

9-7-2016

# The Prognostic Capability and Molecular Function of Duodenal Cytochrome B in Breast Cancer

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# The Prognostic Capability and Molecular Function of Duodenal Cytochrome B in Breast Cancer

David John Lemler, PhD

University of Connecticut, 2016

Iron is an essential growth factor and cofactor for multiple molecular functions in the human body. It is a reactive metal and in excess is capable of participating in Fenton reactions, generating reactive oxygen species and damaging cells. Patients with the iron overload disease hemochromatosis, are at increased risk of hepatic and other cancers. Due to the necessity and toxicity of iron, it is tightly regulated. Cancerous cells have an increased demand for iron and to meet these needs, regulation of iron import (upregulation of transferrin receptor) and export (downregulation of ferroportin) proteins is altered. Differential expression of these iron genes is associated with prognosis. This has led to further analysis of the association of “iron” genes with breast cancer prognosis. The association of duodenal cytochrome b (DCYTB) in breast cancer was identified as part of a 16 gene iron regulatory gene signature (IRGS). DCYTB is a ferrireductase in duodenal enterocyte responsible for reducing dietary iron for cellular uptake.

To further characterize the prognostic capability of DCYTB, we evaluated breast cancer patient microarray data in two combined cohorts totaling over 1600 patients. We found that high DCYTB expression was associated with increased probability of relapse-free survival (both local and distant). Results of array data and breast cancer tissue staining agree that DCYTB expression is reduced with increased tumor grade. We also show that patient with high DCYTB expression are more likely to benefit from Tamoxifen therapy.

This association is quite interesting because previous research has shown that increased iron is associated with poor outcome in cancer. Here, however, we see an iron import protein that is associated with good prognosis. Therefore, we evaluated iron metabolism as a

result of DCYTB knockdown and overexpression. Iron responsive protein expression and labile iron were not altered, suggesting that in breast cancer cell lines DCYTB functions independently of iron. Metabolism of copper and ascorbate, two known substrates of DCYTB, was also evaluated. The results of these experiments were inconclusive, but revealed potential perturbations to reactive oxygen species signaling and regulation of cytochrome p450, which will be the focus of future experiments.

# The Prognostic Capability and Molecular Function of Duodenal Cytochrome B in Breast Cancer

David John Lemler

B.S., University of Wisconsin – Green Bay, 2008

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

at the

University of Connecticut

2016

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APPROVAL PAGE

Doctor of Philosophy Dissertation

The Prognostic Capability and Molecular Function of Duodenal Cytochrome B in Breast Cancer

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## List of abbreviations

|          |  |
|----------|--|
| 101F6    | CYB561D2, TS101F6  |
| AA       | ascorbic acid, ascorbate, vitamin C                        |
| ABCB1    | MDR1/P-glycoprotein  |
| AP-1     | activator protein 1  |
| AR       | ascorbate radical  |
| ATOX1    | antioxidant 1 copper chaperone                             |
| ATP7A    | Menkes ATPase, Cu <sup>2+</sup> transporting P-type ATPase |
| ATP7B    | Wilson ATPase, Cu <sup>2+</sup> transporting P-type ATPase |
| CCS      | copper chaperone for superoxide dismutase                  |
| CDDP     | cis-diaminedichloroplatinum (II)                           |
| CP       | ceruloplasmin  |
| CPX      | ciclopirox   |
| CTR1     | copper transporter 1                                       |
| CYB561A1 | chromaffin granule cytochrome b561, CG561                  |
| CYP1A1   | cytochrome P450, family 1, subfamily A, polypeptide 1      |
| CYCS     | cytochrome c   |
| DCIS     | ductal carcinoma <i>in situ</i>                            |
| DCYTB    | duodenal cytochrome b, CYB561A2, CYBRD1                    |
| DCFH-DA  | 6-carboxy-2',7'-dichlorofluorescein diacetate              |
| DFO      | desferoxamine  |
| DHA      | dehydroascorbic acid                                       |
| DHLA     | dihydrolipoic acid   |
| DMFS     | distant metastasis-free survival                           |
| DMT1     | divalent metal transporter 1                               |

|               |  |
|---------------|--|
| DSS           | disease specific survival                    |
| ER            | estrogen receptor                            |
| FAC           | ferric ammonium citrate                      |
| FTH1          | ferritin heavy chain                         |
| FTL           | ferritin light chain                         |
| FPN           | ferroportin, SLC40A1                         |
| GSH           | glutathione                                  |
| GSSG          | glutathione disulfide                        |
| GULO          | L-gulonolactone oxidase                      |
| HAMP          | hepcidin                                     |
| HIF2 $\alpha$ | hypoxia inducible factor 2 $\alpha$          |
| HMOX1         | heme oxygenase                               |
| HRE           | hypoxia responsive element                   |
| ICP-MS        | inductively coupled plasma mass spectroscopy |
| IL1           | interleukin 1                                |
| IL6           | interleukin 6                                |
| IL8           | interleukin 8                                |
| IRE           | iron responsive element                      |
| IRP1          | iron responsive protein 1                    |
| IRP2          | iron responsive protein 2                    |
| JNK           | MAPK8, mitogen-activated protein kinase 8    |
| HSPA1A1       | heat shock protein 70                        |
| LIP           | labile iron pool                             |
| LN            | lymph node                                   |
| MDR           | multidrug resistance                         |
| MTA2          | metallothionein                              |

|                |  |
|----------------|--|
| NAD(P)H        | nicotinamide adenine dinucleotide phosphate                      |
| NF- $\kappa$ B | nuclear factor kappa B   |
| p38 MAPK       | p38 mitogen-activated protein kinase                             |
| RFS            | relapse-free survival  |
| RNR            | ribonucleotide reductase   |
| ROS            | reactive oxygen species  |
| SPIA           | signaling pathway impact analysis                                |
| STEAP3         | six-transmembrane epithelial antigen of prostate family member 3 |
| TCYTB          | tonoplast-localized cytochrome b561                              |
| TFA            | trifluoroacetic acid   |
| TFRC/CD71      | transferrin receptor   |
| TM             | tetrathiomolybdate   |
| tPMET          | transplasma membrane electron transport                          |
| VEGF           | vascular endothelial growth factor                               |

## **Chapter I**

### **Introduction and Background**



## A. Breast cancer

It is expected that there will be approximately 1.6 million new cases of invasive cancer in the United States in 2015 [1]. Breast cancer is the most common cancer in women, accounting for 29% of new cases and more than 300,000 women are expected to be diagnosed with breast cancer (invasive carcinoma or carcinoma *in situ*) this year [1]. This equates to approximately 1 in 8 women developing invasive breast cancer in their lifetime [1].

Breast cancer incidence rose drastically in the 1980's and 90's [1] This is largely attributed to increased mammography screening, which identified many breast cancers earlier than they otherwise would have been detected. Identifying the disease in this manner, i.e. before symptoms or distant disease are present, has been shown to increase the five-year survival rate [1]. Therefore, the rise in breast cancer incidence was coupled with a decrease of disease specific mortality through application of breast cancer therapies to more susceptible tumors [2].

All breast cancer patients will require surgery [3]. Minimally this will be breast conserving surgery (lumpectomy) and at most, mastectomy. It is recommended that patients who undergo breast conservation surgery receive adjuvant radiation therapy as it reduces the risk of recurrence and mortality [4]. Patients with unresectable disease will receive neoadjuvant therapy in an attempt to reduce the size of the tumor prior to surgery. Both neoadjuvant and adjuvant therapy is given based on the pathological diagnosis of needle biopsies. Estrogen receptor (ER) positive tumors, which include luminal A and B subtypes, are treated with endocrine therapy, such as tamoxifen [3, 4]. Tumors positive for HER2, including some luminal B and HER2-enriched subtypes, are treated with monoclonal antibodies targeting HER2 [4], most commonly trastuzumab. Triple negative tumors, which include the basal-like subtype, are indicated for chemotherapy [3, 4]. Utilizing these treatment methodologies, the five-year relative

survival rate has risen to 91%. However, breast cancer is still the second leading cause of cancer related death among women, with about 40,000 deaths expected this year [1]. To fully treat this disease, it will be necessary to fully understand the interplay of genes expressed by the tumor and effectively target neoplastic cells at multiple points. Alternative therapies, such as iron chelation, are currently being explored in an attempt to identify novel and innovative ways to eradicate this disease.

## **B. Iron metabolism**

Iron is a tightly regulated essential micronutrient. The primary reason for this tight regulation of iron is that there is no efflux mechanism for removing iron from the body [5, 6], thus uptake of body iron is the only avenue of regulation. In the event that there are elevated iron stores in the body, potentially deleterious effects may occur.

### **a. Dietary iron requirements and uptake**

The human body contains 3 to 4 grams of iron present largely in the liver, spleen, erythrocytes and bone marrow [7]. It is recommended that males at 19 and older ingest 8 milligrams of iron daily while females age 19 to 50 are recommended to ingest 18 milligrams of iron daily [8]. After age 50, the recommendation for females decreases to 8 milligrams daily [8]. Dietary iron is largely present in the ferric form, necessitating the expression of a ferrireductase in the digestive tract to prepare dietary iron for uptake. Duodenal cytochrome b (DCYTB) was discovered in the duodenal enterocytes where it acts as the ferrireductase for dietary ferric iron prior to cellular uptake [9] (Figure 1-1). Ferrous iron is imported into the cytoplasm via divalent metal transporter 1 (DMT1) where it joins the metabolically active labile iron pool (LIP) [6, 10, 11]. Labile iron exists loosely bound to the chaperone molecules poly(rC)-binding proteins PCBP1, PCBP2 or citrate [12, 13]. Labile iron is utilized in mitochondrial production of iron-sulfur clusters [14] and is a necessary cofactor in molecules such as ribonucleotide reductase [15] and

prolyl hydroxylases [16]. Excess labile iron can be stored inertly in the 24 subunit ferritin polypeptide (FTH1 and FTL) or exported via ferroportin (FPN, SLC40A1). Expression of ferroportin is regulated by the hepatic peptide hormone hepcidin (HAMP) [17]. Formation of the hepcidin/ferroportin complex results in polyubiquitination and proteasomal degradation of both molecules [17, 18]. Iron exported via ferroportin exists in the ferrous state which is oxidized by the copper-dependent oxidases hephaestin and ceruloplasmin [19]. Two molecules of ferrous iron are then bound by one molecule of apo-transferrin to form holo-transferrin, which delivers iron to peripheral tissues.

### **b. Cellular iron uptake in peripheral cells**

Peripheral cells express the transferrin receptor (TFRC) concordant with iron requirements (see section c). Binding of transferrin to this receptor results in endocytosis of the holoprotein/receptor complex (Figure 1-1). The endosome is acidified by proton pumps, releasing iron from holo-transferrin, which is reduced by STEAP3 and imported via DMT1. This ferrous iron enters the metabolically active labile iron pool where it is utilized, stored or exported, as above.

### **c. Iron-mediated post-transcriptional regulation**

Tight regulation of cellular iron is required as there is no mechanism by which iron is exported from the body [5, 6]. To this end, many proteins involved in iron metabolism contain stem-loop structures present in the 5' or 3'-end of mRNA called iron responsive elements (IRE). Iron responsive proteins (IRP1 and IRP2) bind to the IRE under iron deplete conditions stabilizing transcripts at the 3'-IRE, such as transferrin receptor, and preventing translation by blocking ribosomal binding at the 5'-IRE, e.g. ferritin. The reverse is true under replete conditions where transcripts with a 3'-IRE will be destabilized and degraded while translation will be de-repressed at the 5'-IRE [6, 20].

#### **d. The role of iron in cancer**

In spite of the cellular necessity for iron, it is a potential tumor initiator and growth factor [21, 22]. Metabolically active labile iron will readily undergo Fenton chemistry: oxidation by hydrogen peroxide generating a hydroxyl radical and a hydroxide ion [23]. Reaction of DNA with these radicals can result in DNA adduct formation or strand breaks, potentially resulting in initiation of tumorigenesis [24-26]. In fact, patients with the iron accumulation disorder hemochromatosis are at an increased risk of hepatic and other cancers [27, 28].

Iron is also coordinated in cofactors, iron-sulfur clusters and heme cofactors [14, 29], or directly coordinated by the protein, ribonucleotide reductase and prolyl hydroxylases [15, 16]. Due to its involvement in the functionality of multiple proteins, the necessity of cellular iron increases drastically for rapidly dividing cancer cells. Investigations into iron biology as it relates to cancer have revealed that mice fed iron restrictive diets display a reduced rate of tumor growth [26].

It is possible for cancerous cells to meet their iron requirements by multiple mechanisms [30]. First, cells may increase expression of transferrin receptor. This will result in an increased endocytosis of holo-transferrin and an increased cellular iron. This can occur through c-Myc induced overexpression of transferrin receptor and iron responsive protein 2 (IRP2) and inhibition of FTH1 expression [31-33]. Second, decreasing expression of the iron export protein ferroportin will reduce iron export, bolstering the labile iron pool. Expression of ferroportin can be transcriptionally repressed [34] or post-translationally repressed by hepcidin-mediated degradation [17, 35].

In light of this evidence, several iron chelators have undergone clinical trials as treatments for various cancers [6, 36, 37]. Desferoxamine (DFO), for instance, has been shown to arrest the cell cycle and induce apoptosis [38]. Administration to patients (n= 10) with advanced hepatocellular carcinoma resulted in a partial response or stable disease in 50% of

patients [36]. Another iron chelator, triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone), is potent ribonucleotide reductase (RNR) inhibitor similar to hydroxyurea. It inhibits DNA synthesis by labilizing and chelating ferrous iron from the diferric center of RNR [39]. Further, the triapine-Fe(II) complex participates in Fenton reactions, which is likely involved in cytotoxicity. Triapine has shown little efficacy as a single agent [37], though this study included multiple cancer types from different primary sites. It did, however, provide additional benefit when given in combination with gemcitabine [40]. The iron chelating fungicide, ciclopirox (CPX), decreased cell growth and viability in several different cancer cell lines *in vitro* [41, 42]. Further experimentation revealed that CPX inhibited Wnt signaling *in vitro* and that administration of CPX to AML patients resulted in decreased expression of Wnt target genes [43]. Ultimately, the utilization of iron chelators in cancer therapy requires more research, though the potential exists for therapeutic utility of these compounds. Further, this opens the door for investigation of the role of other iron genes in cancer.

Our laboratory has recently investigated the role of ferroportin expression in breast cancer [35]. *In vitro* cell culture models were used to demonstrate that ferroportin expression was decreased in malignant breast cells compared to non-malignant breast cells and that this correlated with an increase of cellular labile iron in the malignant cells. Using an *in vivo* murine model, we found that tumoral expression of ferroportin resulted in decreased tumor size. We also utilized four publicly available breast cancer patient microarray datasets to evaluate ferroportin as a prognostic indicator of breast cancer. In these cohorts, patients with high ferroportin expression (values above the mean) had a significantly increased distant metastasis-free survival (DMFS) or disease specific survival (DSS) compared to patients with low ferroportin expression.

As our group and others have shown, iron genes are useful for predicting breast cancer endpoints [35, 44, 45]. Gene signatures, such as Oncotype DX and MammaPrint, are clinically available. These signatures are used to predict patient outcome and determine the most

effective therapeutic regimen [4]. To this end, our laboratory wished to determine the utility of multiple iron genes for the prediction of breast cancer outcome. A breast cancer patient meta-cohort consisting of 759 patients was prepared from 6 publicly available breast cancer patient microarray cohorts [46]. Sixty-one iron regulated and related genes were considered for this analysis. A 16 gene signature was identified that accurately predicted metastasis-free survival of breast cancer patients. Included in the signature were transferrin receptor ( $p= 6.16e-04$ , HR= 3.54) and ferroportin ( $p= 7.00e-04$ , HR= 0.76) [46], which correlated as expected from previous research. Unexpectedly, duodenal cytochrome b (DCYTB/cybrd1) was the most significant gene and its expression was associated with increased DMFS ( $p= 1.83e-07$ , HR= 0.60) [46]. This is surprising because DCYTB is an iron import protein and increased iron import typically has beneficial effects for cancer. DCYTB has been extensively studied in the duodenum [9, 47] yet expression in breast and breast cancer has yet to be investigated.

## **C. The discovery and function of duodenal cytochrome b**

It had been hypothesized that a ferrireductase must exist in the digestive tract to facilitate uptake of dietary ferric iron [48]. In 2001, McKie et al. proposed that the newly discovered duodenal cytochrome b (DCYTB), localized in the brush border of duodenal enterocytes, supplied reduced dietary iron to DMT1 [9]. DCYTB appears to be induced by iron deplete conditions as well as hypoxia [9]. No iron responsive elements (IRE) have yet been described, however, hypoxia responsive elements (HRE) have been identified in the DCYTB promoter which bind specifically to hypoxia inducible factor 2 $\alpha$  (HIF2 $\alpha$ ) [9, 49, 50].

