in transcription requires more studies, it is likely that BRCA2 activates transcription by modulating histone acetylation via association with p300/CBP. Moreover, BRCA1 is required for the actions of G2/M checkpoint.[29] Besides, BRCA1 functions in breast epithelial differentiation, causing the transition of ER-negative breast epithelial stem cells to ER-positive luminal progenitors, which may be one of the reasons for the association of BRCA1 dysfunction with basal-like breast cancer (ER-negative phenotype).[31-32] Association of BRCA2 with ER-negative BCs is also reported.[33]

PI3K/AKT pathway plays a key role in many cellular functions and its abnormal activation is a major cause of tumorigenesis, including breast cancer formation.[34] In general, phosphatidylinositol 3-kinase (PI3K) converts PIP2 to PIP3 which recruits AKT and phosphoinositide-dependent kinase 1 (PDK1) to plasma membrane via their pleckstrin homology (PH) or PH-like domain. PDK1 phosphorylates and activates AKT to initiate the downstream signaling cascades.[35] The phosphatase and tensin homolog

deleted from chromosome 10 (PTEN) on the other hand inhibits the effect of PI3K by triggering the conversion of PIP3 to PIP2; PTEN is a critical negative regulator of the PI3K pathway[34-35] and is also subjected to feedback regulations by PI3K pathway. PI3K can regulate PTEN transcription by engaging NF-κB, PPARβ/δ and TNFα; NEDD4-1, an E3 ligase for PTEN, can inactivate PTEN by ubiquitinoylation[36]. There are multiple genomic aberrations facilitating PI3K/AKT pathway activation.[35] In breast cancer, about 21.4% of cases are related to the mutation in PIK3CA (the gene encoding p110α, a subunit of PI3K), especially in the regions involved in Akt activation and downstream signaling[37-39]. PIK3CA mutations occur more commonly in HR+ (34.5%) and HER2+ (22.7%) tumors compared to triple-negative tumors (8.3%). Although the PTEN gene is mutated in only 2.3% of cases, PTEN function is compromised in around 30% of BCs via other mechanisms at the RNA and protein level.[35,40] Furthermore, both AKT1 and AKT2 are amplified in breast cancer. Additionally, PI3K pathway is well known to be activated by cell surface growth factor receptor signaling, including HER2 and other signaling molecules in cells.[35,37] (Fig 1)

Ras/MAPK pathway is another crucial signaling pathway important for cell proliferation. Ras is in an active form when associated with GTP and transmits the growth-promoting signals from the cell membrane to the nucleus. Mutations in the codons of 12, 59, or 61 sustain Ras at the active form and thus cause malignant transformation.[41] Although Ras is infrequently mutated in breast cancer, it is a common

target in other signaling events, including those occurring in EGFR, HER2, and other growth factor receptors. Overexpression of EGFR and HER2 occurs in approximately 20-50% of breast cancer and these events are associated with poor patient survival. Collectively, these observations support a common activation of Ras in breast cancer.[42-44] Besides, the c-FMS/colony stimulating factor-1 receptor signaling through Ras are reported in breast cancer.[45] As a target of RAS, mitogen-activated protein kinase (MAP kinase) is in part activated by the binding of epidermal growth factor (EGF) to its receptor tyrosine kinase (RKT). Following ligand binding, RTKs are dimerized and activated to provide dock sites to downstream proteins, which leads to recruiting effectors to plasma membrane, including the guanine-nucleotide exchange factor son of sevenless (SOS). The presence of SOS promotes the change of GDP to GTP on RAS, resulting in RAF recruitment to plasma membrane where RAF activates MAPK kinases via phosphorylation. MAPK kinases then phosphorylate and activate ERK1/2 which subsequently promotes cell cycle progression and tumor survival by regulating cytosolic proteins and transcriptional factors.[46-47] (Fig 1) Approximately 50% of MAP mutations are hyperactive in BC.[43,48] MAP activation is associated with estrogen signaling. In ER+ BC, MAPK pathway promotes cell proliferation in part via activation of ER independent of estrogen. Also, MAPK pathway can be activated in BC by estrogen and progesterone through their non-genomic association with membrane-associated receptors like G-protein coupled receptors.[49] Moreover, Ras mutations can result in hyperactivity of MAPK pathway.[42,47] On the other hand, tuberous sclerosis

complex 1 and 2 (TSC1/2) tumor suppressors, which integrates the information from PTEN, PI3K, and Akt, Ras/MAPK pathway can be linked with PI3K/AKT pathway and regulate cell survival and proliferation.[48] MAPK pathway can cross talk to PI3K pathway in which the mammalian target of rapamycin (mTOR) signaling is involved. (Fig 1) As the regulation loops in these two pathways are complicated and mutations of key components in PI3K and MAPR often link to each other, the single blockade of one pathway may cause the activation of another and tumors can also escape from the treatment by this strategy.[49]

1.2. MAPR family and CYB5D2

1.2.1. MAPR family

Membrane associated progesterone receptors (MAPRs) are a group of small and partially homologous proteins with a transmembrane domain (TM) and cytochrome b5 domain (CYB5), a heme binding motif. MAPRs include four members: progesterone membrane component 1 (PGRMC1), PGRMC2, neudesin (NENF), and neuferricin (CYB5D2). These proteins share structural similarities, have different expression pattern, and possess multifunctional properties, from cholesterol/ steroid biosynthesis, drug metabolism, to tumorigenesis.[50-51] Especially, MAPR family proteins show a particular expression pattern in breast cancer.[51]

1.2.1.1. PGRMC1

PGRMC1 is the most studied member in the MAPR family. The protein has 195 amino acids and is mainly expressed in the kidney and the liver in mammals but can also be detected in other organs, like breast, heart, lung, skeletal muscle, pancreas etc. The localization of PGRMC1 can be the nucleus, cytoplasm, plasma membrane, smooth endoplasmic reticulum, and mitochondria.[51-53] Although as a member of MAPR family, PGRMC1 does not interact with progesterone directly.[54] Functionally, PGRMC1 is involved in divergent cellular activities, including cholesterol/steroid biosynthesis and metabolism by activating the P450 protein Cyp51/lanosterol demethylase, iron homeostasis and heme trafficking, promotion of autophagy, regulation of cell cycle,

proliferation, cell death, cell migration, cell invasion, cancer development and others.[52-53,55-56] In addition, some reports also suggest PGRMC1 being a potential drug target as a binding site of sigma-2 receptor, a biomarker for tumor cell proliferation and an crucial molecular target of radiotracers for tumors imaging.[57-59] PGRMC1 is a signaling adaptor in processes driven by kinase or ligand.[60]

PGRMC1 is overexpressed in a set of different cancers, such as breast, ovarian, colon, lung, neck and oral cancers.[61] In cancers, dioxin can induce PGRMC1 to facilitate tumorigenesis (actually, PGRMC1 was originally discovered through a screen for dioxin-inducible genes[58]); PGRMC1 is part of a six-gene signature associated with nongenotoxic carcinogens.[62-63] The reduction of PGRMC1 caused a series of changes in several mouse xenograft cancer models, including decreases in tumor numbers and size, attenuation of tumor growth, and lessening of metastasis.[55] In vitro, PGRMC1 enhances tumor growth through the progestogen norethisterone (NET). In vitro, it facilitates MCF7 cell proliferation via estrogen actions.[64-65]

PGRMC1 contributes to cancer cell chemoresistance by its dimerization mediated by Y113 after binding with heme, which leads to activation and interaction with CYP450. In doxorubicin resistance, PGRMC1 facilitated drug degradation and conversed it to an inactive status. Similar effects might also exist in other resistance against chemotherapeutics. Moreover, PGRMC1 interacts with EGFR directly,

modulates the phosphorylation of EGFR in response to ligand EGF and the down-stream target, such as AKT and ERK etc. and therefore mediates EGFR signaling pathway.[66-67] In addition, elevated expression of PGRMC1 by ERα implicates the association between PGRMC1 and ER signaling pathway. In fact, estrogen-dependent PGRMC1 function in cancer is consistent with the changes in PGRMC1 phosphorylation between ERα+ and ERα- breast cancer.[68] Through forming a complex with the sterol-regulatory element-binding protein (SREBP), SREBP cleavage-activating protein (SCAP) and insulin-induced gene 1 (INSIG1) in ER, PGRMC1 regulates steroidogenesis and cell metabolism, which is important for cancer cells growth.[67]

Both PGRMC1 and PGRMC2 function in M phase progression. PGRMC1 stabilizes spindle in a P4-dependent manner[69-70] and co-localizes with the mitotic kinase aurora kinase B at the midbody of the meiotic spindle in oocytes at metaphase II of meiosis.[71-72] In addition, PGRMC1 is involved in cell signaling in BC via facilitating Akt and NFκB activation.[68,73]

1.2.1.2. PGRMC2

PGRMC2 is much less studied compared with PGRMC1. Both proteins share a high degree of homology and likely they were diverged from a common gene.[74] Nonetheless, there are structural differences between both proteins. PGRMC1 has a

SH3 target sequence sitting between the TM domain and the CYB5 motif and a consensus CK2 (Casein Kinase Ⅱ, a member of the superfamily of eukaryotic protein kinases) site. These structural elements are absent in PGRMC2.[60] PGRMC2 has 247 amino acids in length[74] and is localized in endoplasmic reticulum in SKOV-3 ovarian cancer cells.[75] PGRMC2 resides in the cytoplasm of epithelial cells of functional and basalis glands in the endometrium of macaques under physiological conditions and partially relocates to the nucleolus under endometriosis.[76] This feature implies an association of PGRMC2 with endometriosis. PGRMC2 is expressed in multiple tissues, especially in the placenta, with different expression profiles. In breast cancer, PGRMC2 is elevated in later stage compared to the earlier one.[74] Functionally, a recent study reveals that PGRMC2 plays a crucial role in adipocyte function as an intracellular heme chaperone. PGRMC2 transfers heme from mitochondria-bound PGRMC1 to endoplasmic reticulum. Through regulation heme trafficking, PGRMC2 is involved in the regulation of mitochondrial function in brown adipocytes and affects cell metabolism. The activation of PGRMC2 can reverse the metabolic syndrome caused by the deficiency of PGRMC2 via increasing heme flux to the nucleus.[77] In cancers, unlike PGRMC1 being oncogenic, PGRMC2 is likely a tumor suppressor. PGRMC2 is commonly lost in nodal metastasis of endocervical adenocarcinomas, suggesting that PGRMC2 suppresses endocervical adenocarcinoma metastasis.[78] These observations are in agreement with a negative impact of PGRMC2 but not PGRMC1 in regulating SKOV-3 cell migration.[75]

1.2.1.3. Neudesin

Neudesin was firstly identified as a secreted protein with neurotrophic activity from mouse embryos and thus named as neuron-derived neurotrophic factor (NENF).[79] Neudesin is preferentially expressed in the brain and spinal cord in mouse embryo but is also expressed in different postnatal tissues, including brain, adipose tissue, heart, lung, and kidney. Human neudesin is a secreted protein with 172 amino acids in length.[80] It plays a role mainly in neuronal function, energy metabolism, and tumorigenesis. Knockout of neudesin causes anxious behavior in mice.[81] The expression of neudesin in the hypothalamus could be impacted by brain-derived neurotrophic factor (BDNF, a sort of a key regulator of appetite), and the addition of neudesin led to reductions in food intake and body weight.[74] With respect to energy metabolism, neudesin is abundantly expressed in mouse white adipose tissue and remarkably suppresses adipogenesis in 3T3-L1 preadipocytes, suggesting that neudesin is a regulator of adipogenesis.[82] Moreover, knockout of neudesin in mice resulted in increases in sympathetic activity and energy expenditure, heat production, and fatty acid oxidation in brown adipose tissue and enhanced lipolysis in white adipose tissue.[83] These observations indicate neudesin as a regulator of energy homeostasis and food intake. A recent study containing 15 obese diabetic patients shows that circulating neudesin concentrations was significantly elevated after endoscopic duodenal-jejunal bypass liner (DJBL, an intervention affecting energy homeostasis) implantation with weight loss and glucose control. This study reveals the possibility of the existence of

the relationship between neudesin level and energy homeostasis.[84] Neudesin plays a role in tumorigenesis. Its upregulation was observed in carcinomas of the breast, uterine cervix, colon, lung, and skin as well as in leukemia and lymphoma. Of note, neudesin protein was expressed at higher levels in ER+/PR+ tumors than ER+/PR-tumors. Neudesin promotes the invasiveness of MCF7 cells in vitro and the cell's ability of forming tumors in vivo likely via affecting the MAPK and PI3K pathways. Besides, silencing of neudesin inhibited the proliferation and invasion of cancer cells and suppressed the actions of several signaling pathways, including those of AKT, WNT, and MAPK.[85] In addition, Neudesin Quotient value (cerebrospinal fluid neudesin concentration divided by serum neudesin concentration) could be a new biomarker in astrocytic brain tumor, as it was statistically higher value in astrocytic brain tumor patients and influenced by fewer factors, like sex.[86]

Neudesin is involved in the MAPK signaling pathway as well as PI3K pathway and can enhance the phosphorylation of extracellular signal-regulated kinase (ERK)1/2. This effect may be related to the Gi/Go-protein-coupled signaling pathway.[80] In neural precursor cells, the activity of neudesin may be influenced by the Gs protein-coupled signaling pathway, causing increases in cAMP. Overall, the mechanism of neudesin's effects is exerted via the G protein-coupled signaling pathway.[87]

1.2.2. CYB5D2

Cytochrome b5 domain containing 2 (CYB5D2, also called neuferricin), was first reported as heme-binding protein. The CYB5D2 gene is located at 17P13. 2, where its loss or alterations often occurred in several cancers, including breast cancer. Human CYB5D2 consists of 264 amino acids with a N-terminal TM domain and a C-terminal CYB5 motif.[88] There are three polymorphisms in CYB5D2, R7P, R7G (in colon cancer), and Q167K (from Mammalian Gene Collection).[89-90] We and others have shown that CYB5, especially the residue D86 in this region is required for heme binding and the mutant D86G is heme-binding defective. CYB5D2 binds to type b heme via its heme binding region.[91] As a member of MAPR family, similar with PGRMC1, CYB5D2 interacts with cytochrome P450 reductase (CYPOR), a common redox enzyme required by microsomal cytochrome P450 monooxygenases for their catalytic activities.[91] In high five cells, recombinant CYB5D2 was secreted into media, implying CYB5D2 might be an extracellular protein. CYB5D2 suppressed the proliferation of Neuro2a cells and enhanced neurogenesis by suppressing the self-renewal of undifferentiated neural cells.[92] The potential mechanisms governing CYB5D2-mediated suppression of cell proliferation may be associated with Bcl-2, an anti-apoptotic protein. [92] Moreover, the heme-binding region (residues 55-98) of CYB5D2 was deemed to be important for inhibition of Neuro2a cells survival.[88,92] Since murine CYB5D2 is a secreted protein when produced in high five cells, we also examined the association of its secretory

ability with heme-binding function. According to the results in 293T cells, the secretion of ectopic CYB5D2 required heme-binding but not transmembrane domain.[91]