The predicted protein sequence indicated that DCYTB was a member of the cytochrome b561 protein family [9]. This family of proteins has been broken down into seven subfamilies of di-heme, transplasma membrane electron transporters [51, 52]. The crystal structure of cytochrome b561 (CYB561A1) has been determined in *Arabidopsis thaliana* [53] revealing a

homodimer. Based on these studies, it is likely DCYTB exists as a homodimer as well.

The prototypical chromaffin granule cytochrome b561 (CYB561A1) utilizes intracellular ascorbate as a source of reducing equivalents for the reduction of intravesicular ascorbate radical (AR) generated in the biosynthesis of dopamine [54]. It is now known that many members of the cytochrome b561 family are known to accept electrons from intracellular ascorbate [52]. DCYTB has been shown to mediate levels of extracellular ascorbate independently of iron in erythrocytes [55], though the intracellular ascorbate binding site is only partially conserved [9]. Other substrates, such as flavonoids and dihydrolipoic acid (DHLLA) represent potential alternative sources of reducing equivalents for b561 cytochromes [56, 57].

Regardless of substrate specificity, DCYTB has been shown to reduce iron in a pH-dependent manner [9, 47, 58, 59]. This reaction was found to be ascorbate-dependent in MDCK cells heterologously expressing a Tet-repressible DCYTB-EGFP fusion protein [59]; however, in human bronchial epithelial cells this reaction was found to be ascorbate-independent [60]. In both cases, DCYTB expression resulted in increased levels of cellular iron [59, 60].

Ascorbate-dependent copper reduction by DCYTB has also been shown in the MDCK cells described above [59]. This is a pH-independent reaction [59]. Cuprous copper is also a substrate for import by DMT1 [61], emphasizing the potential for DCYTB to mediate copper uptake in addition to iron.

To understand DCYTB expression in the breast, we adopted a two-pronged approach wherein DCYTB was investigated from a bioinformatic as well as a molecular biology standpoint.

## **D. Rationale & Aims**

- a. Aim 1: The prognostic significance of duodenal cytochrome b expression in breast cancer**

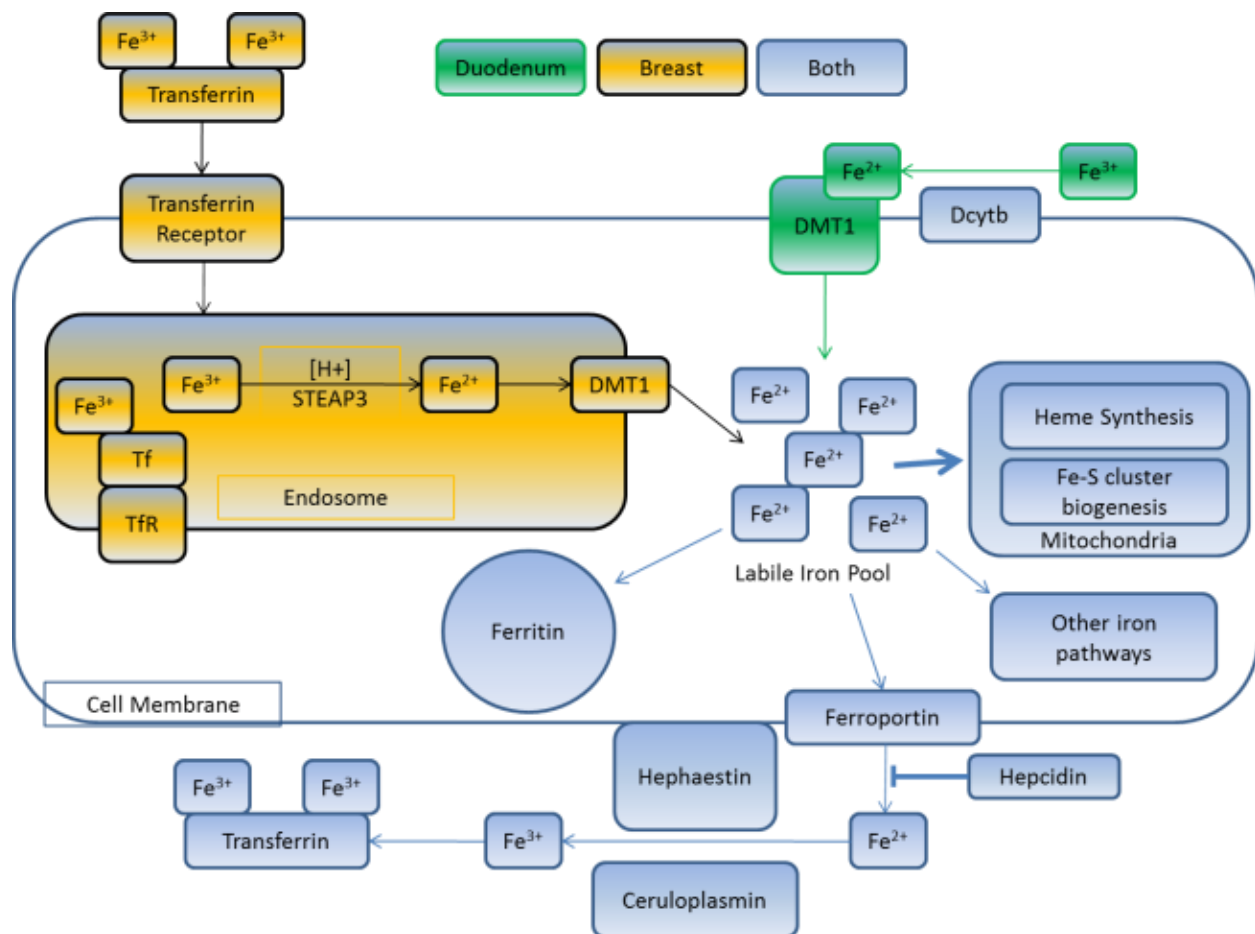
We will first adopt a bioinformatic approach to evaluate the prognostic capabilities of DCYTB expression in breast cancer outcome. DCYTB expression has been shown to be associated with distant metastasis-free survival (DMFS) of breast cancer patients [46]. Further evaluations of the prognostic implications of DCYTB expression on breast cancer subgroups have yet to be completed. We hypothesize that utilizing DCYTB to predict breast cancer outcome will provide additional diagnostic information. We will utilize the National Center for Biotechnology Information Gene Expression Omnibus [62] to identify publicly available breast cancer patient microarray cohorts, in addition to the combined cohort described in Miller et al., 2011 [46]. These cohorts will be analyzed using R [63] in accord with the available phenotypic data. Our goal is to evaluate DCYTB compared to current prognostic indicators, such as molecular subtype and ER status. This will ultimately allow for more accurate evaluation and therapeutic treatment of patients.

## **b. Aim 2: The molecular function of duodenal cytochrome b expression in breast cancer**

We will next use a molecular biology approach to evaluate the function of DCYTB in breast cancer. Our laboratory and others have identified changes to ferroportin and transferrin receptor expression as mechanisms by which tumors accumulate iron [22, 35]. The function of DCYTB in iron homeostasis is clearly understood in duodenal enterocytes [9, 47, 59], though no research to date has investigated DCYTB in breast cells. This is, perhaps, not surprising as iron import in breast cells mainly relies on the transferrin/transferrin receptor system. Furthermore, while increased intracellular iron in breast cells has been correlated with breast cancer prognosis [35], it is unlikely that cellular labile iron will be influenced by DCYTB expression due to lack of available substrate [64]. We hypothesize that expression of DCYTB will affect intracellular iron differently in the breast than duodenal enterocytes. With the potential for negative effects perpetuated by the cellular labile iron pool (LIP), such as DNA damage through



generation of oxygen radicals, it is important to understand how DCYTB influences breast cell iron homeostasis.



**Figure 1-1. Cellular iron homeostasis.**

Dietary iron is imported into duodenal enterocytes via DMT1 (divalent metal transporter 1) following reduction by the transmembrane ferrireductase Dcytb (duodenal cytochrome b). Cellular labile iron (metabolically active iron) may be inertly stored in ferritin, utilized for cellular processes or exported to systemic circulation via ferroportin. Exported iron is oxidized and bound to transferrin for systemic transport where it binds to transferrin receptor. The complex is endocytosed and iron is imported into the labile iron pool where it is stored, utilized or exported.

## **Chapter II**

### **DCYTB is a predictor of outcome in breast cancer that functions via iron-independent mechanisms**

(to be submitted to Breast Cancer Research)

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Center, Farmington, Connecticut.

## A. Contributions

The majority of this work was completed by David J. Lemler in Suzy V. Torti's laboratory. This includes biochemical analysis of ferriredutase activity, labile iron pool assays, qRT-PCR, monitoring of cell growth and preparation of samples for the ICP-MS analysis performed by OHSU. Breast tissue staining by immunohistochemistry was performed by David J. Lemler with pathological assessment by Poornima Hegde. All Western blots were completed by David J. Lemler, except figures 2-9 and S2-10a which were performed by Lia Tesfay. DCYTB expression vectors were prepared by Zhiyong Deng and Bibbin T. Paul. Gene expression analyses included in this manuscript were performed by David J. Lemler in collaboration with and under the supervision of Miranda L. Lynch.

The manuscript was written and edited by David J. Lemler, Miranda L. Lynch, Suzy V. Torti and Frank M. Torti.

## B. Abstract

**Background:** Duodenal cytochrome b (DCYTB) is a ferriredutase that functions together with divalent metal transporter 1 (DMT1) to mediate dietary iron reduction and uptake in the duodenum. We previously identified DCYTB as a member of a 16 gene Iron Regulatory Gene Signature (IRGS) that predicts metastasis-free survival in breast cancer patients. The association of increased DCYTB with reduced risk was surprising, since the expression of genes that increase iron uptake or retention have generally been associated with increased risk. To better understand the relationship between DCYTB and breast cancer, we explored in detail both the prognostic significance and molecular function of DCYTB in breast cancer.

**Methods:** The prognostic and predictive significance of DCYTB expression independent of other iron genes was evaluated using publicly available microarray data from breast cancer tissue. Molecular function of DCYTB was assessed by modulating DCYTB expression in breast

cancer cells, determining the location of DCYTB in breast tissue, and assessing the effect of DCYTB on iron uptake and metabolism using breast cancer patient tissues and cell lines.

**Results:** High DCYTB expression was associated with prolonged recurrence-free survival in two large independent cohorts, together totaling 1610 patients (cohort #1,  $p = 1.6 \times 10^{-11}$ ,  $n = 741$ ; cohort #2,  $p = 1.2 \times 10^{-5}$ ,  $n = 869$ ; log-rank test). DCYTB also predicted response to tamoxifen and chemotherapy in subsets of these cohorts. Immunohistochemistry revealed that endogenous DCYTB is localized on the plasma membrane of breast epithelial cells, particularly on the surface of the ductal lumen, and that expression is dramatically reduced in high grade tumors. DMT1 was also expressed in breast cancer tissue, but did not co-localize with DCYTB. Surprisingly, neither overexpression nor knockdown of DCYTB affected levels of ferritin H, transferrin receptor or labile iron in breast cancer cells. The absence of an effect on cellular iron accumulation was further confirmed by ICP-MS, which showed no difference in total cellular iron regardless of the level of DCYTB expression.

**Conclusion:** We conclude that DCYTB is expressed in breast tissue but does not affect intracellular iron accumulation in breast cancer cells. DCYTB is an important predictor of outcome and response to therapy in breast cancer patients.

## C. Background

Iron has been implicated in both the initiation and progression of cancer. Due to its ability to catalyze the formation of oxygen free radicals, iron can facilitate DNA damage and lead to potentially mutagenic changes in DNA [65]. Iron can also act as a tumor growth factor, potentiating the growth of numerous tumors, including breast tumors, in animal models [26, 66]. Consistent with these laboratory studies, epidemiologic studies have linked excess iron and cancer [67-70]. For example, subjects with increased levels of circulating iron are at increased risk of cancer [21, 27, 28], and conversely, subjects who have undergone phlebotomy for iron

reduction are at decreased cancer risk [70].

The major mechanism of iron import in both normal and malignant cells is the transferrin/transferrin receptor endocytic pathway. Two molecules of ferric iron bound to transferrin are endocytosed upon transferrin receptor binding. Iron is released in the acidified endosome, reduced, and imported into the cytosol, where it enters a low molecular weight, metabolically active labile iron pool (LIP). Excess iron in the cytosol is stored in ferritin or exported via the iron exporter, ferroportin [71]. Other mechanisms of iron import include uptake of heme, ferritin, and import of siderophore-bound iron by proteins such as Lipocalin 2 (LCN2, NGAL), a secreted glycoprotein [72-75].

In the duodenum, where uptake of dietary iron occurs, an additional mechanism for iron import has been described that involves duodenal cytochrome b (DCYTB) [9, 47, 59]. Dietary iron is largely present in an oxidized form (ferric iron,  $\text{Fe}^{+3}$ ). DCYTB acts as a ferrireductase, reducing ferric iron to ferrous iron to permit iron uptake by divalent metal transporter 1 (DMT1). Identified in 2001 [9], DCYTB is a member of the cytochrome b561 protein family of di-heme, transplasma membrane electron transporters [51, 52]. Reduction of iron by DCYTB is pH-dependent and ascorbate-dependent in duodenal enterocytes [9, 47, 58, 59], but ascorbate-independent in bronchial epithelial cells [60]. Copper is also a substrate for reduction by DCYTB, a reaction that occurs in a pH-independent, ascorbate-dependent manner [59]. Additionally, DCYTB expression has been shown to maintain extracellular levels of ascorbate [55].

Cancer cells exhibit an enhanced requirement for iron compared to their normal counterparts. To meet the increased metabolic demand for iron, breast and other cancer cells frequently increase expression of the iron importer transferrin receptor [31-33]. Alternatively or additionally, cancer cells suppress expression of the iron efflux protein ferroportin. Although retained iron is sequestered in ferritin, this nevertheless results in an increase in labile iron [17, 34, 35].

Measurements of the expression of genes of iron metabolism are strong predictors of patient prognosis. For example, breast cancer patient microarray data demonstrated that increased transferrin receptor expression [44, 45] or decreased ferroportin expression in breast tumors is associated with poor prognosis [35]. Tumoral expression of LCN2 is also associated with poor prognosis and increased metastasis in breast cancer [76, 77].

To ascertain which components of iron metabolism most influence breast cancer prognosis, our group studied the association of 61 “iron” genes with breast cancer patient outcome [46]. From this, an “iron gene regulatory signature” was derived, consisting of 16 genes whose expression best predicted breast cancer patient outcome. Of these 16 genes, duodenal cytochrome b (DCYTB, CYBRD1, CYB561A2) was the most significantly associated with distant metastasis-free survival (DMFS), with high expression (values above the mean) associated with a HR of 0.6 ( $p = 1.8 \times 10^{-7}$ ). Since DCYTB facilitates iron import, its association with improved outcome was surprising. The expression of this gene in the breast was also unanticipated, since its best-known function involves uptake of dietary iron.

We sought to understand in greater depth the nature of the association of DCYTB with breast cancer, and to explore the role of DCYTB in the breast. We assessed the ability of DCYTB to predict patient survival and response to therapy utilizing two large independent gene expression datasets obtained from breast cancer patients. We also investigated whether DCYTB expression influenced iron homeostasis in malignant breast cells. Our results indicate that DCYTB expression is closely tied to patient outcome and response to therapy. We also demonstrate that DCYTB does not affect intracellular iron in breast cancer cells. These results uncouple DCYTB from iron metabolism in breast cancer tissue and provide an explanation for the paradoxical association between increased DCYTB expression and favorable prognosis in breast cancer patients.

## **D. Results**

### **a. DCYTB as a prognostic indicator of breast cancer**

#### *Expression of DCYTB predicts metastasis/relapse-free survival*

We first focused on the prognostic significance of DCYTB when considered as a single gene rather than as part of the larger IRGS gene signature [46]. Analysis of the combined cohort of 741 breast cancer patients that was used in the design of the IRGS [46] (herein termed cohort #1), revealed that high DCYTB expression (values above the mean) was an excellent overall predictor of distant metastasis-free survival ( $p= 1.6e-11$ ,  $n= 741$ , log-rank test; Figure 2-1a).

We then validated and expanded our results using additional datasets not included in cohort #1, which we combined into a new cohort of 869 patients (cohort #2). To construct this cohort, we selected all of the larger datasets ( $n > 100$ ) with sufficient event rates to meaningfully separate patients by outcome (Table 2- 1). Datasets that did not meet these criteria (e.g. GSE19615 [78], TCGA) were excluded. In cohort #2, DCYTB expression above the mean was again associated with increased relapse-free survival (RFS) ( $p= 1.2e-05$ ,  $n= 869$ ; log-rank test; Figure 2-1b). One of the datasets used to construct cohort #2 contained information on bone-specific RFS; analysis of this subgroup ( $n=272$ ) further revealed that DCYTB expression was associated with bone-specific RFS (Figure 2-1c). Consistent with the association of high DCYTB with favorable prognosis, we further observed that expression of DCYTB was higher in tumors that expressed estrogen receptor (ER+) than in ER- tumors (Supp Figure 2-1). Additionally, DCYTB expression decreased with increased tumor grade (Supp Figure 2-2). In aggregate, these results indicate that high DCYTB expression predicts a more favorable prognosis in breast cancer patients.

We next tested whether DCYTB retained its prognostic value in known breast cancer prognostic groups. We first examined whether DCYTB expression was predictive in both estrogen receptor positive (ER+) and ER- cohorts. As shown in Figure 2-2, Kaplan-Meier



survival analysis of the cohort #1 indicated that DCYTB significantly predicted DMFS independently of estrogen receptor status ( $p = 1.3e-10$  and  $p = 0.03$ , log-rank test, Figure 2-2a & b). Similarly, analysis of cohort #2 revealed that high DCYTB expression was associated with increased relapse-free survival of both ER+ and ER- patients ( $p = 0.004$  and  $p = 0.01$ , log-rank test, Supp Figure 2-3a & b).

We also tested whether DCYTB had predictive value in patients whose disease remained confined to the breast (LN-) and patients whose disease had spread to adjacent lymph nodes (LN+). In cohort #1, DCYTB expression was capable of predicting DMFS independently of LN status ( $p \leq 0.0001$ , log-rank test Figure 2-2c & d). The association of elevated DCYTB expression with prolonged relapse-free survival was also observed in LN+ and LN- patients of cohort #2 ( $p = 0.02$  and  $p = 0.0001$ , log-rank test, Supp Figure 2-3c & d). Collectively, these data indicate that high DCYTB expression is associated with a more favorable prognosis in breast cancer patients, independent of their ER or LN status.

#### *DCYTB expression correlates with better prognosis breast cancer molecular subtypes*

We then investigated the expression of DCYTB within breast cancer intrinsic molecular subtypes. These subtypes are now commonly used to divide patients into prognostic subgroups based on gene expression profiles [79, 80]. When cohort #1 was divided into intrinsic subtypes, the expected prognostic associations with patient outcomes were observed [79, 81]: Luminal A and Normal-like demonstrated better outcomes, and Luminal B, Basal and Her2 had less favorable survival (Supp Figure 2-4). We found that DCYTB expression was higher in subtypes with more favorable prognoses: Luminal A subtype had significantly higher DCYTB expression than all other subtypes ( $p \leq 0.0028$ , pairwise t-test) while Normal-like subtype had significantly higher DCYTB expression than all other subtypes with less favorable prognosis ( $p \leq 2.8e-15$ , pairwise t-test) and Basal subtype had significantly reduced DCYTB expression compared to all other subtypes ( $p \leq 0.0027$ , pairwise t-test, Figure 2-3). Subtype information was also available

for a subset of patients from cohort #2 (Supp Figure 2-5a). Similar to what we observed in cohort #1, in this subset, patients of Luminal A subtype had significantly more DCYTB expression compared to Luminal B, Her2 and Basal subtypes and the Normal-like subtype was significantly increased compared to Luminal B and Basal (Supp Figure 2-5b). Thus high DCYTB expression is associated with subtypes that have better outcome.

#### *Expression of DCYTB predicts response to therapy*

Finally, we asked whether DCYTB expression was predictive of response to therapy. To address this question, we first examined a subset of ER+, LN- patients from cohort #1 that were histologically similar and had been treated with tamoxifen monotherapy (n= 263) [46]. DCYTB expression significantly predicted DMFS in this group ( $p= 5.7\text{e-}05$ , log-rank test; Figure 2-4a). To determine whether DCYTB also predicted response to chemotherapy, we then examined a subset of cohort #2. This group consisted of 310 patients who were ERBB2- (HER2-) and either ER+ or ER- and had been treated with taxane-anthracycline neoadjuvant chemotherapy (and tamoxifen if ER+) followed by surgery (GSE25055) [82]. We found that DCYTB was also predictive of improved RFS in this group ( $p= 0.003$ , log-rank test, Figure 2-4b). Thus DCYTB predicts response to chemotherapy as well as hormone therapy.

#### **b. DCYTB expression and localization in normal and malignant breast tissue**

We next investigated the level of DCYTB in normal and malignant breast tissue. This analysis was restricted to cohort #2 because only cohort #2 contained normal breast samples. We observed that normal breast tissue exhibited significantly higher levels of DCYTB mRNA than malignant tissue (Supp Figure 2-6).

We then assessed the cellular distribution and localization of DCYTB in breast tissue using immunohistochemical analysis of a tissue microarray of 75 cases and controls. Our

objective was to assess whether DCYTB was limited to breast epithelial cells or was present in immune, endothelial, adipose and other cell types that constitute tumor tissue. We also expected to gain information on the potential function of DCYTB by assessing its intracellular distribution. In the duodenum, where DCYTB functions in iron import, DCYTB is localized to the brush border, on the surface of the enterocyte [9]. However, there are reports of DCYTB in the membrane of intracellular vesicles in both esophageal carcinoma and in normal and malignant colon [83, 84]. Further, other members of the cytochrome b561 family, which function in vesicular catecholamine synthesis and lysosomal degradation, are expressed in the membrane of intracellular organelles [52, 54, 85].

We observed that DCYTB was present on the luminal surface of epithelial cells in breast ducts and on the cell membrane of myoepithelial cells in normal breast tissue (Figure 2-5a). Consistent with previous reports, erythrocyte membranes also stained positive for DCYTB [55]. Cribriform-type DCIS showed intense staining along the luminal surfaces, similar to normal tissue, with additional faint cytoplasmic staining (Figure 2-5b). Higher grade invasive tumors displayed reduced gland/tubule formation [86, 87], with a corresponding reduction in epithelial cells with membrane expression of DCYTB (Figure 2-5c & d).

In addition to its role in intestinal iron uptake, DCYTB has been suggested to detoxify excess iron in bronchial epithelial cells through a mechanism involving DCYTB-mediated ferrireduction, uptake of divalent iron by divalent metal transporter 1 (DMT1), and storage in ferritin [60]. Since a role for DCYTB in either iron import or detoxification requires DMT1, we performed immunohistochemical analysis of DMT1. As expected, in control duodenal tissue, expression of DCYTB and DMT1 overlapped (Figure 2-5i & j), consistent with the functional partnership of DCYTB and DMT1 in iron reduction and import in this tissue [9, 10]. In contrast, in the breast, expression of DMT1 was predominantly cytoplasmic, with minimal membrane staining (Figure 2-5e-h). Collectively, these data suggest that DCYTB expressed in breast tissue may not function in its typical iron import role.

### **c. Effects of DCYTB expression on iron metabolism in breast cancer cells**

To directly test whether DCYTB affects iron metabolism in breast cells, we selected breast cell lines with high and low expression of DCYTB. As shown in Supp Figure 2-7a, Western blot analysis indicated that T47D ductal carcinoma cells exhibited high basal expression of DCYTB, whereas MCF7 breast cancer cells exhibited substantially lower DCYTB expression. Because we had observed that DCYTB was higher in ER+ than in ER- tumors using both microarray (Supp Figure 2-1) and immunohistochemical analysis (Figure 2-5a-d), we explored whether DCYTB was directly regulated by estrogen in these cells. We found that DCYTB was neither induced by estrogen nor inhibited by tamoxifen (Supp Figure 2-7).

To determine whether DCYTB played a role in iron import in breast cancer cells, we over-expressed and knocked down DCYTB and assessed effects on parameters of iron metabolism. We first constitutively overexpressed DCYTB in MCF7 cells, which express low levels of endogenous DCYTB (Figure 2-6a). To confirm that this exogenous DCYTB was functional, we measured its enzymatic activity using a ferriredutase assay. Tet-off DCYTB-EGFP MDCK cells, which have been previously shown to express doxycycline-regulated functional DCYTB with ferriredutase activity [59], were used as a control. As seen in Figure 2-6b, MCF7 cells over expressing DCYTB had significantly higher ferriredutase activity than cells transfected with empty vector. Control DCYTB-EGFP MDCK cells exhibited the expected doxycycline-regulated ferriredutase activity (Figure 2-6b). Thus exogenous DCYTB is expressed and functional in MCF7 cells.

We then tested whether DCYTB modulated iron import by examining transferrin receptor 1 (TFRC) and ferritin H (FTH1), two sensitive indicators of intracellular iron [88-90]. Expression of these proteins is post-translationally regulated by iron: transferrin receptor expression is increased in iron deplete conditions and decreased in iron replete conditions, while the opposite is true of ferritin H. Thus, high TFRC expression coupled with low FTH1 is indicative of a state of

decreased cellular iron, whereas low TFRC and high FTH1 indicates elevated levels of cellular iron. We observed no difference in transferrin receptor or ferritin H expression in MCF7 cells expressing DCYTB when compared to cells infected with the empty vector (Figure 2-6a), suggesting that exogenous DCYTB does not affect levels of intracellular iron.

To further investigate the effects of DCYTB, we performed the converse experiment by knocking down DCYTB in T47D cells, which express high levels of endogenous DCYTB (Figure 2-7a). DCYTB was significantly reduced by transfection of targeted siRNA (Figure 2-7a); however, ferritin H and transferrin receptor were not affected. Consistent with these results, measurement of the labile iron pool revealed no change in labile iron as a function of DCYTB expression (Figure 2-7b). To confirm these results, we also assessed total cellular iron by ICP-MS in DCYTB knockdown T47D cells and DCYTB overexpressing MCF7 cells. Treatment with iron was used as a control. In both cell types, levels of intracellular iron were comparable, regardless of the level of DCYTB expression (Figure 2-8a & b). This suggests that modulation of DCYTB expression does not significantly influence overall levels of cellular iron.

However, it was possible that DCYTB might facilitate iron uptake under the specific condition of iron excess. To explore this, we used T47D and MCF7 cells expressing a Tet-inducible DCYTB expression vector, which enabled us to modulate DCYTB expression over a more graded range than that obtained using constitutive overexpression (Supp Figure 2-8). We found that in both T47D and MCF7 cells, basal levels of ferritin H were unaffected by DCYTB expression, regardless of the levels of DCYTB induction, supporting results obtained with constitutive expression of DCYTB (Supp Figure 2-8). We then compared the effect of DCYTB on the response of cells to exogenous iron (ferric ammonium citrate, FAC). In all cases, iron induced ferritin H and increased the labile iron pool to a similar extent (Figure 2-9). Thus, in both T47D and MCF7 cells, there was a 3-4-fold increase in ferritin with 200  $\mu$ M FAC, regardless of the level of DCYTB (Figure 2-9a and c). Similarly, labile iron in both T47D and MCF7 cells was unchanged by DCYTB expression (Figure 2-9b & d). Consistent with these results, ICP-MS

analysis of cells cultured for 24 hours in 200  $\mu$ M FAC revealed no effect of DCYTB status on total cellular iron (Figure 2-8a & b).

#### **d. Signaling Pathway Impact Analysis**

Although we observed that DCYTB was capable of reducing iron (Figure 2-6b), expression of DCYTB had no measurable effect on iron levels in breast cancer cells (Figure 2-6, 7, 8 & 9). We therefore sought to identify other molecular functions of DCYTB that might be responsible for its positive association with prognosis. To accomplish this, we compared expression profiles from patients that expressed the highest ( $\geq$  90th percentile) and lowest ( $\leq$  10th percentile) levels of DCYTB in cohorts #1 and #2. We used the Signaling Pathway Impact Analysis (SPIA) package [91] in the statistical software environment “R” [63] to identify functionally altered pathways, accounting for differentially expressed genes and pathway topology. In both cohorts, two pathways were significantly altered: cell cycle and focal adhesion (Supplemental Table 2- 1). These pathways converge, since increased signaling of the focal adhesion pathway can stimulate the cell cycle [92]. We therefore assessed the effect of knockdown or overexpression of DCYTB on cell proliferation. We observed that the rate of increase in cell number was the same in T47D cells treated with siDCYTB or control siRNA, and was also unchanged in MCF7 cells that overexpressed DCYTB when compared to controls (Figure 2-10a & b). We also investigated the effect of DCYTB expression on progression through the cell cycle in T47D cells treated with siGAPDH or siDCYTB (Supp Figure 2-9a & b). There was no appreciable difference in cell cycle distribution regardless of siRNA (Supp Figure 2-9a & b). Thus, expression of DCYTB does not appear to directly affect cell cycle progression or proliferation of breast cancer cells.

### **E. Discussion**

DCYTB was identified as one of 16 genes comprising an Iron Regulatory Gene

Signature (IRGS) that is predictive of breast cancer patient survival [46]. In the IRGS, high expression of DCYTB was associated with improved distant metastasis-free survival. This was unexpected, because in the duodenum, DCYTB acts in conjunction with DMT1 to promote iron uptake, and an extensive literature links enhanced iron uptake with increased rather than decreased cancer risk [21, 26-28, 66-70]. Our results resolve this apparent paradox between the anticipated role of DCYTB and its association with favorable prognosis by revealing that in breast cancer cells, DCYTB does not play a role in iron acquisition.

We used immunohistochemical analysis to confirm the expression of DCYTB protein in breast tissue and to assess its cellular and subcellular localization (Figure 2-5). We observed that DCYTB is present on the cell surface of epithelial and myoepithelial cells, and is particularly abundant at the luminal surface of ducts. DCYTB did not co-localize with DMT1, the transport protein with which DCYTB partners for uptake of iron, casting doubt on a role for DCYTB in iron transport or detoxification in breast cells (Figure 2-5). We therefore used cell culture experiments to directly test the ability of DCYTB to impact iron metabolism in breast cancer cells.

Neither DCYTB overexpression nor DCYTB knockdown altered parameters of iron metabolism in breast cancer cells. Exogenously expressed DCYTB exhibited ferrireductase activity (Figure 2-6b), indicating that the function of the transfected gene was preserved. However, basal levels of ferritin, an iron storage protein that is translationally regulated by iron, and transferrin receptor, an iron import protein that is also post-transcriptionally regulated by iron, were unchanged following either overexpression of DCYTB in MCF7 cells (Figure 2-6a) or knockdown in T47D breast cancer cells (Figure 2-7a). DCYTB overexpression also did not affect the response of cells to excess exogenous iron (Figure 2-8, 9a & c), the intracellular labile iron pool (Figure 2-7b, 9b & d), or total cellular iron (Figure 2-8).

There are several potential alternative roles for DCYTB. DCYTB is expressed in numerous cell types that are not directly involved in dietary iron uptake, such as erythrocytes,

airway epithelial cells, astrocytes, and HepG2 cells [55, 60, 93-95]. This has led to the suggestion that DCYTB functions as a general oxidoreductase rather than a specific ferrireductase [96]. Since DCYTB belongs to the cytochrome b561 family of proteins involved in ascorbate regeneration [52], contains predicted ascorbate binding domains [9], uses ascorbate as a reductant [58], and has been suggested to function in maintenance of extracellular ascorbate in erythrocytes [55], the primary role of DCYTB in breast tissue may lie in the maintenance of tissue redox balance. Alternatively, DCYTB is capable of reducing copper [59], and this activity may be associated with its protective role in breast cancer, albeit by an unknown mechanism.

We used Signaling Pathway Impact Analysis (SPIA) to identify potentially novel functions of DCYTB in breast cancer. We observed that cell cycle and focal adhesion pathways were significantly different in breast tumors that express the highest versus the lowest levels of DCYTB (Supplemental Table 2- 1). However, DCYTB did not directly affect the proliferation of breast cancer cells in tissue culture (Figure 2-10), suggesting that although expression of DCYTB is associated with perturbation of the cell cycle in breast tumors, this may not be a causal relationship. Future experiments will test whether DCYTB influences the focal adhesion pathway, particularly focal adhesion kinase (FAK), which regulates cell survival and motility [92, 97] and is often aberrantly expressed in cancer [98, 99].