CYB5D2 enhanced Hela cells survival of etoposide (ETOP, topoisomerase II poison)-mediated cytotoxicity but did not affect ETOP-induced DNA damage response (DDR) as ectopic CYB5D2 might not activate G2/M arrest induced by ETOP. On the other hand, CYB5D2 could not protect cells from UV-induced cytotoxicity and TNFα-induced apoptosis, indicating that CYB5D2 has no impact on the UV-involved mitochondrial pathway and TNFα-associated death receptor, the central parts of apoptosis. This result is consistent with the protection effect provided by PRGMC1 against doxorubicin toxicity (also a kind of topoisomerase II poison). Both the TM and CYB5 domains are necessary for CYB5D2 to confer cells resistance to ETOP.[93] Notably, ectopic CYB5D2 enhanced Hela cells resistance to paclitaxel (mitotic inhibitor) and cisplatin (DNA cross-linking inducer) treatment. In comparison to D86G mutant defective in heme binding, association with heme was required for CYB5D2 in sustaining Hela cells survival from chemotherapeutic agents.[91] CYB5D2 suppresses HeLa cell-derived oncogenesis in vitro and in vivo.[94] In vitro, CYB5D2 reduced HeLa cell invasion, in which the residue R7 was required.[94] This residue borders the tentative TM domain of CYB5D2 at the N-terminus, indicating the involvement of this potential TM motif in CYB5D2-derived inhibition of HeLa cell invasion.[94] In comparison, the D86G CYB5D2 mutant retains full capacity in inhibiting HeLa cell invasion[94],

suggesting heme binding being dispensable in the inhibition. Downregulation of CYB5D2 in cervical cancer tissues compared to normal cancer tissue was demonstrated, supporting CYB5D2 being a tumor suppressor of cervical cancer. [94]

The *CYB5D2* gene resides in a region which is often lost in breast cancer; this genetic knowledge together with CYB5D2-derived suppression of in cervical cancer, we reasoned a similar tumor suppression role of CYB5D2 in breast cancer. This possibility is consistent with decreases in CYB5D2 mRNA expression in breast cancers compared to normal breast tissues based on cancer genomic/gene expression data available in the Oncomine database.[2] Furthermore, within BC, there was a reducing CYB5D2 expression in the more aggressive subtype, like ER-, PR-, and HER2+ or TN subtype. BC patients with reduced CYB5D2 expression had a significant reduction in overall survival (OS).[2] CYB5D2 suppressed cell proliferation in both MCF7 (ER+) and HCC1954 (HER2+) cells. Transient expression of CYB5D2 decreased ER-derived transcription.[2] In HCC1954 cells, the potential mechanism might be related to apoptosis induction and G1 arrest. Again, we analyzed the expression of CYB5D2 mRNA and found its downregulation in ER+, HER2+, and triple-negative types of breast cancer. Besides, we found the downregulation being related to mutations in PIK3CA, GATA3, MAP3K1, CDH1, TP53, and RB1.[2,88]

1.3. Tet-On System

Investigations of tumor suppressors are more challenging compared studies of oncogenes owing to the intrinsic inhibitory activities of tumor suppressors on cell proliferation. A solution to this hurdle is to control or induce tumor suppressor expression. Accordingly, we chose the tetracycline (tet) inducible system to examine CYB5D2-mediated suppression of breast cancer. In Escherichia Coli, Tet repressor protein (TetR) represses TetA expression via binding to its operator (tetO); TetA is a membrane pump that effluxes tetracycline (Tc) and its derivatives doxycycline (DOX). Binding of Tc or DOX to TetR prevents TetR to interact with TetO, allowing TetA expression and thus conferring E. Coli resistance to tetracycline.[95-97] This setting is known as Tet-off. In the Tet-On inducible system, rTetR (a mutant from TetR) adopts confirmation to bind tetO following its association with tetracycline or DOX; the binding of rTetR-tet (or DOX) complex to tetO induces TetA expression. To utilize this property to regulate gene expression in eukaryotic cells, the transcription activation domain (AD) of the herpes simplex virus (VP16) protein was fused to rTetR, which yielded the tetracycline-controlled reverse transcriptional activator (rtTA). Then, rtTA could bind with Ptet (tetracycline-responsive promoter, produced by fusing tetO sequence with the TATA-box) to activate the transcription of design genes.[97-98]

1.4. Hypothesis and research aims

1.4.1. Central Hypothesis

Based on the effect of CYB5D2 on cervical cancer and our previous work in breast cancer, we hypothesize that CYB5D2 suppresses breast cancer.

1.4.2. Objectives

Aim#1

To characterize CYB5D2-induced inhibition of HCC1954 cell proliferation using the Tet-ON inducible system.

Aim#2

To examine CYB5D2-derived inhibition of on HCC1954 cell-produced xenograft growth in NOD/SCID mice.

Aim#3

To analyze a potential relationship between CYB5D2 and PTEN, which may shed light on the mechanisms by which CYB5D2 inhibits breast cancer tumorigenesis.

Chapter 2: Materials and Methods

2.1. Cell culture

293T cells were purchased from American Type Culture Collection (ATCC) and cultured in DMEM media supplemented with 10% FBS (Sigma Aldrich) and 1% Penicillin-Streptomycin (Thermo Scientific Fisher). HCC1954 Tet-ON and HCC1954 Tet-CYB5D2 cells were constructed before[88] and cultured in RPMI1640 media with 10% FBS (Sigma Aldrich) and 1% Penicillin-Streptomycin (Thermo Scientific Fisher).

2.2. Colony formation assay

HCC1954 Tet-ON and HCC1954 Tet-CYB5D2 cells were seeded in 6-well tissue culture plate at 1×10^3/well. Cells were incubated in DOX containing medium for 11 days (except control cells) at 37°C and 5% CO_2, changing fresh media with or without doxycycline every day. Cells were fixed for 20 minutes in a solution containing 2% formaldehyde, 0.2% glutaraldehyde. Then, cells were stained with crystal violet solution (0.5% crystal violet, 20% methanol, 150 mM NaCl) for 20 minutes. Plates were rinsed with water after staining and air-dried. Colonies were then quantified. Both Tet-ON and Tet-CYB5D2 cell lines were treated by 1μg/ml of doxycycline with different length.

2.3. Western blot analysis

Cell lysates were prepared in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM b-glycerophosphate, 0.1 mM sodium orthovanadate, 1mM PMSF, 10 μg/ml aprotinin and 2 μg/ml leupeptin. 50 μg of cell lysate proteins were separated by SDS-PAGE and subsequently transferred onto Amersham hybond ECL nitrocellulose membranes (Amersham, Baie d'Urfe, QC). Membranes were then blocked by 5% skim milk for one hour at room temperature followed by incubation with serum or primary antibodies overnight at 4°C. Signal was developed by incubating membrane with corresponding secondary antibody and an ECL Western Blotting Kit (Amersham, Baie d'Urfe, QC). Antibodies used in Western blot were: CYB5D2 serum (1:200), anti-PTEN (Cell Signaling, 1:1000), M2-flag (1:1000; Sigma Aldrich), anti-HA (1:1000; Sigma Aldrich), β-actin (1:1000; Santa Cruz), anti-rabbit IgG (1:3000; Sigma Aldrich), and anti-mouse IgG (1: 3000; Sigma Aldrich). Note: indicate which secondary antibodies are conjugated with what.

2.4. Co-immunoprecipitation (Co-Ip) of CYB5D2 and PTEN

1 mg cell lysate and rProtein G Agarose beads (Invitrogen) with either 1μg of antibody of interest or control IgG were incubated in Co-Ip buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA and 0.1% Triton X-100 overnight at 4°C followed by

wash for 8 times with Co-Ip buffer. The antibodies used for immunoprecipitation of tagged proteins were anti-Flag (M2, Sigma Aldrich) for PTEN or anti-HA (Sigma Aldrich) for CYB5D2 and its mutants and mouse IgG (Sigma Aldrich) as negative control. The antibodies used for immunoprecipitation of endogenous PTEN were anti-PTEN (Cell Signaling) or mouse IgG (Sigma Aldrich) as negative control. The cell lysates then resuspending in protein sample buffer (PSB). Either CYB5D2 or PTEN was detected via Western blot analysis.

2.5. Immunofluorescence

CYB5D2 and PTEN were co-transfected into 293T cells. Cells on chambers were fixed after 48h of transfection followed by blocking for one hour at room temperature in a buffer containing 3% donkey serum and 3% BSA in TBST. Double immunofluorescence staining was carried out using the following antibodies: anti-Flag (M2, 1:500, Sigma Aldrich) for PTEN or anti-HA (1:200, Sigma Aldrich) for CYB5D2, FITC-Donkey anti-rabbit IgG (1:200; Jackson Immuno Research) and Rhodamine-Donkey anti-mouse IgG (1:200; Jackson Immuno Research) were used as secondary antibodies. 293T nucleus were counterstained with DAPI. Images were captured using Axiovert 200 M confocal microscope and AxioVision 3 software.