Despite clear evidence that DCYTB does not modulate iron levels in breast cancer cells, our data demonstrate that DCYTB is nonetheless a strong predictor of outcome and response to therapy in breast cancer patients. Analysis of two combined cohorts that together total 1610 breast cancer patients revealed that high DCYTB expression was associated with longer distant metastasis-free survival and longer relapse-free survival (both local and distant) (Figure 2-1). DCYTB was capable of further stratifying patients into groups with significantly different outcomes regardless of ER or LN status (Figure 2-2 and Supp Figure 2-3).

Breast cancer patients have been successfully classified into outcome groups based on



molecular profiling [79, 80], and several platforms for patient classification have been developed, including Oncotype Dx, Mammaprint, PAM50, and EndoPredict [100, 101]. DCYTB is not included in these currently available commercial and research-based classification systems. However, we observed that DCYTB expression increased in molecular subtypes with more favorable prognosis (Figure 2-3 and Supp. Figure 2-5), demonstrating that as a marker, DCYTB exhibits behavior that is consistent with known molecular markers of breast cancer.

Although estimating patient prognosis is helpful to physicians and patients, predicting the probability of response to therapy is critical to clinical decision-making, and remains a challenge in breast cancer [100, 102, 103]. To test whether DCYTB could be used in predicting response to therapy, we assessed the association of DCYTB expression with outcome in two different cohorts. The first cohort consisted of women with ER+ tumors who had been treated with tamoxifen monotherapy (Figure 2-4a), and the second was a population of women with ERBB2-tumors treated with neoadjuvant chemotherapy (Figure 2-4b). We observed a significant association of DCYTB expression with patient response to therapy. In both cases, patients with low DCYTB expression were less likely to respond to treatment than those with high DCYTB expression (Figure 2-4a & b). These results suggest that measurement of DCYTB expression may be useful in tailoring therapy: for example, it could help guide a subset of ER+ patients to more aggressive therapy, or alternatively, identify those for whom the risks of chemotherapy are less warranted.

## **F. Conclusion**

Our results demonstrate that DCYTB is a strong predictor of outcome and response to therapy in breast cancer patients. Although DCYTB can reduce iron and facilitate iron uptake in other tissues, DCYTB appears to function via an iron-independent mechanism in the breast.

## **G. Methods**

### **a. Cell Culture & Reagents**

17- $\beta$ -estradiol (Sigma, E2758), Tamoxifen (4-hydroxy-TEMPO) (Sigma, 176141), FerroZine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate) (Sigma, 160601), ferric ammonium citrate (Sigma, F5879), Doxycycline hyclate (Sigma, 9891), FuGENE® HD Transfection Reagent (Promega, E2311), Hydroxyurea (Sigma, H8627)

Cell culture: T47D in RPMI-1640 with 10% FBS, MCF7 in EMEM with 10% FBS and 10 U/mL insulin, MCF10A in MEGM Bulletkit™ with 100 ng/mL cholera toxin (Sigma) and MDCK in DMEM with 10% TET-free FBS (Clontech or Hyclone) and puromycin (1.0 ng/mL) [59]. All basal media supplied by LONZA and FBS supplied by GEMINI unless otherwise specified.

### **b. Overexpression cell lines**

#### *Constitutive DCYTB expression vector*

DCYTB coding sequencing was amplified from cDNA of U138MG cells, which was then cloned into BamHI and XbaI sites of pSL2 vector, a lentiviral overexpression vector with an EGFP selection marker [104]. The cloning primers were: DCYTB-F (5' tcgggatccgcatggagggctactggcgct 3') and DCYTB-R (5' tagtctagatcacatggtagatctctgcccag 3'). Sequence comparison with the reference gene in NCBI database revealed that the cloned DCYTB cDNA was a polymorphism variant (S266N, rs10455 [105]). To obtain the wide-type DCYTB expression vector, we rectified the pSL2-DCYTB (S266N) variant using a site-direct-mutagenesis cloning method. Sequence authentication for all of the vectors was done by DNA sequencing.

#### *Inducible DCYTB expression vector*

Primers were designed to amplify human DCYTB cDNA from pSL2-DCYTB plasmid. Forward (5' CCCTCGTAAAGAATTCGCCACCATGGCCATGGAGGGCTACTGG 3') and

reverse (5' GAGGTGGTCTGGATCCTTACATGGTAGATCTCTGCCCAGCC 3') primers contained restriction enzyme sequence for EcoR1 and BamH1 respectively. The PCR product of DCYTB (861 bp) was restriction digested and inserted between the EcoR1/BamH1 sites in the pLVX-TetOne-Puro (Takara-Clontech, Mountain View, CA). Plasmids were subsequently purified and sequenced. Cells were transfected using FuGENE® HD transfection reagent followed by 2 weeks of puromycin selection.

### **c. siRNA**

All reagents obtained from Dharmacon. Transfections performed according to manufacturer's recommendations with Dharmafect #1 transfection reagent.

### **d. Western Blotting**

For DCYTB analysis, non-reduced samples were used; other samples were reduced. Western blots were probed with antibodies to DCYTB (Sigma), transferrin receptor (Invitrogen), ferritin H (Abcam) or  $\beta$ -actin (Sigma).

### **e. mRNA Expression**

qRT-PCR was performed essentially as described [106], except that RNA was isolated and purified using the High Pure RNA Isolation Kit (Roche Diagnostics) and RT-qPCR was carried out using 2X SYBR® Green PCR Master Mix (BioRad) in a ViiA7 cyclor (Applied Biosystems). Primers for PCR were designed with IDT PrimerQuest software (Integrated DNA Technologies, Inc.): DCYTB forward 5'-TGCATACAGTACATTCCCGCCAGA-3', DCYTB reverse 5'-ATGGAACCTCTTGCTCCCTGTTCA-3', ACTB forward 5'-TTGCCGACAGGATGCAGAAGGA-3', ACTB reverse 5'-AGGTGGACAGCGAGGCCAGGAT-3'. GREB1 primers were extracted from Stossi et al. [107].

### **f. Immunohistochemistry**

Breast tissue microarrays were obtained from US Biomax. Antigen retrieval was performed using 0.05% citraconic anhydride (Acros Organics) at pH 7.4 prior to immunostaining with a rabbit anti-DCYTB antibody (Sigma) or rabbit anti-DMT1 antibody (Sigma). Slides were counterstained with hematoxylin (Poly Scientific).

#### **g. Measurement of the labile iron pool - LIP Assay**

Cells were siRNA transfected or doxycycline treated in 96-well plates and treated with 200  $\mu$ M ferric ammonium citrate (Sigma) for 24 hours prior to assay. Cells were washed, incubated with 2  $\mu$ M calcein acetoxymethyl ester (Life Technologies) for 15 to 30 minutes at 37°C, washed with phenol-free EMEM, and 100 $\mu$ M starch-conjugated deferoxamine (DFO; a generous gift of Biomedical Frontiers, Inc., Minneapolis, MN) was added. Fluorescence was measured at 485 nm excitation and 535 nm emission (BioTek Synergy 2). Following stabilization of the fluorescence signal, 10  $\mu$ M salicylaldehyde isonicotinoyl hydrazone (SIH) was added for several minutes. The change in fluorescence following the addition of SIH ( $\Delta F$ ) was used as a measure of the labile iron pool.

#### **h. Cell cycle analysis**

Cells were synchronized with a 24-hour treatment of hydroxyurea. Following release from synchronization, cells were removed from the culture dishes and washed several times in PBS containing FBS and EDTA and fixed in 70% ethanol overnight. Propidium iodide staining and RNase treatment was done with FxCycle™ PI/RNase Staining Solution (ThermoFisher Scientific, F10797). Fluorescence intensity was collected using a MACSQuant Analyzer. ModFit software was used to calculate cell cycle histograms.

#### **i. Microarray Data Sets**

Cohort #1 [46] was downloaded in October 2013 from Cancer Research [108] as a

preprocessed file. Individuals with missing data (event data was unavailable for 18 patients) were excluded from the analysis. For construction of cohort #2, four publicly available breast cancer patient datasets met our criteria: (i) 303 (Discovery, GSE25055) and 193 (Validation, GSE25065) patients from a prospective study at M.D. Anderson Cancer Center that identified a predictive signature of response to neoadjuvant chemotherapy [82]; (ii) retrospective study of frozen tissue of 272 lymph node negative patients from Rotterdam, Netherlands who did not receive systemic adjuvant or neoadjuvant therapy (GSE2034) [109]; and (iii) 101 cancer and 14 normal patient samples from Dublin, Ireland resected prior to hormone or chemotherapy (GSE42568) [110]. GSE25055 was downloaded April 2015 and GSE25065, GSE2034 and GSE42568 datasets were downloaded May 2015 from the National Center for Biotechnology Information Gene Expression Omnibus [62, 111] along with clinical and follow-up data. Where possible, CEL files were downloaded, preprocessed and RMA normalized. Surrogate variable analysis (SVA package) was used to batch correct cohort #2 [112, 113].

#### **j. Statistical analysis**

Analysis of microarray patient datasets was done with R: A Language and Environment for Computing using the affy [114], survival [115, 116], limma [117] and SPIA [91] packages. The DCYTB probe with the greatest expression in each cohort was used for analysis. Kaplan-Meier (KM) survival analysis was used to determine distant metastasis-free survival (DMFS), relapse-free survival (RFS) (both local and distant) and bone-specific (RFS). Significance of KM plots was determined by the log-rank test. We used the signaling pathway impact analysis (SPIA) algorithm [91], implemented in R, to identify significantly activated or inhibited pathways ( $p\text{FWER} < 0.05$ ), using information from KEGG pathway annotations and differentially expressed genes ( $p < 0.05$ ) between high and low DCYTB expressing groups.

#### **k. ICP-MS**

All containers used for sample digestion and preparation were pre-treated with trace-metal grade HNO<sub>3</sub> to remove metal contaminations. Protein samples were digested in 100 µl HNO<sub>3</sub> (trace metal grade, Fisher Scientific) in polypropylene reagent tubes (Sarstedt) in a heating block at 90° C for 3 hours after which 100 µl of 10 M H<sub>2</sub>O<sub>2</sub> (trace metal grade, Fisher Scientific) was added to the solution. The digested sample was further diluted to 2 ml total volume with 1% HNO<sub>3</sub> and stored in pre-cleaned polypropylene tubes until measurement. To ensure elemental recovery of >90%, NIST reference material (freeze-dried, powdered bovine liver, SRM 1577c) as well as the common elemental standard mix (VHG laboratories) were simultaneously digested by the same method. To determine background contamination from the tubes an empty tube was treated with 1 ml HNO<sub>3</sub> and prepared concomitantly with the samples. Inductively coupled plasma mass spectroscopy (ICP-MS) analysis was performed using an Agilent 7700x equipped with an ASX 250 autosampler. The system was operated at a radio frequency power of 1550 W, an argon plasma gas flow rate of 15 L/min, Ar carrier gas flow rate of 1.04 L/min. Elements were measured in kinetic energy discrimination (KED) mode using He gas (4.3 ml/min). Data were quantified using a 9-point (0, 0.5, 1, 2, 5, 10, 50, 100, 1000 ppb (ng/g)) calibration curve with external standards for Mg, Mn, Fe, Cu, and Zn. For each sample, data were acquired in triplicates and averaged. A coefficient of variance was determined from frequent measurements of a sample containing 10 ppb of all elements analyzed. An internal standard (Sc, Ge, Bi) introduced with the sample was used to correct for detector fluctuation and to monitor plasma stability. Elemental recovery was evaluated by measuring NIST reference material (water SRM 1643e) and found to be >90% for all determined elements.

## **H. Funding**

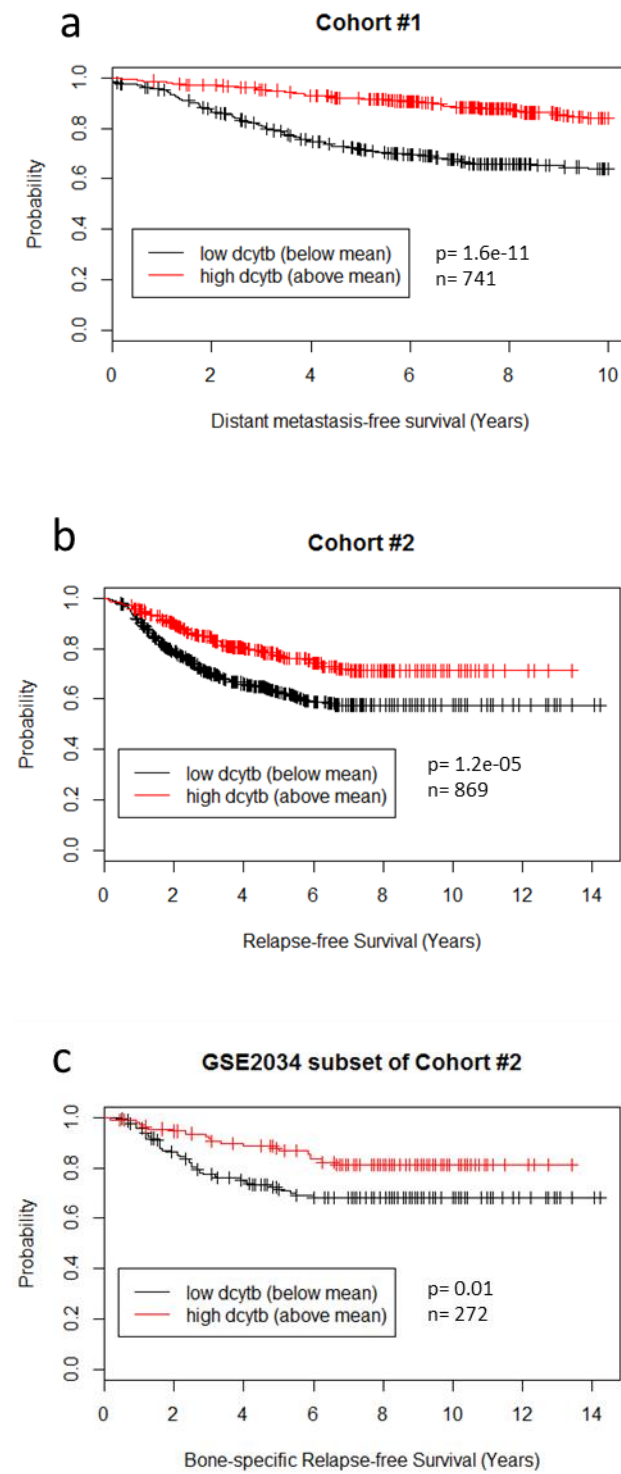
Supported in part by NIH grants R01 CA171101 (F.M.T) and R01 CA188025 (S.V.T). ICP-MS measurements were performed at the OHSU Elemental Analysis Core with partial

support from NIH core grant S10RR025512.

## **I. Acknowledgments**

We thank Dr. Andrew McKie for the Tet-off DCYTB-EGFP MDCK cells and for valuable discussion.