2.6. Production of xenograft tumors

10^6 HCC1954 Tet-ON or HCC1954 Tet-CYB5D2 cells were resuspended into a RPMI1640/Matrigel mixture (1:1 volume), followed by injection of 0.1 ml of cell solution subcutaneously into the flank of NOD/SCID mice (5 mice/per line). Tumor volume was calculated according to the formula $L \times W^2 \times 0.52$ (L and W represents for the longest and shortest diameters, respectively). Drinking water containing DOX (2 mg/ml) was given to mice when tumor size reached at 200 mm^3 (Week 3-Week4). Two groups of mice were sacrificed together at the end of Week 8. All animal work was performed according to protocols approved by the McMaster University Animal Research Ethics Board.

2.7. Real time PCR analysis

RNA was isolated from frozen mice tumor tissue using TRIZOL (Life Technologies, Burlington, ON). Reverse transcriptions of RNA were carried out by using superscript III reagents (Life Technologies, Burlington, ON) according to the manufacturer's instructions. In brief, 2 μg of RNA was converted to cDNA through PCR program: 65 °C for 6 minutes, iced for 2 minutes, 25 °C for 11 minutes, 50 °C for 60 minutes and 70 °C for 15 minutes. Real time PCR primers used include CYB5D2 (F: 5′-GACCGGGGACTGTTCTGAAG-3′; R: 5′-TAGAACCGTCCTGTCACCCT-3′) and Actin (F: 5′-ACCGAGCGCGGCTACAG-3′; R: 5′-CTTAATGTCACGCACGATTT

CC-3′). Reactions were performed with the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Burlington, ON) in the presence of SYBR-green. All samples were run in triplicate.

2.8. Statistical analysis

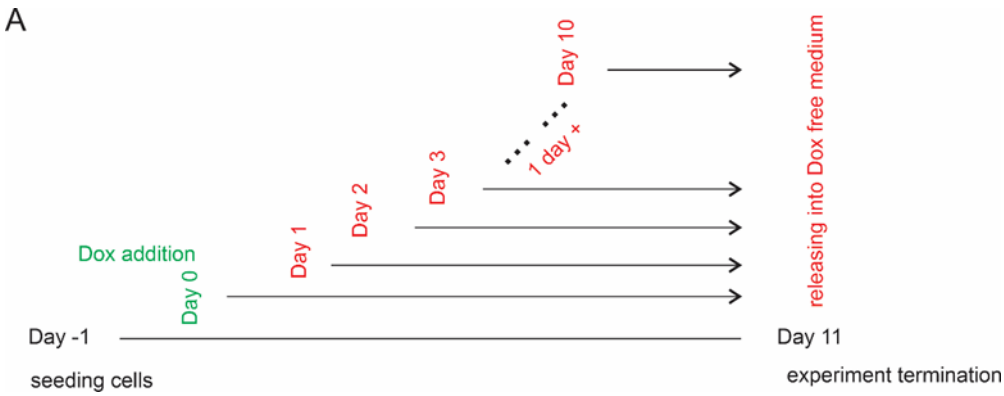
The two-tailed Student's t-test and two-way ANOVA (GraphPad Prism 8.0) were used for statistical analysis. A value of $p < 0.05$ is considered statistically significant. Data are presented as mean ± SEM.

Chapter 3: Results

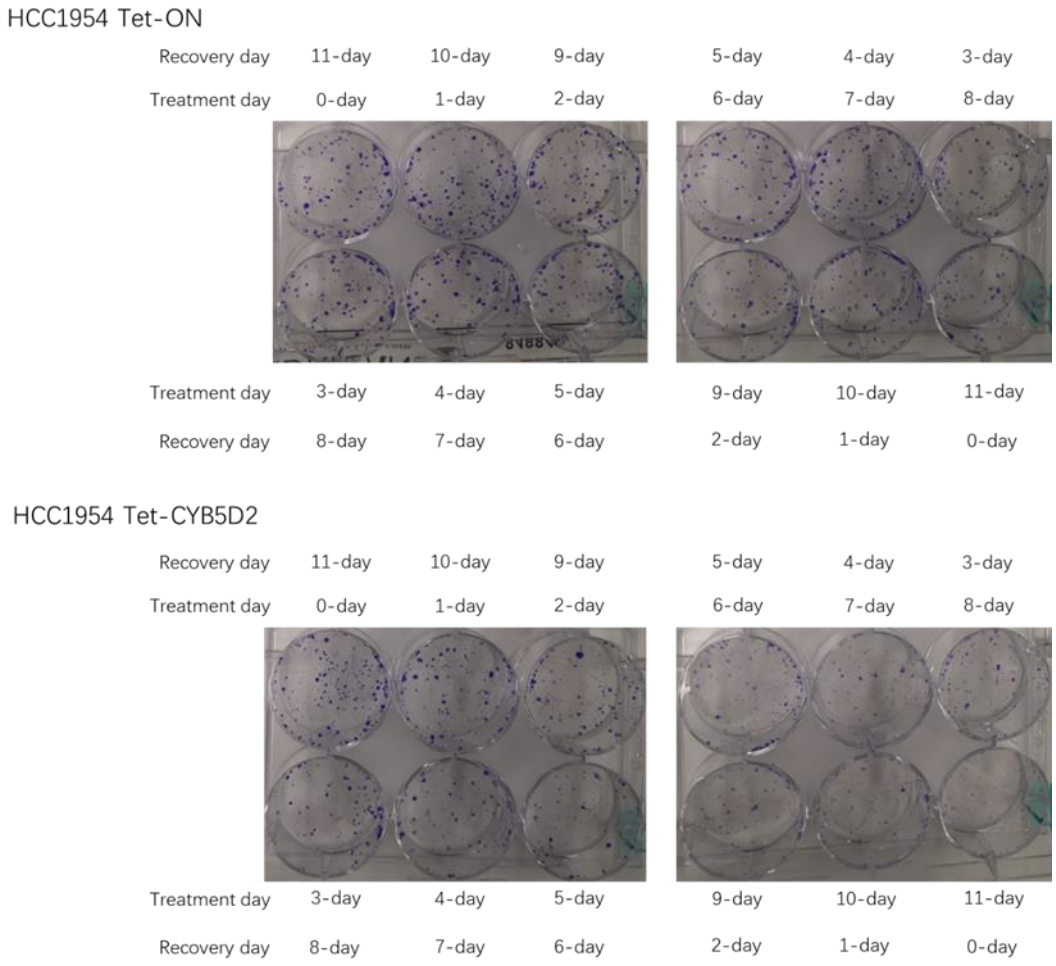
3.1. Characterization of CYB5D2-mediated suppression of cell proliferation

We have previously established a tetracycline-inducible CYB5D2 expression line in HCC1954 HER2+ BC cells (HCC1954 Tet-CYB5D2) and demonstrated that CYB5D2 suppressed the proliferation of HCC1954 Tet-CYB5D2 cells after induction of CYB5D2 by doxycycline (DOX). To characterize this process, I have conducted an experiment with different lengths of CYB5D2 induction in HCC1954 Tet-CYB5D2 cells in a duration of 11 days (Fig 2A); in this setting, cells were seeded at day -1 with addition of DOX to all cells (except control) at day 0, followed by releasing cells from the induction condition into normal culture (without DOX) medium at day 1, day 2 and up to day 10 (Fig 2A). This release setting will test the cell's ability to recover from CYB5D2-derived growth inhibition (Fig 2A). In comparison to control cells (HCC1954 Tet-ON) that were similarly treated with DOX, the number of colonies formed in HCC1954 Tet-CYB5D2 cells was negatively correlated with the length of DOX treatment or CYB5D2 induction (Fig 2B, C). In comparison to no treatment (day 0), one day (addition of DOX at day 0 and released into normal medium at day 1, see Fig 2A) induction of CYB5D2 inhibited HCC1954 Tet-CYB5D2 cells to form colonies even with a period of 10-day recovery (Fig 2B). This trend holds at cells treated with 2, 3, 4, and 5 days respectively coupled with the respective releasing period of 9, 8, 7, and

6 days (Fig 2B). This data indicates that although cells are able to recover from CYB5D2-induced inhibition, the recovery was not 100% (Fig 2B). Longer treatments were associated with reduced recovery abilities (Fig 2B). While the detail mechanisms under these observations remain to be determined, it is possible that CYB5D2 induction initiates a network changes which could be persistent even with CYB5D2 induction being removed. This scenario is supported by the appearance of a number of small colonies formed by cells induced for CYB5D2 expression for 7 and more days (Fig 2B).



B


HCC1954 Tet-ON
Recovery day 11-day 10-day 9-day 5-day 4-day 3-day
Treatment day 0-day 1-day 2-day 6-day 7-day 8-day
Treatment day 3-day 4-day 5-day 9-day 10-day 11-day
Recovery day 8-day 7-day 6-day 2-day 1-day 0-day
HCC1954 Tet-CYB5D2
Recovery day 11-day 10-day 9-day 5-day 4-day 3-day
Treatment day 0-day 1-day 2-day 6-day 7-day 8-day
Treatment day 3-day 4-day 5-day 9-day 10-day 11-day
Recovery day 8-day 7-day 6-day 2-day 1-day 0-day

C

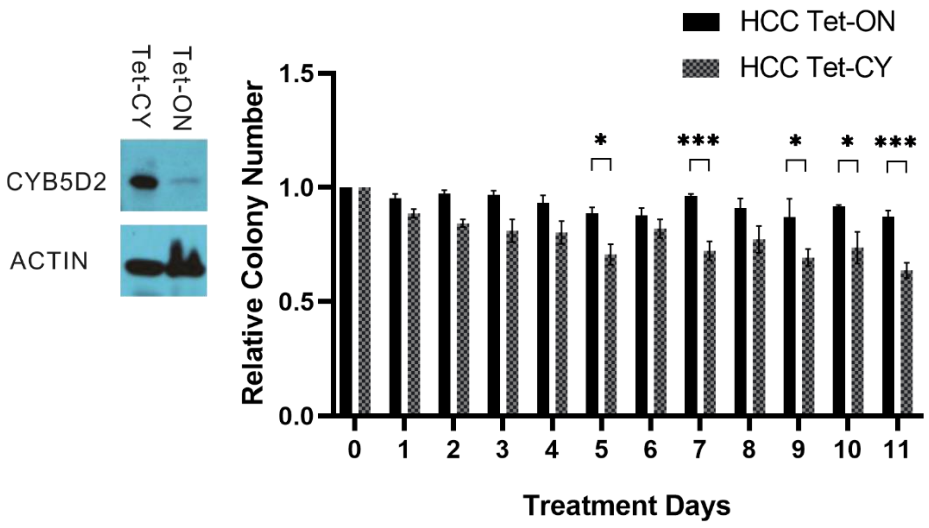

Tet-CY
Tet-ON
CYB5D2
ACTIN
HCC Tet-ON
HCC Tet-CY
Relative Colony Number
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 10 11
Treatment Days
* *** * * ***

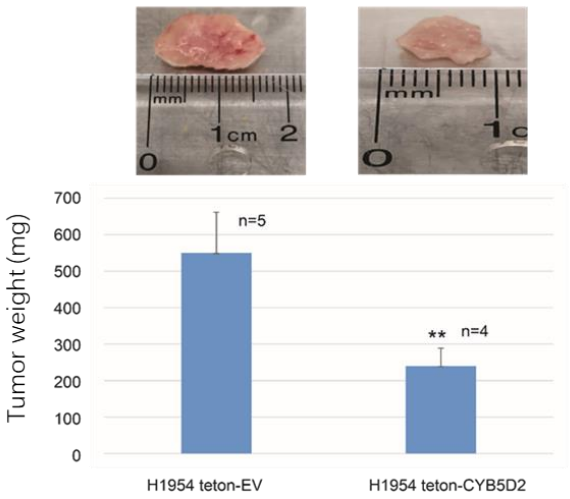
Figure 2. CYB5D2 inhibits HCC1954 cell proliferation.

(A) Schematic outlines of experimental design. HCC1954 Tet-ON or HCC1954 Tet-CYB5D2 cells (10^3 cells/well) were seeded in 6-well plate at day-0; doxycycline (DOX) was added at day 1 for all wells except control. Cells were then released into normal or DOX-free medium at day 1, day 2, day 3 and till day 10 at one day apart as indicated. Experiments were terminated at day 11; colonies formed were then stained with crystal violet. (B) Typical colony formation for the indicated cells. (C) The colony number of indicated cells were counted. The colony number at 0-day treatment well was set as 1; the relative colony numbers in individual treatments were normalized to the number of colonies in 0-day treatment. Experiments were repeated 3 times; means ± SEM are graphed. Statistical analysis was performed using one-way ANOVA, followed by post-hoc analysis using Sidak (*$p < 0.05$, ***$p < 0.001$). GraphPad Prism 8.3.0 was used for statistical analyses.

3.2. CYB5D2 inhibits breast cancer growth in vivo

Following our in vitro analysis of CYB5D2-mediated inhibition of HCC1954 cell proliferation, we have conducted an animal experiment to examine whether CYB5D2 reduces tumor growth in vivo. My colleague, David, injected HCC1954 EV (HCC1954 Tet-ON cells, control group) and HCC1954 Tet-CYB5D2 cells into NOD/SCID mice (5 animals each for EV group and 4 for CYB5D2 group) for xenograft tumor formation. Mice were given DOX through drink when tumors were at 200 mm^3 at time between weeks 3 and 4 post tumor implantation. All mice were sacrificed at week 8. In comparison to tumors formed by HCC1954 Tet-ON cells, induction of CYB5D2 significantly reduced the growth of xenografts produced by HCC1954 Tet-CYB5D2 cells (Fig 3A); I subsequently confirmed the expression of CYB5D2 in HCC1954 Tet-CYB5D2 cell-derived tumors treated with DOX (Fig 3B). Collectively, we provide an in vivo evidence for CYB5D2 in inhibition of the growth of HER2+ BC cell-derived tumors.

A



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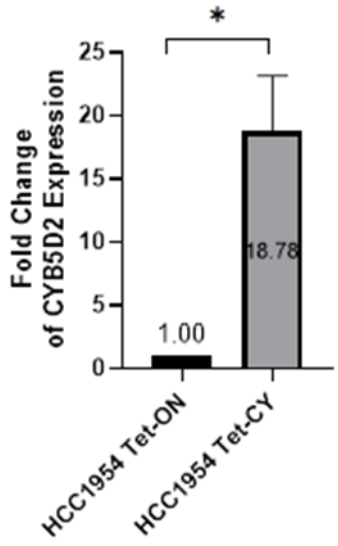


Figure 3. CYB5D2 reduces the growth of breast cancer in vivo.

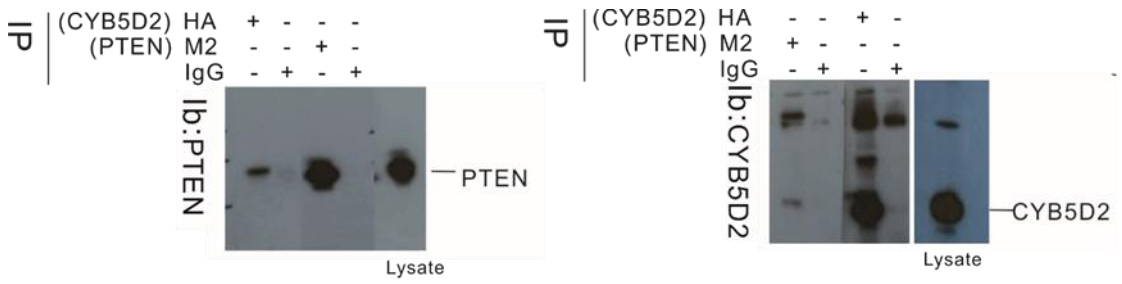
10^6 HCC1954 Tet-ON or HCC1954 Tet-CYB5D2 cells were implanted into each NOD/SCID mice. Both CYB5D2 (HCC1954 Tet-CYB5D2) and EV (HCC1954 Tet-ON) mice were fed with DOX water from week 3-4. Tumors were harvested at week 8. (A) Typical size of EV and CYB5D2 tumors (up panel). Tumor weights for EV and

CYB5D2 cell-derived tumors were analyzed (bottom panel). **$p < 0.01$ in comparison to the EV tumors by 2-tailed Student's t-test. (B) Fold change of CYB5D2 mRNA expression in the indicated tumors by qRT-PCR. Means ± SEM are graphed and analyzed by GraphPad Prism 8.3.0. *$p < 0.05$ by 2-tailed Student's t-test.