## J. Figures and Tables





**Figure 2-1. High DCYTB expression is associated with increased recurrence-free survival in breast cancer**

High DCYTB expression is associated with increased recurrence-free survival in breast cancer

Breast cancer patient microarray data was subsetted into high and low DCYTB expression

groups (above and below the mean). a. Cohort #1 distant metastasis-free survival ( $p= 1.6e-11$ ,

$n= 741$ , log-rank test); b. Cohort #2 relapse-free survival (both local and distant) ( $p= 1.2e-05$ ,  $n=$

869, log-rank test); c. Subgroup of cohort #2 (GSE2034) bone-specific relapse-free survival

( $p=0.01$ ,  $n= 272$ , log-rank test)

**Table 2-1. Characteristics of patients and samples used in microarray analysis**

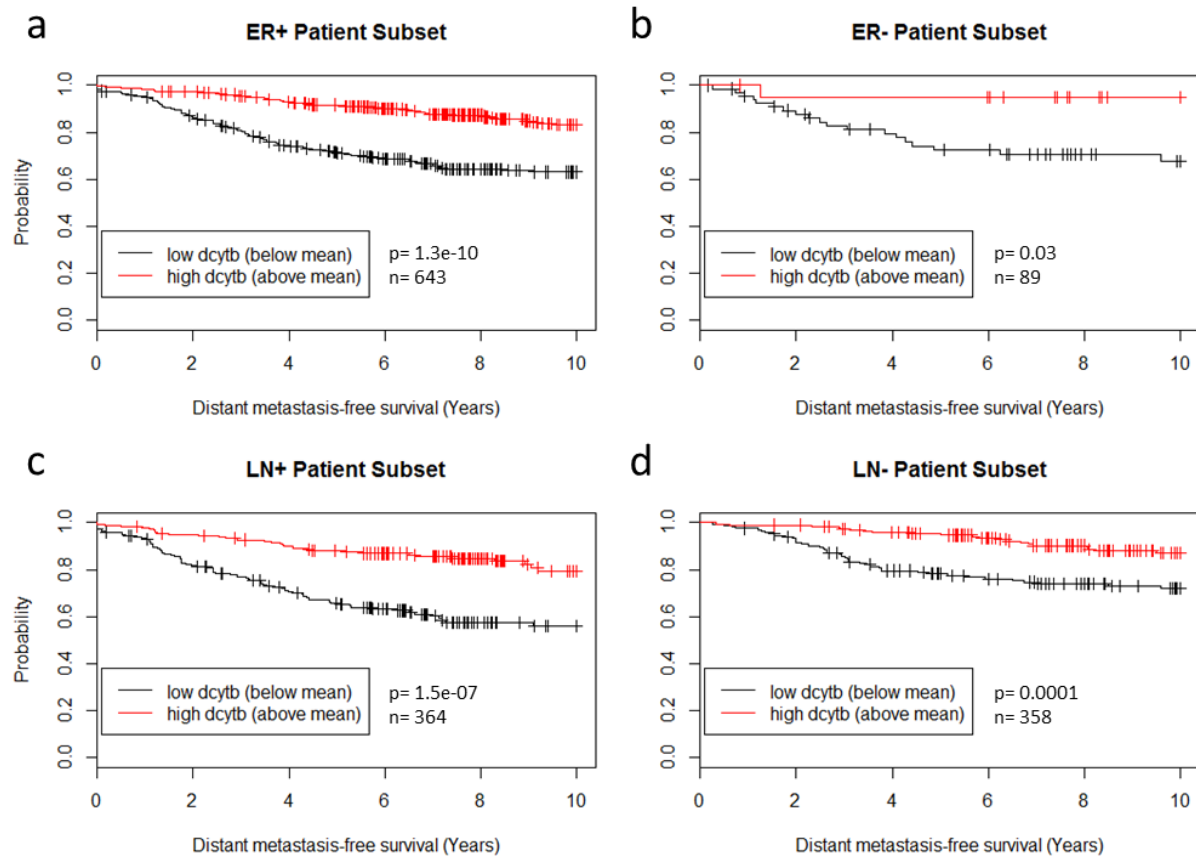
| Name                                     | n   | Event (%) | Median Recurrence-free Survival (Years) | Median Follow-up (Years) | Mean Age, years (SD) | ER+ | ER- | LN+ | LN- | Her2 | PR+ | PR- | Grade 1/2/3 | Normal Tissue |
|--|-----|-----------|---|--------------------------|----------------------|-----|-----|-----|-----|------|-----|-----|-------------|---------------|
| Cohort #1*                               | 741 | 167 (22)  | 7.8                                     | 8.7                      | 60.8 (12.5)          | 643 | 89  | 364 | 358 | NA   | NA  | NA  | 153/314/188 | NA            |
| Cohort #2**                              | 869 | 251 (29)  | 4.3                                     | 4.5                      | 51 (11.1)            | 486 | 276 | 342 | 426 | 6    | 239 | 250 | 43/209/309  | 14***         |
| Hatzis et al. [82] Discovery (GSE 25055) | 303 | 63 (21)   | 2.4                                     | 2.6                      | 50 (10.3)            | 170 | 133 | 217 | 86  | 4    | 140 | 157 | 19/113/149  | NA            |
| Hatzis et al. [82] Validation (GSE25065) | 193 | 42 (22)   | 3.2                                     | 3.7                      | 49.2 (10.6)          | 120 | 72  | 125 | 68  | 2    | 99  | 93  | 13/59/107   | NA            |
| Wang et al. [109] (GSE2034)              | 272 | 100 (37)  | 7.2                                     | 8.6                      | 54 (12)              | 196 | 76  | 0   | 272 | NA   | NA  | NA  | NA          | NA            |
| Clarke et al. [110] (GSE42568)           | 101 | 46 (46)   | 5.3                                     | 6.2                      | 58.6 (11.6)          | 65  | 33  | 58  | 43  | NA   | NA  | NA  | 11/37/53    | 14***         |

\* constructed from data in caArray: mille-00271; GEO: GSE1456, GSE6532, GSE9195

\*\* constructed from data in GEO: GSE25055, GSE25065, GSE2043, GSE42568

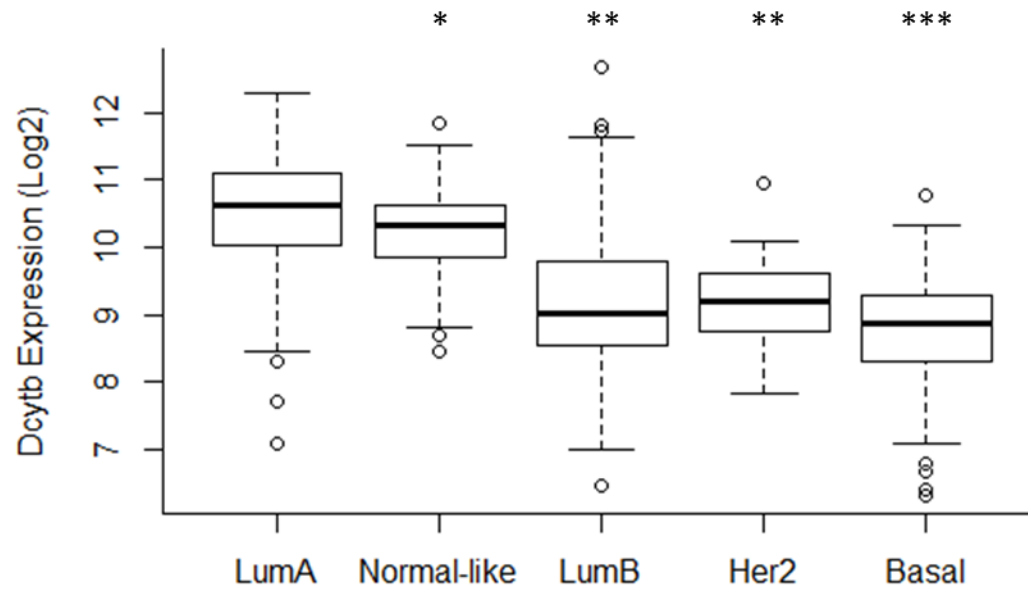
\*\*\* Normal tissue samples are not included in the number of patients (n).

Complete datasets were not available for all patients. Discrepancies between total number of patients in cohorts and the number of patients evaluated based on individual characteristics (ER status, LN status, Her2, PR, grade) are due to missing data in each of these categories.



**Figure 2-2. DCYTb predicts outcome independent of ER and LN status**

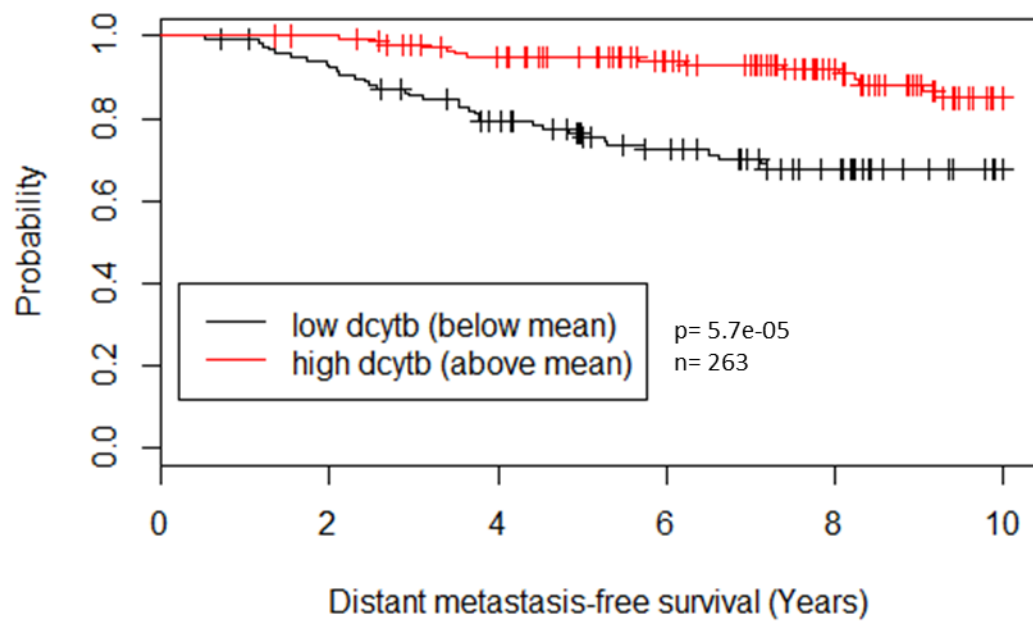
Kaplan-Meier analysis of patients in cohort #1 subsetted by high and low DCYTb expression and a. ER+ ( $p = 1.3 \times 10^{-10}$ ,  $n = 643$ ), b. ER- ( $p = 0.03$ ,  $n = 89$ ), c. LN+ ( $p = 1.5 \times 10^{-7}$ ,  $n = 364$ ), d. LN- ( $p = 0.0001$ ,  $n = 358$ ) status



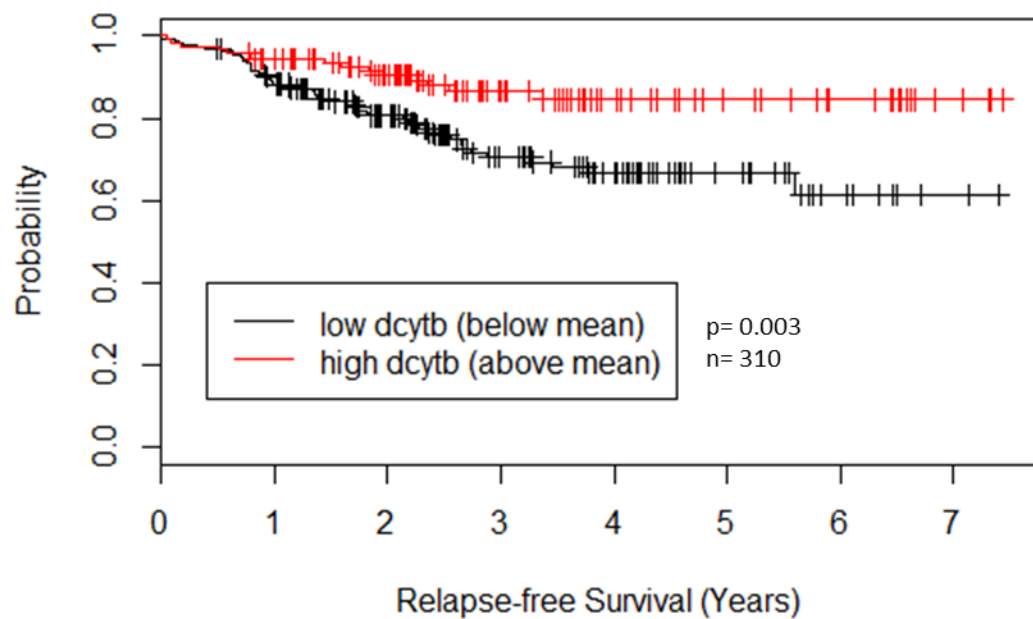
**Figure 2-3. Increased DCYTB expression in molecular subtypes with better outcome in cohort #1**

DCYTB expression in each breast cancer molecular subtype of cohort #1. Luminal A, n= 252; Normal-like, n= 154; Luminal B, n= 136; Her2, n= 61; Basal, n= 104. \* $p \leq 0.0028$  vs LumA, \*\* $p \leq 2.8e-15$  vs LumA and Normal-like cohorts, \*\*\* $p \leq 0.0027$  vs all other cohorts

**a Tamoxifen Monotherapy**

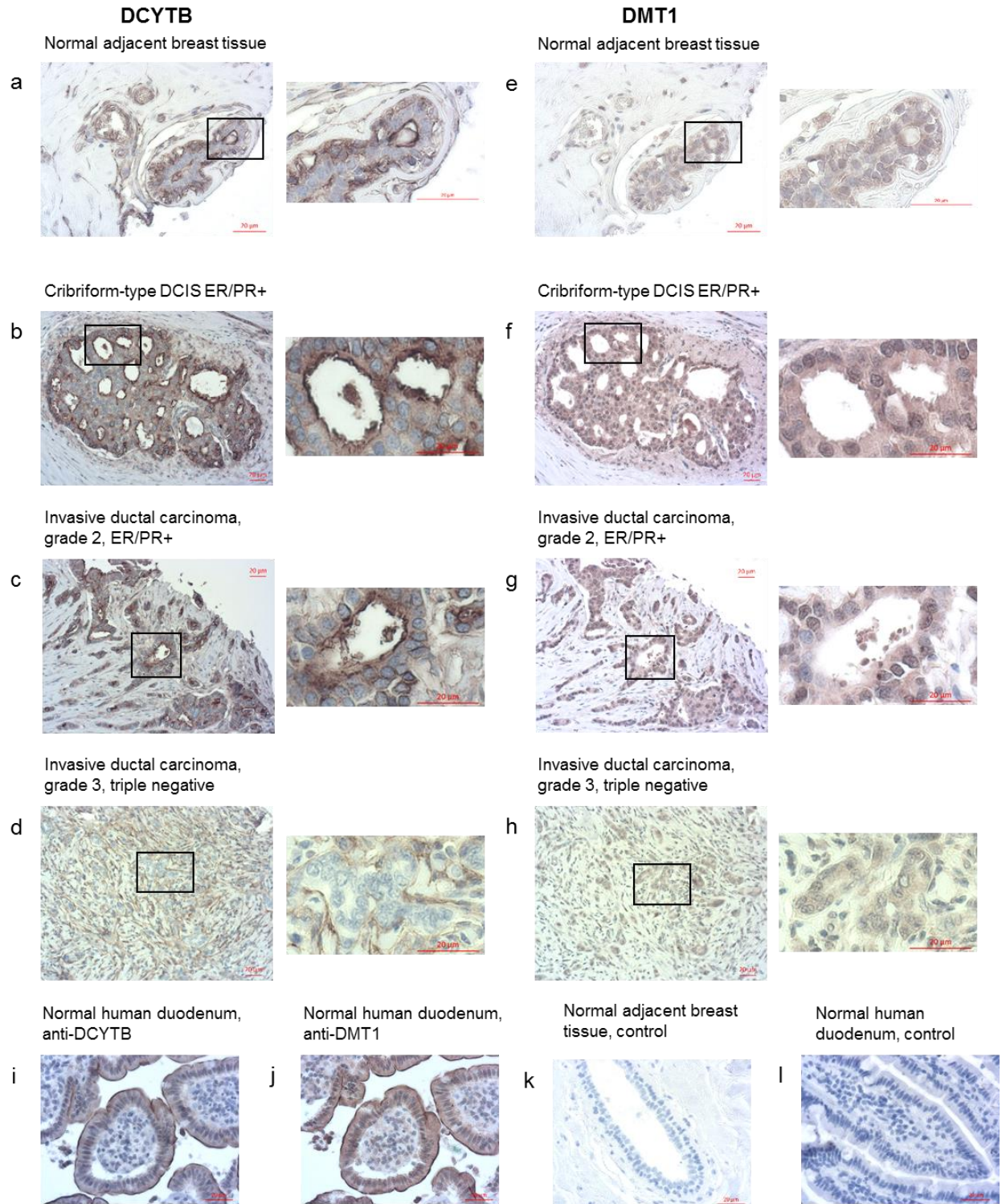


**b Neoadjuvant Therapy**



**Figure 2-4. DCYTB predicts treatment outcome in Tamoxifen and chemotherapy treated cohorts**

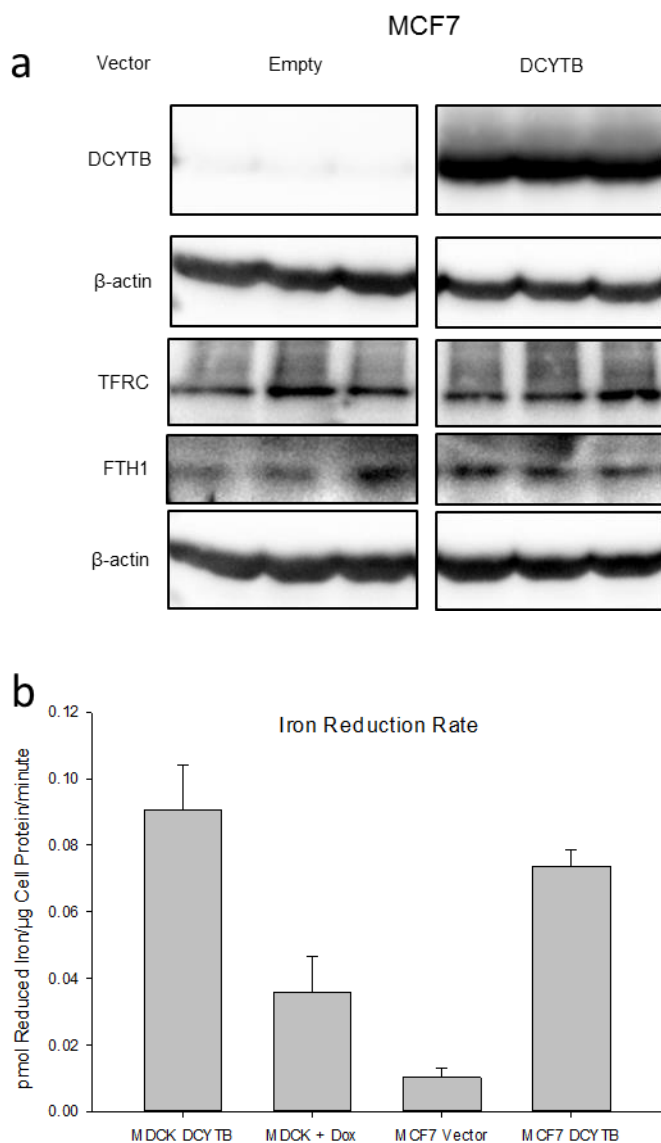
a. Kaplan-Meier analysis of ER+ patients in cohort #1 who received Tamoxifen monotherapy subsetting by high and low DCYTB expression,  $p = 5.7 \times 10^{-5}$ ,  $n = 263$ , log-rank test. b. Kaplan-Meier analysis of patients who were ERBB2- (HER2-) and either ER+ or ER- and had been treated with taxane-anthracycline neoadjuvant chemotherapy (and tamoxifen if ER+) followed by surgery (GSE25055) [82],  $p = 0.003$ ,  $n = 310$ , log-rank test.



**Figure 2-5. Tissue expression of DCYTB & DMT1.**

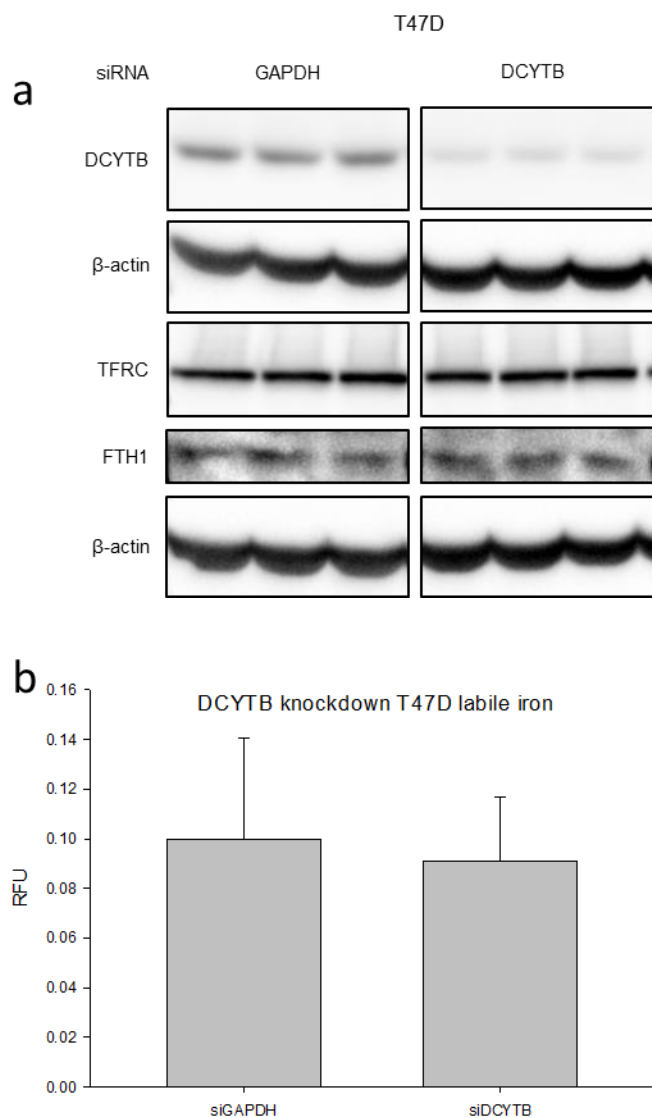
Representative images from breast tissue microarray (20x magnification) and deidentified duodenal tissue from UConn Health Center Department of Pathology. a;f. Normal adjacent breast tissue; b;g. Cribriform-type DCIS ER/PR+; c;h. Invasive ductal carcinoma, grade 2, ER/PR+; d;i. Invasive ductal carcinoma, grade 3, triple negative; e;j. Normal human duodenum, 40x; k. Normal adjacent breast tissue, control stained with secondary antibody only, 40x; l. Normal human duodenum, controls stained with IgG instead of primary antibody, 40x. The box in the series of images to the left, a-d and e-h, represent the location of the magnified image to the right. Scale bar = 20  $\mu$ m





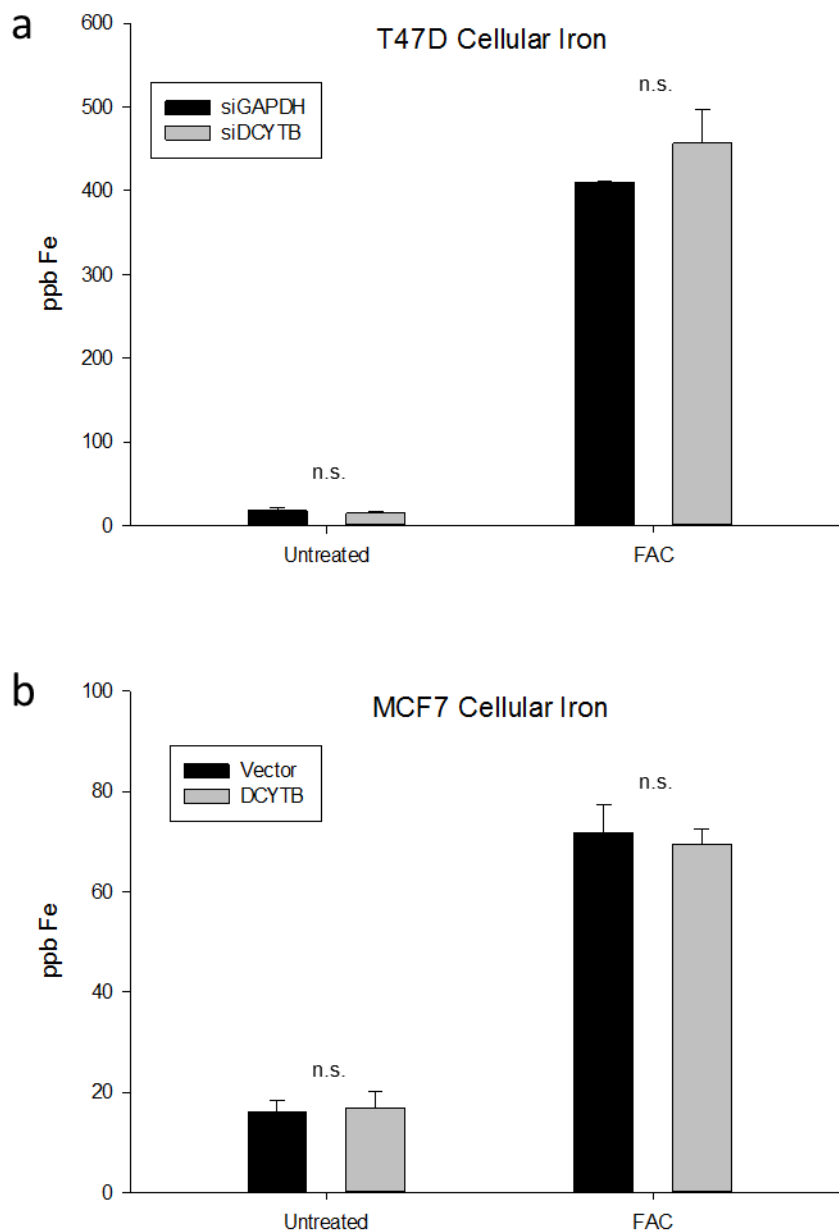
**Figure 2-6. Expression and activity of DCYTB in cultured breast cells does not affect iron metabolism**

a. Western blot of constitutive DCYTB expressing MCF7 cell iron responsive protein expression, triplicate samples are shown. b. FerroZine assay at pH 6.4 of indicated cells, results are the mean and standard deviation of triplicate samples.



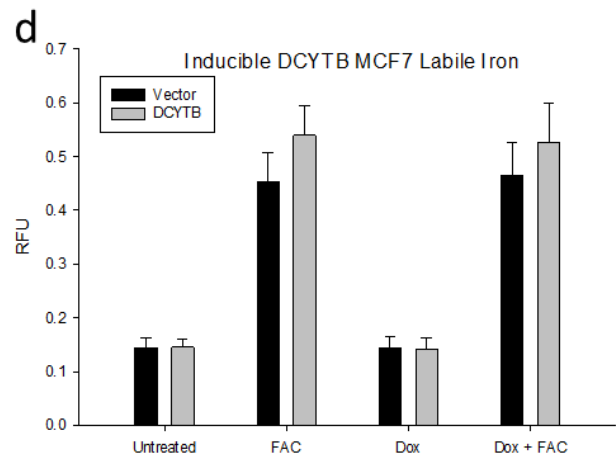
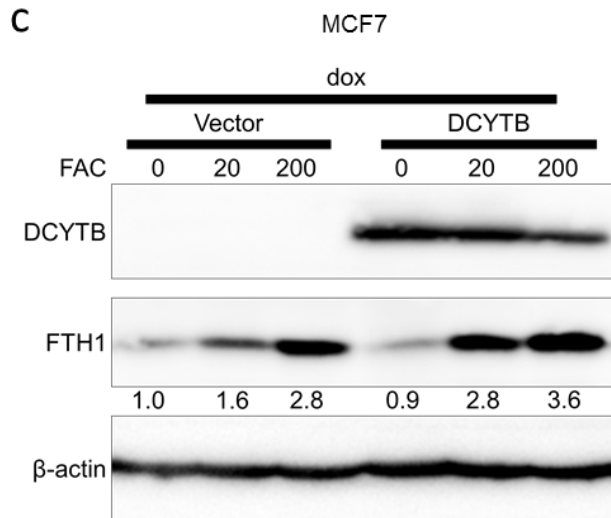
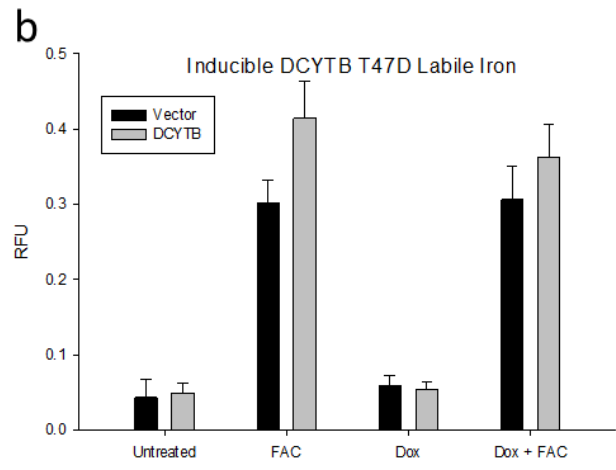
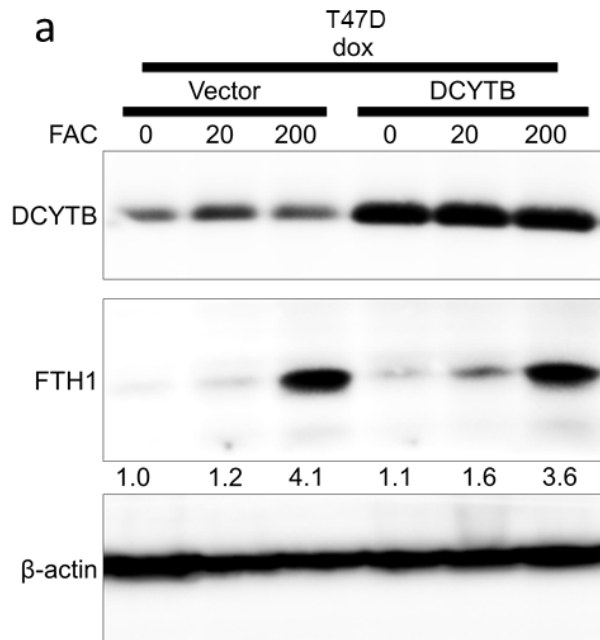
**Figure 2-7. Knockdown of DCYTB in T47D cells does not affect proteins of iron metabolism**

a. Western blot of T47D cells with siRNA-mediated knockdown of DCYTB or GAPDH (control), triplicate samples are shown. b. Labile iron pool of DCYTB knockdown T47D cells. Results represent the mean and standard deviation of at least 14 replicate samples.



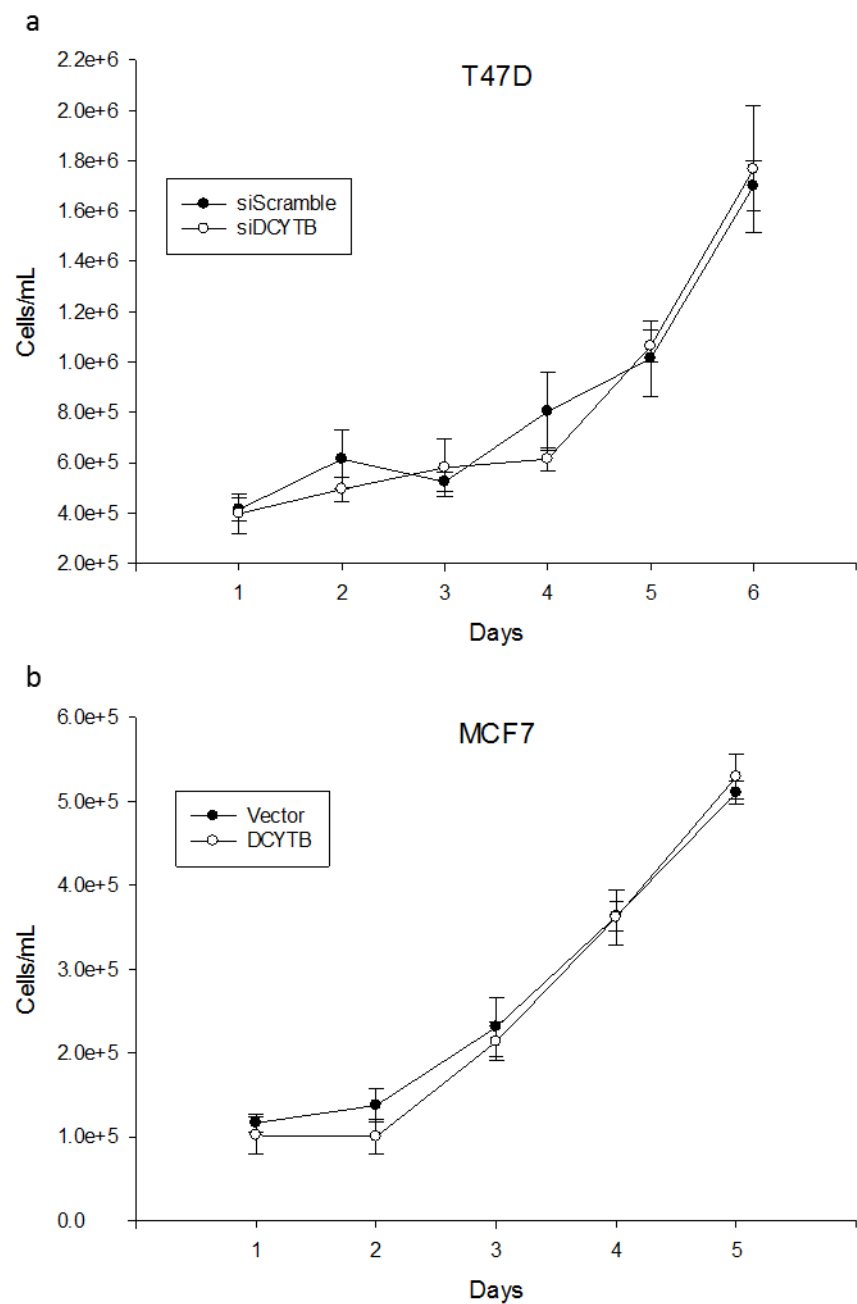
**Figure 2-8. DCYTB expression does not affect total cellular iron**

a. ICP-MS analysis of total cellular iron in DCYTB knockdown T47D cells and b. Constitutive DCYTB expressing MCF7 cells. Cells were either untreated or exposed to 200  $\mu$ M ferric ammonium citrate (FAC) in growth medium for 24 hours. Results represent the mean and standard deviation of 3 replicates.