3.3. Interaction of CYB5D2 with PTEN

PTEN is an established tumor suppressor, playing a key role in the regulation of cell proliferation, apoptosis, cancer metastasis etc. The possibility of the interaction between PTEN and CYB5D2 implies a potential mechanism responsible for CYB5D2 to exert its tumor suppression function. To examine this possibility, CYB5D2 and PTEN were transiently co-transfected in 293T cells and a complex containing both CYB5D2 and PTEN was immunoprecipitated through either protein (Fig 4A). Additionally, the complex was formed between endogenous PTEN and ectopic CYB5D2 in HCC1954 Tet-CYB5D2 cells following induction of CYB5D2 by DOX (Fig 4B). The formation of CYB5D2-PTEN complex might be an artifact caused by cell lysate preparation as the manipulation would bring two proteins together which may facilitate complex formation. To exclude this possibility, I was able to show co-localization between CYB5D2 and PTEN in 293T cells when both were co-transfected in the cells (Fig 4C). Taken together, evidence supports that CYB5D2 interacts with PTEN.

A



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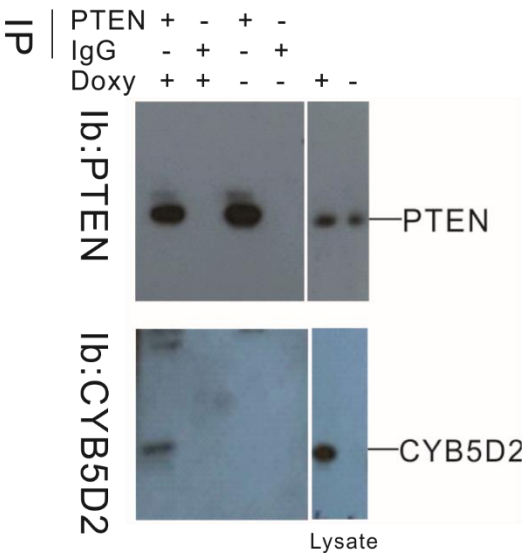

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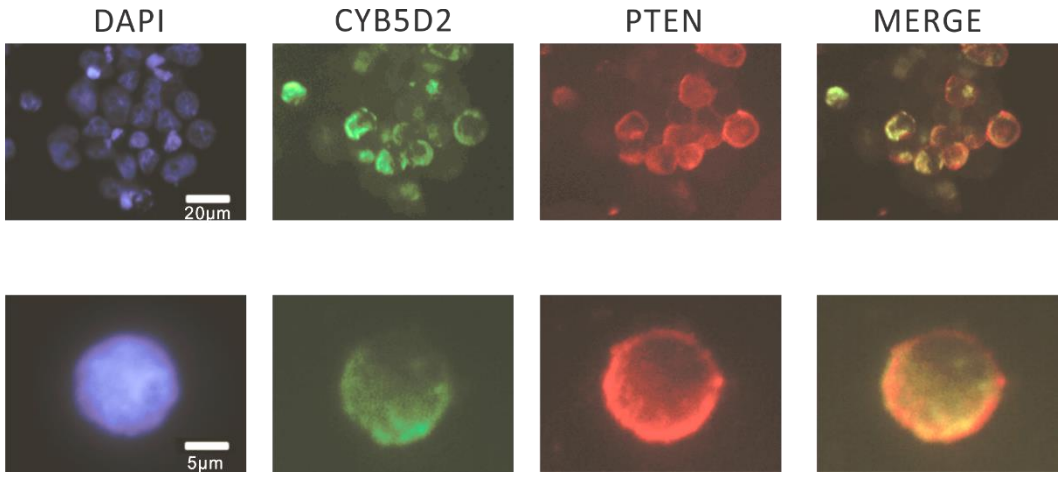


Figure 4. CYB5D2 binds to PTEN.

(A) 293T cells were transiently transfected with HA-tagged CYB5D2 and FLAG-tagged PTEN. Cell lysates were prepared and immunoprecipitated with anti-HA and anti-FLAG (M2) antibodies. The precipitates and lysates were analyzed by Western blot (Ib: immunoblotting) using the indicated antibodies. (B) HCC1954 Tet-CYB5D2 and HCC1954 Tet-ON cells were treated by DOX. Cell lysates were prepared and

immunoprecipitated with anti-PTEN antibodies. The precipitates and lysates were analyzed by Western blot using anti-PTEN antibody and anti-CYB5D2 serum. (C) 293T cells were co-transfected with CYB5D2 and PTEN, followed by immunofluorescent (IF) staining for CYB5D2 and PTEN as indicated. Nuclei were counter-stained with DAPI (blue). Scale bars represent 20 μm and 5 μm respectively.

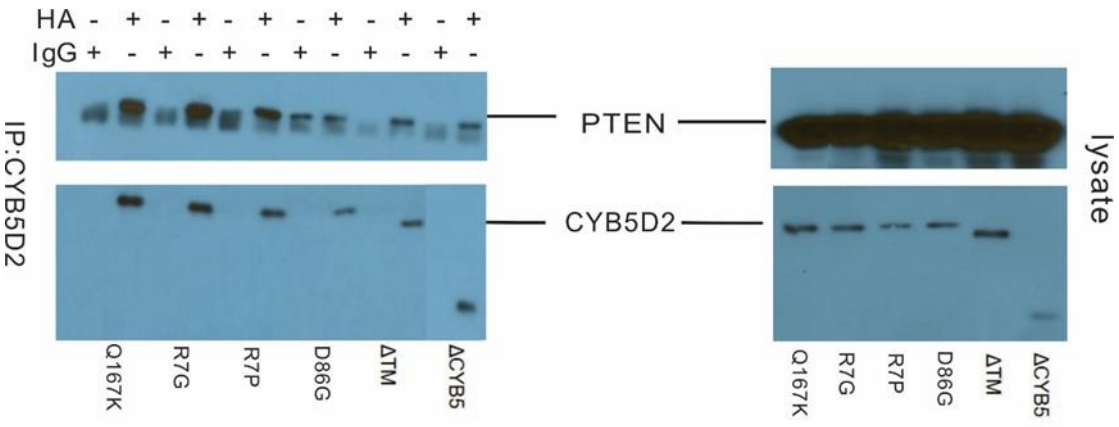
3.4. Characterization of the association between CYB5D2 and PTEN

To characterize the interaction between CYB5D2 and PTEN, I examined the role of the structural features of CYB5D2 in its binding to PTEN. CYB5D2 contains a putative transmembrane domain (reside 7-29), CYB5 domain (reside 35-134), heme binding residue D86, and Q167 (Fig 5A). In view of these structural features, the following CYB5D2 mutants were used: ΔTM (deletion of the transmembrane domain), ΔCYB5 (deletion of the heme-binding domain), and a set of site-directed mutants: R7G, R7P (reported in colon cancer), D86G (heme-binding defective), and Q167K (from Mammalian Gene Collection) (Fig 4A). Using the 293T cell-based co-transfection system, PTEN could be immunoprecipitated through CYB5D2 mutants R7P, R7G, Q167K, ΔCYB5 and ΔTM, while PTEN could not be immunoprecipitated by D86G (Fig 5B). On the other hand, immunoprecipitation of PTEN could co-precipitate wild-type CYB5D2 and the D86G mutant but not other mutants (Fig 5C). These observations suggest that specific structures facilitating the complex formation could be interfered via unique manner used to immunoprecipitate the complex. For instance, the CYB5D2-D86G and PTEN complex can be precipitated via PTEN but not through CYB5D2 mutant; the complexes of PTEN with other CYB5D2 mutants could only be immunoprecipitated through the respective mutants but not PTEN (Fig 5B, C). Collectively, these observations support the formation of CYB5D2 and PTEN complex being independently of these CYB5D2 structural features (Fig 5A). Therefore, heme binding may not be required for CYB5D2 to associate with PTEN.

A


TM domain
CYB5 domain
Binding to PTEN
7 2935 86 134 167
1 264
R D Q
CYB5D2 +
7 2934 135
CYB5D2-ΔCYB5 +/-
35 134
30 264
CYB5D2-ΔTM +/-
7
P/G
CYB5D2-R7P/R7G +/-
86
G
CYB5D2-D86G +/-
167
K
CYB5D2-Q167K +/-

B


HA
IgG
IP:CYB5D2
PTEN
CYB5D2
lysate
Q167K
R7G
R7P
D86G
ΔTM
ΔCYB5

C

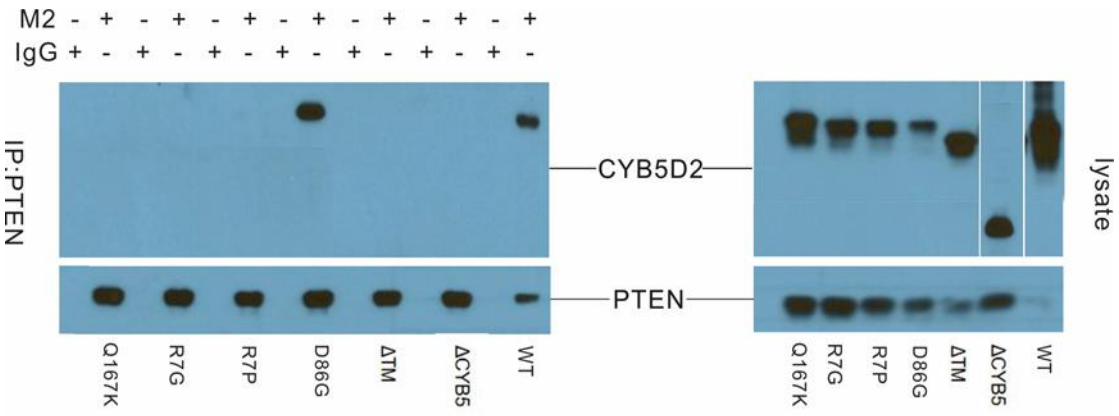

M2
IgG
IP:PTEN
CYB5D2
PTEN
lysate
Q167K
R7G
R7P
D86G
ΔTM
ΔCYB5
WT

Figure 5. CYB5D2 might interact with PTEN independently of heme-binding and transmembrane domain.

293T cells were transiently transfected with PTEN, wild type CYB5D2, CYB5D2 (ΔCYB5), CYB5D2 (ΔTM), CYB5D2 (D86G), CYB5D2 (R7P), CYB5D2 (R7G) and CYB5D2 (Q167K) for 48 hours followed by immunoprecipitation analyses. (A) Summary of CYB5D2 and its mutants for binding to PTEN. +: binding being confirmed by 2 direction co-IP. i.e. precipitation of CYB5D2-PTEN complex via CYB5D2 and PTEN; +/-: binding of the indicated CYB5D2 mutants to PTEN was based on one direction IP. (B) Immunoprecipitations were performed using antibody against CYB5D2, followed by Western blot analyses for CYB5D2 and PTEN. (C) The complexes of PTEN and CYB5D2 or its mutants were immunoprecipitated through PTEN, followed by Western blot analyses as indicated.

Chapter 4: Discussion

Management of BC patients has been steadily improved owing to continuously research efforts. Nonetheless, BC remains a major malignancy and a top cause of cancer death among women worldwide. Clearly, our understanding on BC etiology and factors underlying the disease progression needs significant improvement. BC is a complex disease with multiple genes and pathways being involved and unknown factors to be discovered. My work supports CYB5D2 as a novel tumor suppressor of breast cancer, evident by its activities in inhibiting HCC1954 HER2+ cell proliferation in vitro and the cell-derived xenograft in vivo as well as its association with PTEN, a well-established tumor suppressor.

CYB5D2 was reported to suppress cervical cancer, a concept that was supported by in vitro, in vivo, and clinical evidence.[95] We have previously shown CYB5D2 as a candidate tumor suppressor in breast cancer, a conclusion based on 1) the genetic knowledge of deletion of 17P.13.2-17P.13.3 being detected in over 50% BCs, including the CYB5D2 locus (17P13. 2)[2]; 2) reductions of CYB5D2 mRNA expression in BC vs normal breast tissue and in aggressive BC subtypes[2]; 3) association of CYB5D2 downregulation with shortening in overall survival of BC patients[2]; 4) induction of apoptosis in MCF7 ER+ breast cancer cells[2], and 5) induction of CYB5D2 in HCC1954 Tet-CYB5D2 cells, which resulted in inhibition of HCC1954 cell proliferation[88]. My

research confirmed CYB5D2-derived inhibition of HCC1954 cell proliferation. Furthermore, my work revealed that the inhibition could not be fully recovered even with one day induction of CYB5D2 in HCC1954 cells. The mechanisms underlying this observation could be complex. It is possible that the CYB5D2 protein can exist for a long period of time even after removing the induction condition; it can also be envisaged that CYB5D2 could initiate a network event and this network could inhibit cell proliferation without the continuous presence of CYB5D2. While which of these mechanisms was playing a major in sustaining inhibition of HCC1954 cell proliferation remains to be determined, it is likely that both mechanisms were involved.

The concept of CYB5D2 utilizing a network for inhibition of HCC1954 cell proliferation is consistent with relationship of CYB5D2 downregulation with mutations in genes relevant to BC, including PIK3CA, GATA3, MAP3K1, CDH1, TP53, and RB1. [2,88] Furthermore, the concept is also supported by my study for an interaction of CYB5D2 with PTEN. Although the functionalities of this interaction in tumor suppression via either CYB5D2 or PTEN remain to be explored in future, the fact of both possessing tumor suppression function suggests that the interaction would enhance tumor suppression. Nonetheless, this needs to be studied in future. Should this be confirmed, it will advance our understanding of BC etiology, which offers a potential mechanism for CYB5D2-derived tumor suppression.

Other mechanisms may also contribute to CYB5D2 as a tumor suppressor of BC. While there is no evidence for CYB5D2 to modulate progesterone signaling, this potential may not be excluded in view of the negative impact of progesterone signaling and CYB5D2 on BC. Furthermore, CYB5D2 could be a secretory protein; whether this aspect is relevant in BC and tumorigenesis in general also needs to be considered.

Regardless of what might be the mechanisms underpinning the tumor suppression actions of CYB5D2 in BC, the observed inhibition of CYB5D2 towards HCC1954 cell-generated xenografts significantly enhances the candidacy of CYB5D2 as a new tumor suppression of breast cancer. This suggests a possibility of restoring CYB5D2 function as a therapeutic option in treating breast cancer.

Chapter 5: Conclusions

Besides of in cervical cancer, our previous research and ongoing research support that CYB5D2 suppress breast cancer cell growth in vitro and in vivo.[88,94] My work contributes to CYB5D2 as a novel tumor suppressor of BC in three aspects:

- HCC1954 cells can be partly recovered from CYB5D2-mediated inhibition in vitro, suggesting the involvement of network changes in CYB5D2-mediated inhibition of HCC1954 cell proliferation;
- CYB5D2 inhibits HCC1954 cell derived xenografts growth in vivo;
- CYB5D2 interacts with PTEN and this interaction might be independently of heme binding ability.