**Figure 2-9. Iron responsive protein expression and cellular labile iron in response to DCYTB induction**

Iron responsive protein expression in a. T47D and c. MCF7 cells induced with doxycycline for 72 hours. Transferrin receptor and ferritin expression quantified with Fiji ImageJ [118] and normalized to uninduced cells expressing the DCYTB containing vector. Labile iron pool measurement of b. T47D and d. MCF7 cells induced with doxycycline for 72 hours and iron treated for 24 hours. Results represent the mean and standard deviation of at least 15 replicate samples.



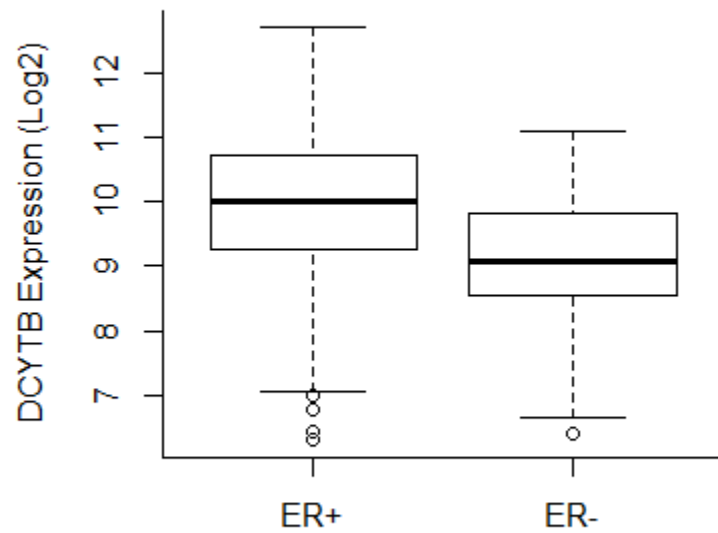
**Figure 2-10. Modulation of DCYTB expression does not affect proliferation of cancerous breast cells**

a. Proliferation of DCYTB knockdown T47D cells monitored by trypan blue exclusion, b.

Proliferation of constitutive DCYTB expressing MCF7 cells monitored by Coulter Counter.

Results represent the mean and standard deviation of 3 replicate samples.

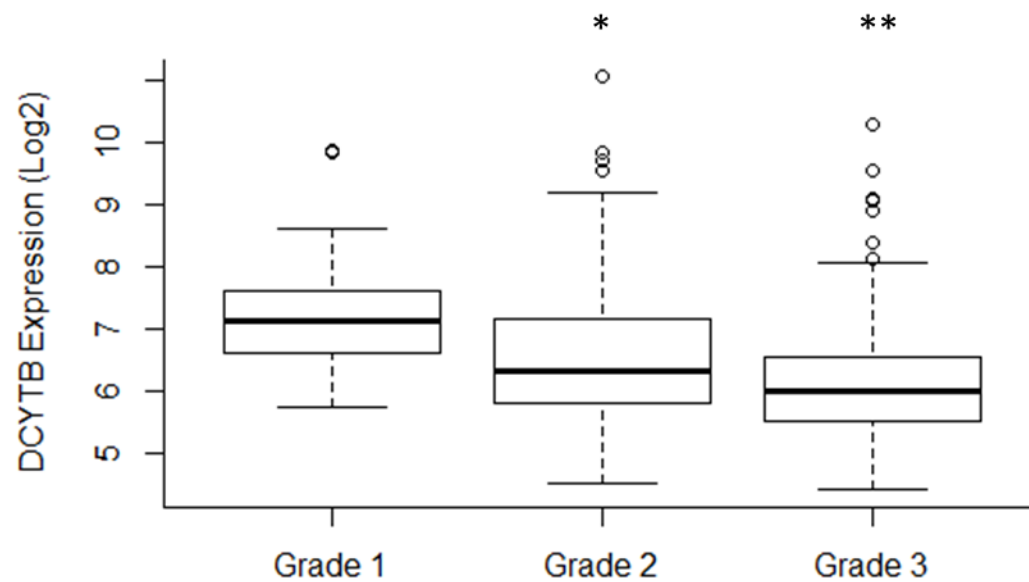
## K. Supplemental Figures and Tables



### Supplemental Figure 2-S1. DCYTB expression is higher in ER+ than ER- patients

Microarray analysis of DCYTB expression in ER+ (n= 643) and ER- (n= 89) patients of cohort

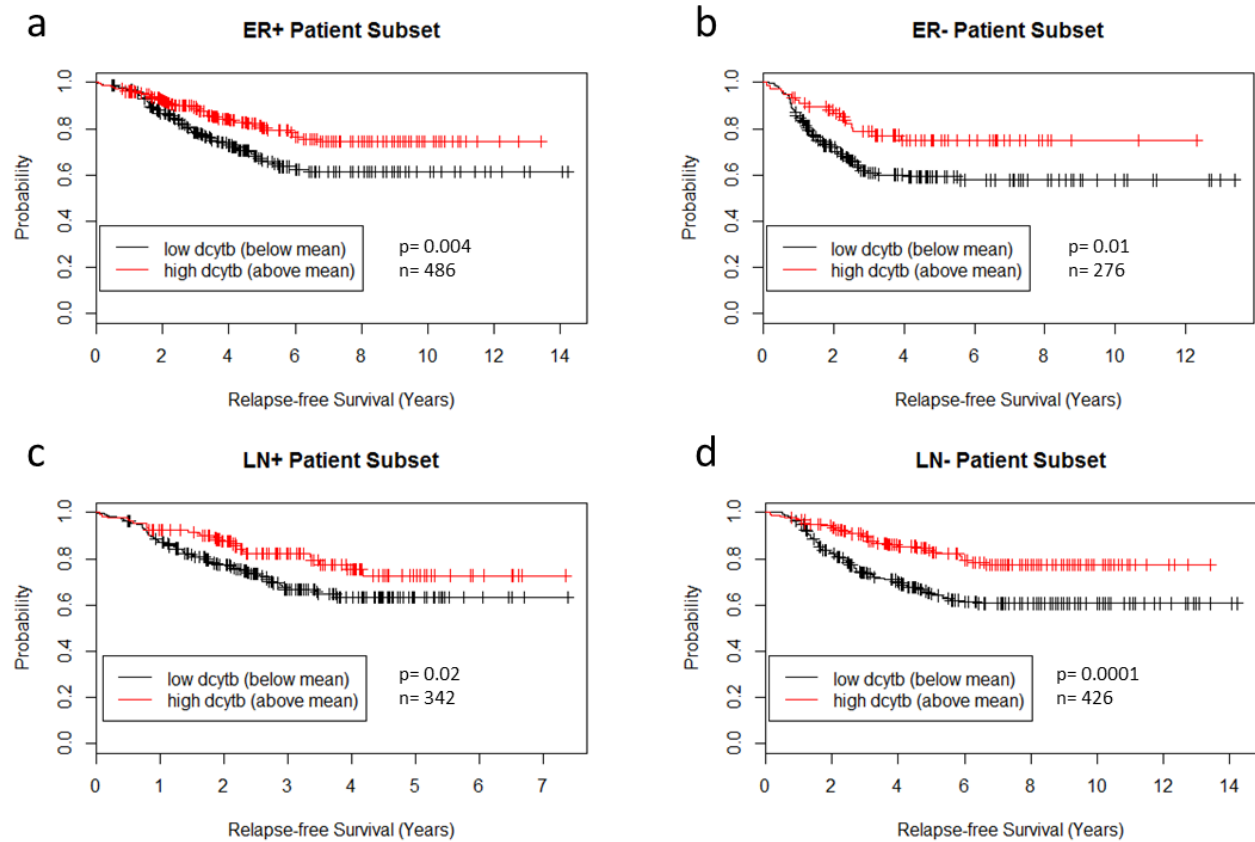
#1,  $p= 2.1e-12$



**Supplemental Figure 2-S2. DCYTB expression decreases with increased tumor grade**

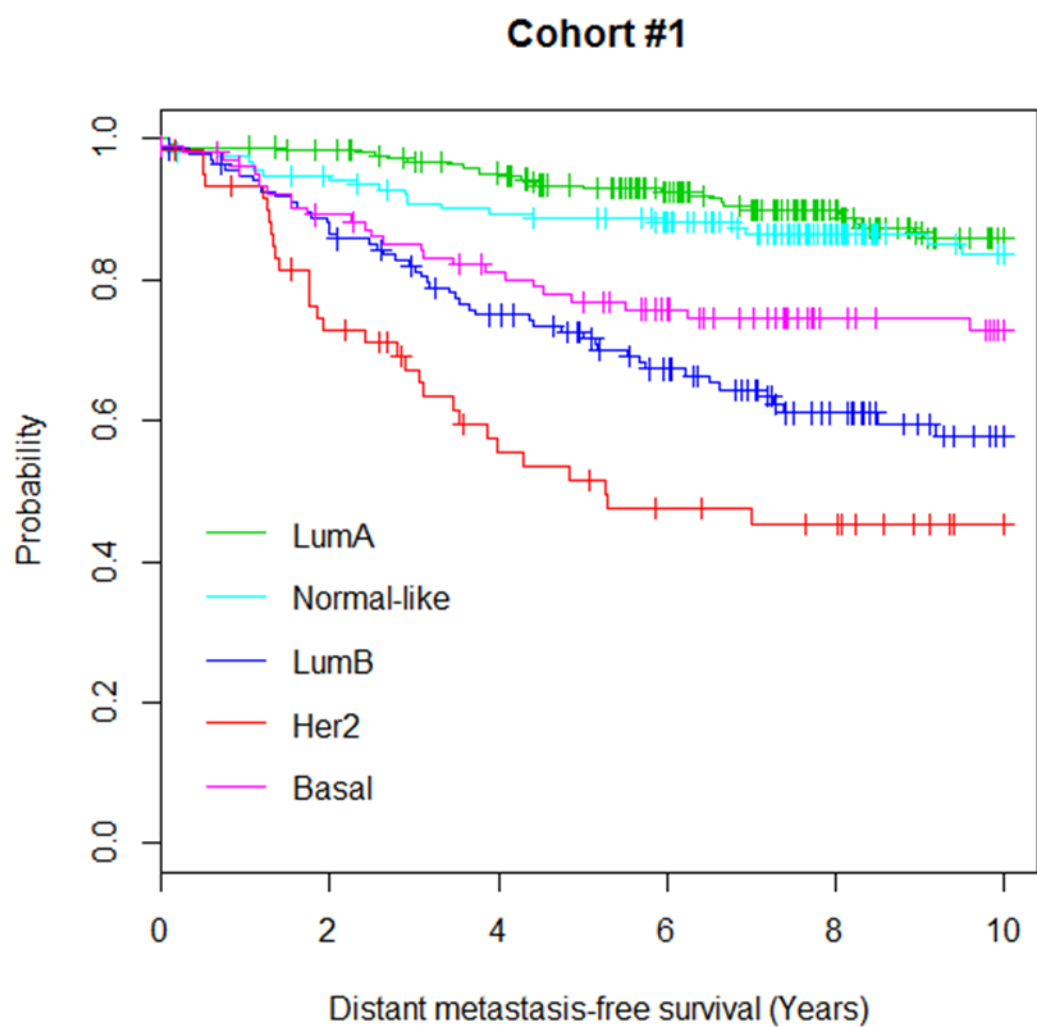
Microarray analysis of DCYTB expression by breast cancer grade in cohort #2. Grade 1, n= 43; grade 2, n= 209; grade 3, n= 209. \*p= 6.4e-05 vs grade 1, \*\*p≤ 5.9e-07 vs grade 1 & 2.





### Supplemental Figure 2-S3. DCYTB predicts outcome independent of ER and LN status

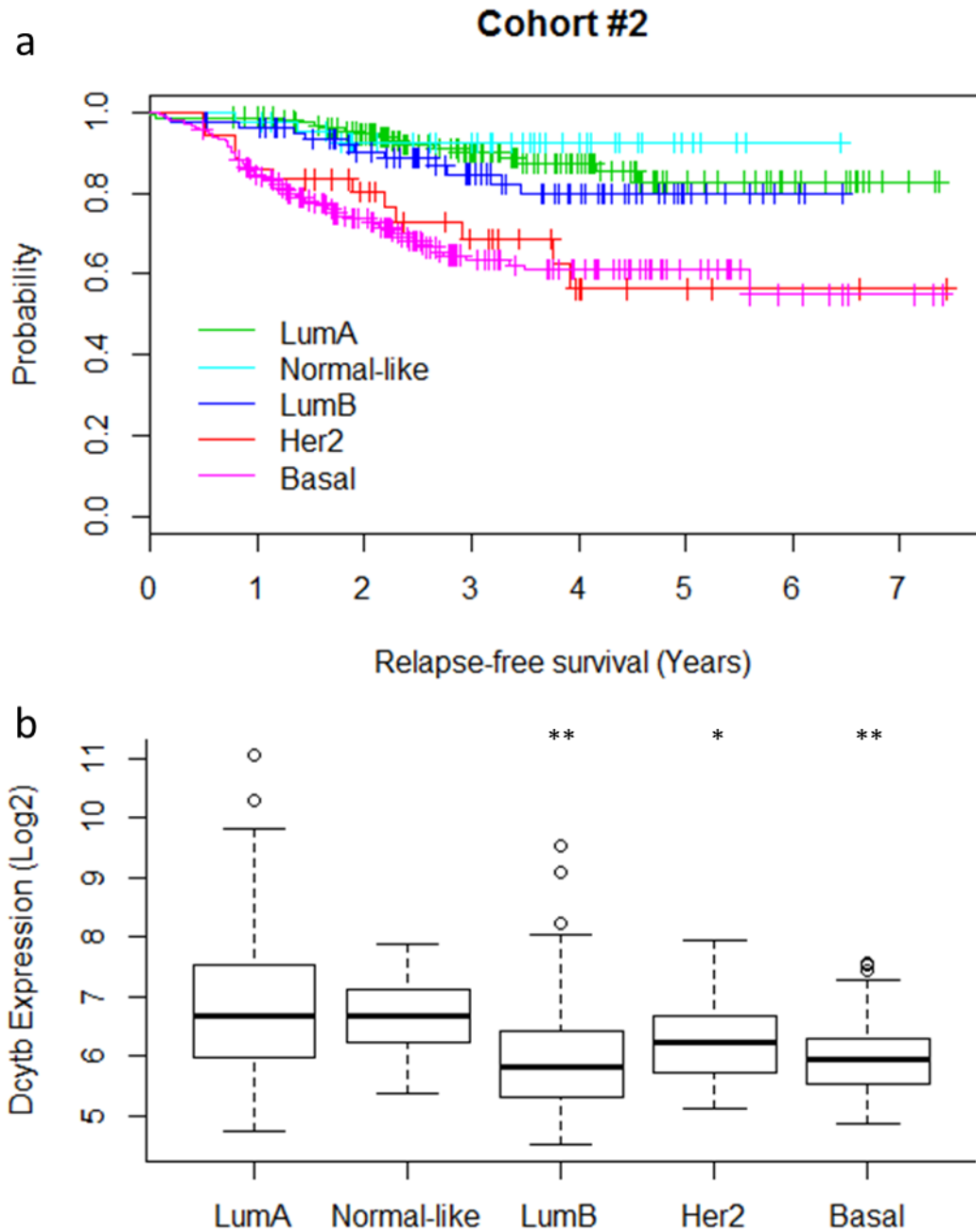
Kaplan-Meier analysis of patients in cohort #2 subsetted by high and low DCYTB expression and a. ER+ (p= 0.004, n= 486), b. ER- (p= 0.01, n= 276), c. LN+ (p= 0.02 n= 342), d. LN- (p= 0.0001, n= 426) status



**Supplemental Figure 2-S4. Survival by molecular subtype in cohort #1**

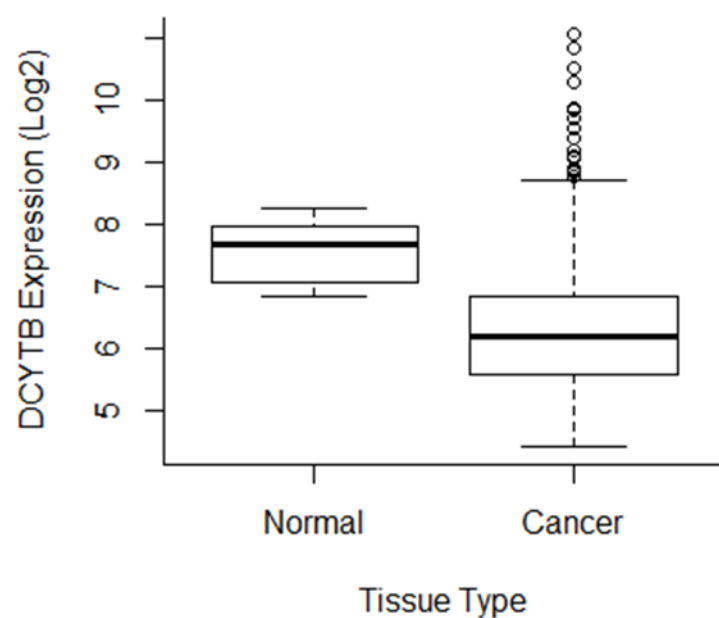
Kaplan-Meier analysis by molecular subtype in cohort #1. Subtypes determined by PAM50 [81]:

Luminal A, n= 252; Normal-like, n= 154; Luminal B, n= 136; Her2, n= 61; Basal, n= 104.