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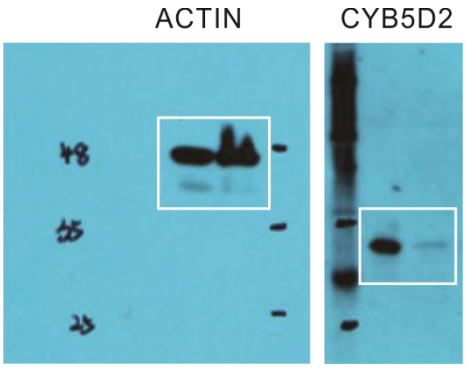
Appendix

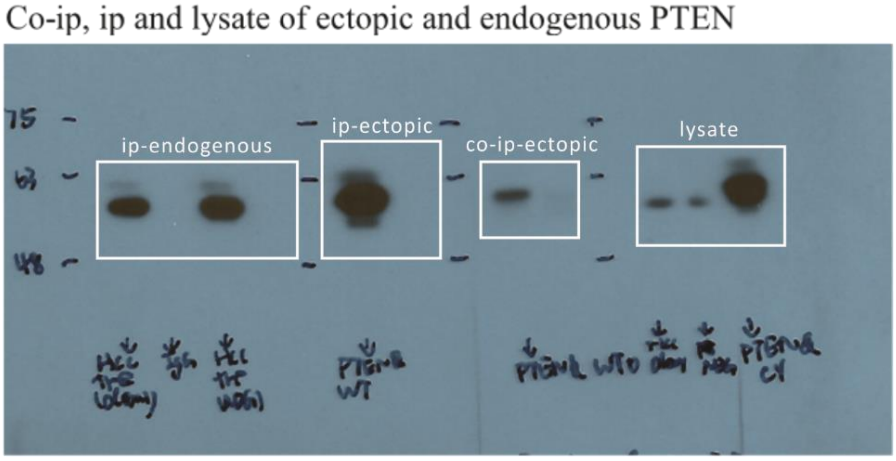
Figure S1.



Uncut gel for Figure 2C. The films used to edit for Figure 1C are indicated.

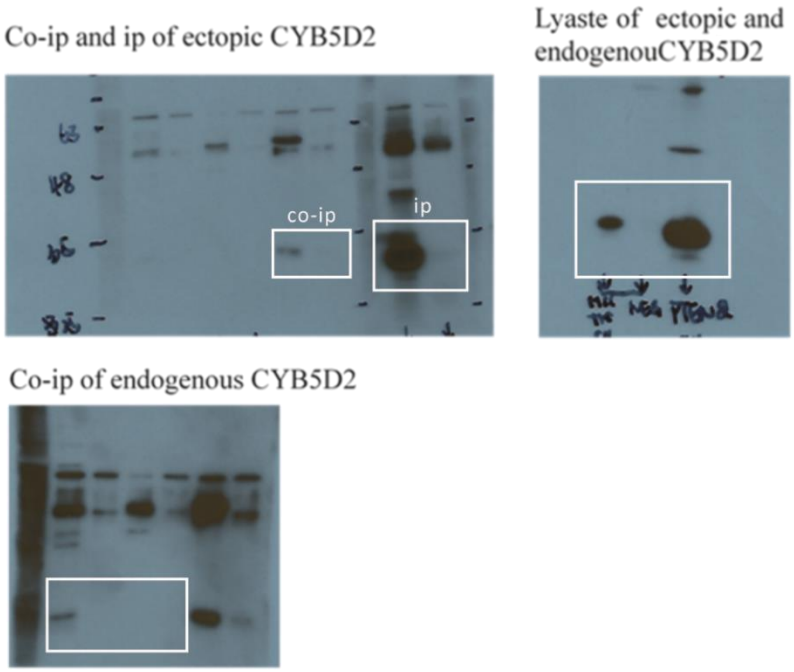
Figure S2.

A



Uncut gel for Fig 4A, 4B. The lanes used to edit for Fig 4A and 4B are indicated.

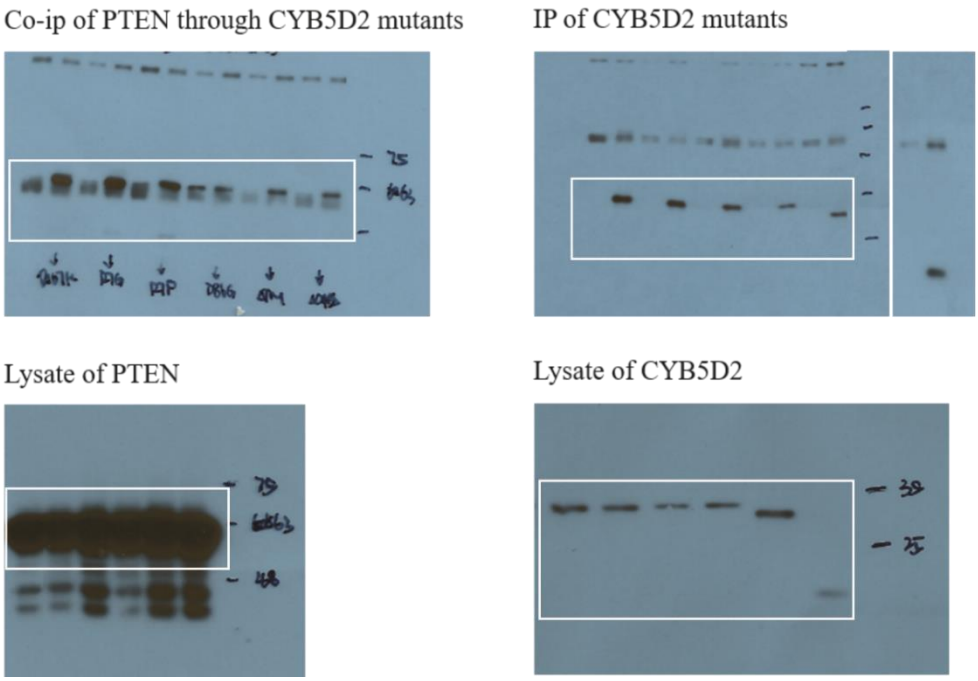
B



Uncut gel for Fig 4A, 4B. The films used to edit for Fig 4A and 4B are indicated

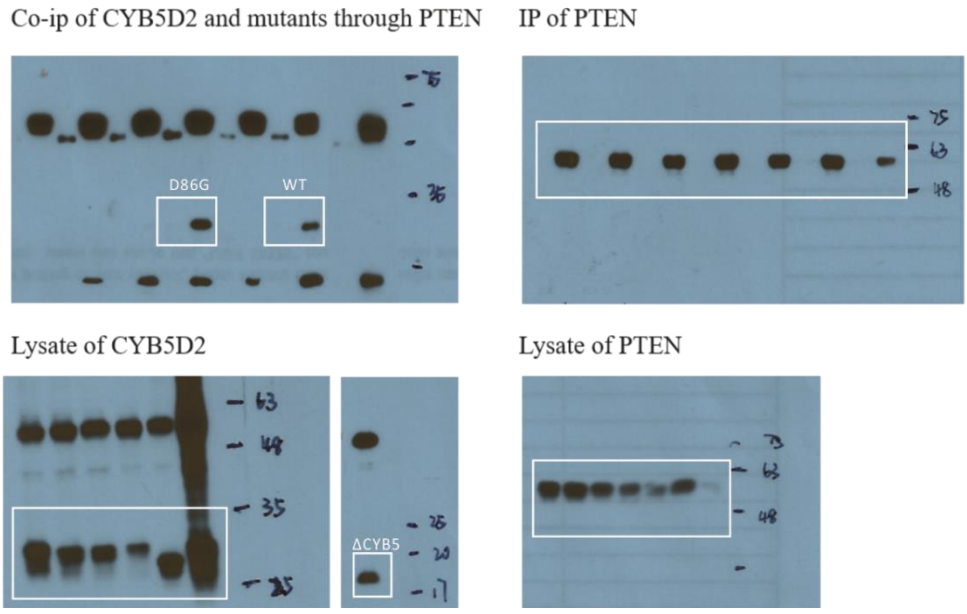
Figure S3.

A



Uncut gel for Fig 5B. The films and lanes used to edit for Fig 5B are indicated

B



Uncut gel for Fig 5C. The films used to edit for Fig 5C are indicated