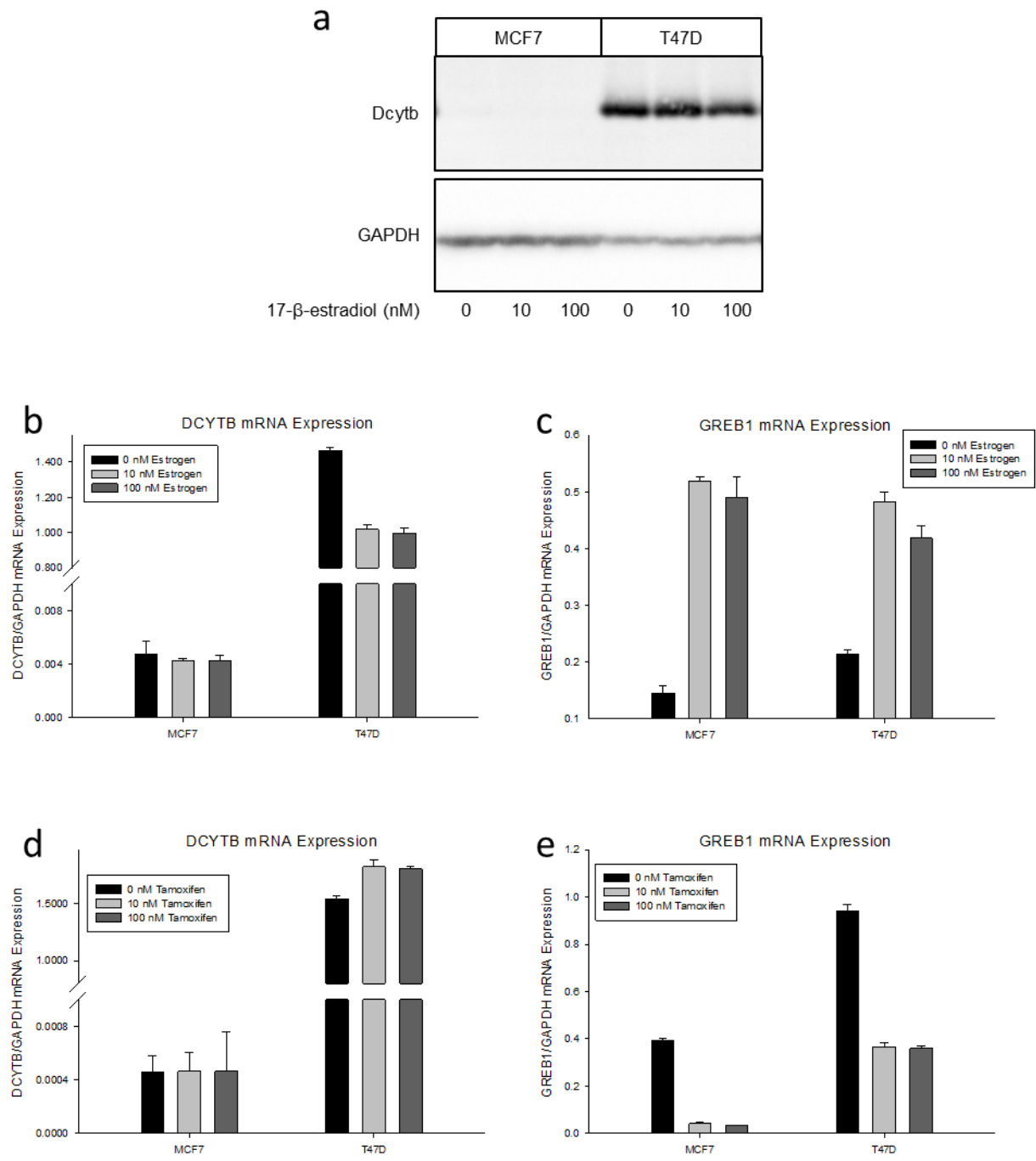
**Supplemental Figure 2-S5. Increased DCYTb expression in molecular subtypes with better outcome in cohort #2**

a. Kaplan-Meier analysis by molecular subtype in cohort #2, b. DCYTb expression in each breast cancer molecular subtype of cohort #2. Subtypes determined by PAM50 [81]: Luminal A, n= 165; Normal-like, n= 42; Luminal B, n= 78; Her2, n= 36; Basal, n= 184. \* p= 0.0021 vs LumA, \*\*p< 0.0025 vs LumA & Normal-like.



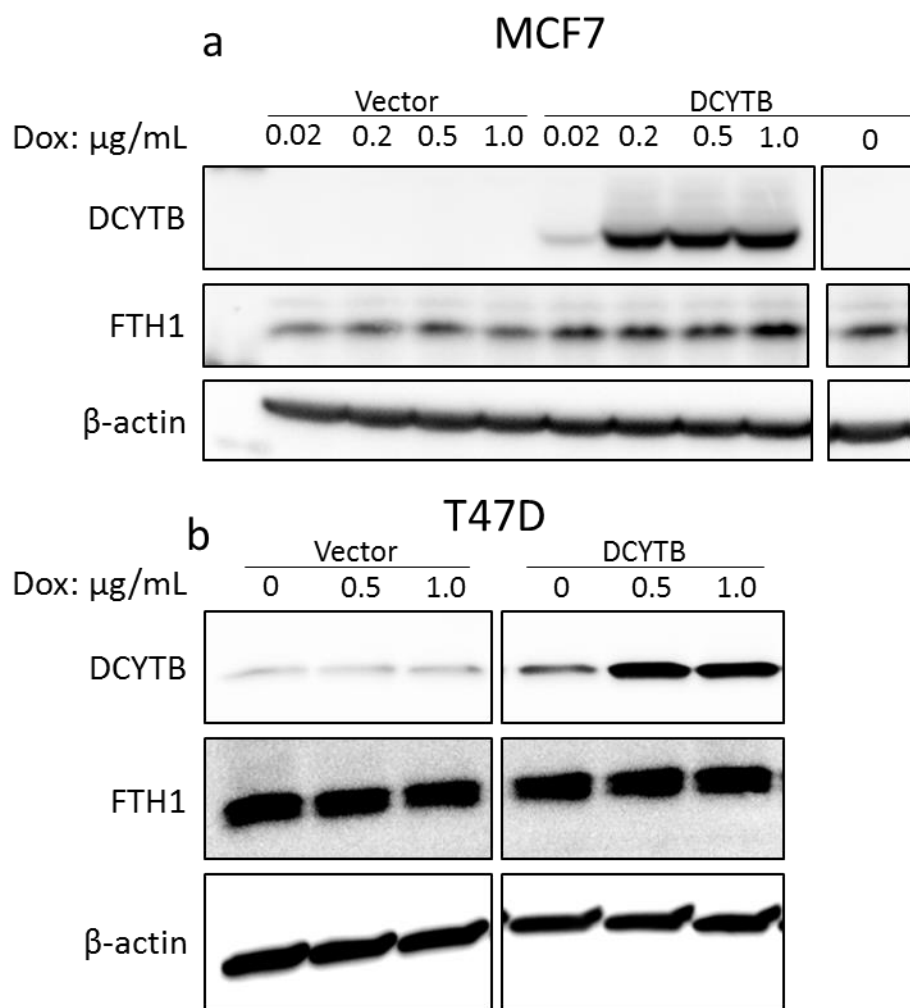
**Supplemental Figure 2-S6. DCYTB expression is decreased in cancerous tissue**

Microarray analysis of DCYTB expression in normal breast (n= 14) and breast tumor tissue (n= 869) in cohort #2,  $p= 9.7e-08$



**Supplemental Figure 2-S7. DCYTB expression is not regulated by estrogen**

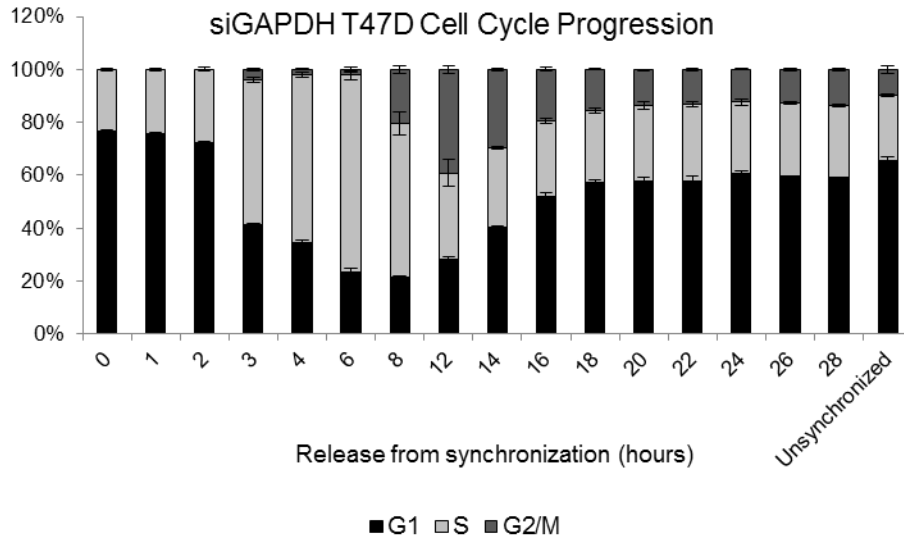
a. Western blot of DCYTB expression in response to 24 hours of 17- $\beta$ -estradiol treatment, b. DCYTB and c. GREB1 (growth regulation by estrogen in breast cancer 1) mRNA expression in response to 24 hours of 17- $\beta$ -estradiol treatment, d. DCYTB and e. GREB1 mRNA expression in response to 24 hours of Tamoxifen treatment. Results represent means and standard deviations of 3 replicates.



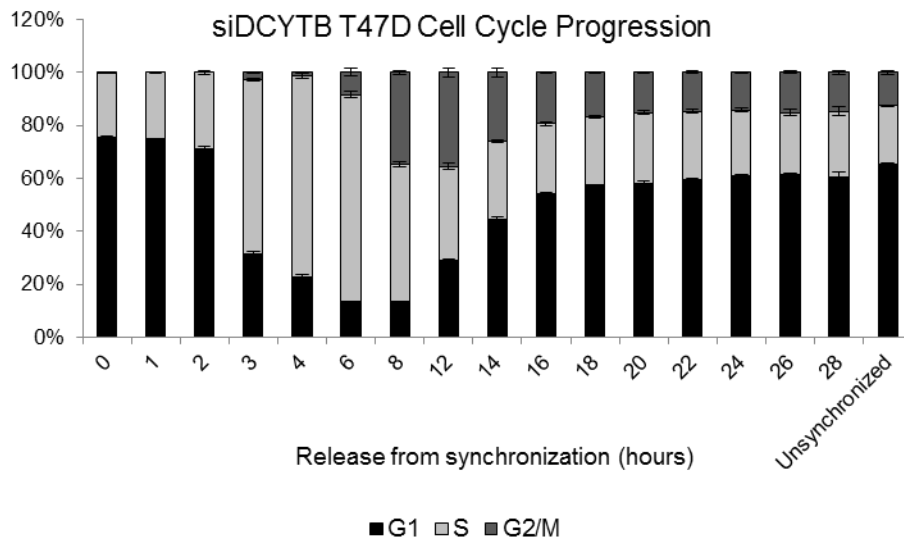
**Supplemental Figure 2-S8. Induction and activity of Tet-on DCYTB expression vector**

Western blot analysis of DCYTB and ferritin in a. T47D and b. MCF7 cells treated for 72 hours with doxycycline.  $\beta$ -actin was used as a loading control.

a



b





**Supplemental Figure 2-S9. Knockdown of DCYTB expression does not affect progression through the cell cycle**

Cell cycle progression of hydroxyurea synchronized T47D cells transfected with a. siGAPDH (control) or b. siDCYTB. Cells were released from synchronization and ethanol fixed at the indicated times. Cell cycle distribution was determined with ModFit software analysis of propidium iodide fluorescence. Results represent means and standard deviations of 3 replicates.

**Supplemental Table 2-S1. Perturbed pathways identified by Signaling Pathway Impact Analysis (SPIA)**

| Pathway Name             | KEGG Pathway ID | Differentially expressed genes | p-value  |
|--------------------------|-----------------|--------------------------------|----------|
| <b>Cohort #1 (n=152)</b> |                 |                                |          |
| Focal adhesion           | 4510            | 131                            | 0.000178 |
| Cell cycle               | 4110            | 93                             | 0.000781 |
| ECM-receptor interaction | 4512            | 52                             | 0.002462 |
| <b>Cohort #2 (n=178)</b> |                 |                                |          |
| Cell cycle               | 4110            | 63                             | 0.000393 |
| Focal adhesion           | 4510            | 60                             | 0.005646 |
| RNA transport            | 3013            | 62                             | 0.040746 |

Data were subsetted into high and low DCYTB expression groups (> 0.9 quantile of expression, or < 0.1 quantile). The signaling pathway impact analysis (SPIA) algorithm [91], implemented in R, was used to identify significantly activated or inhibited pathways (pFWER< 0.05), using information from KEGG pathway annotations and differentially expressed genes (p< 0.05) between high and low DCYTB expressing groups.

## **Chapter III**

### **The effects of DCYTB expression on copper and ascorbate metabolism (unpublished data)**

## **A. Copper induced gene expression in DCYTB expressing cells**

### **a. Copper metabolism**

Copper is a redox active metal necessary for scavenging of free radicals, cellular respiration and iron homeostasis. It is incorporated into many proteins including: superoxide dismutase (SOD1), cytochrome c (CYCS) and ceruloplasmin (CP) [119]. SOD1 participates in the neutralization of ROS by converting superoxide into peroxide. CYCS is the terminal enzyme in the electron transport chain, converting molecular oxygen to water [120]. CP is the main source of copper in plasma, containing anywhere from 60-95% of copper in the serum [119, 121, 122]. It is also a ferroxidase necessary for iron loading of transferrin [123].

Like iron, dietary copper enters the body through the intestine and the recommended intake is about 1.0 mg per day [124]. Transport across the membrane is mediated by copper transporter 1 (CTR1) though reports indicate that DMT1 is also capable of copper transport [61, 124-126]. Import of copper via CTR1 is unregulated although high cellular copper concentrations result in endocytosis of CTR1, preventing further import [124, 127, 128]. Excess copper can be eliminated from the body and it is predominantly excreted in the bile [124]. ATP7A, one of the copper transporting P-type ATPases, is necessary for export of copper to the plasma. Copper in the plasma is transported bound to albumin,  $\alpha$ 2-macroglobulin or histidine and other amino acids [124, 126]. This copper is bound in the cupric state and must be reduced prior to import [124].

The chaperone proteins: copper chaperone for superoxide dismutase (CCS) and antioxidant 1 copper chaperone (ATOX1) transport copper throughout the cytosol, delivering it to the mitochondria, golgi or the nucleus [128, 129]. CCS is responsible for delivering copper to SOD1 in the cytosol and mitochondria [130]. ATOX1 delivers copper to the trans-golgi network and the Cu-ATPases, ATP7A and ATP7B, for efflux or incorporation into copper-dependent

proteins [131]. ATP7A is ubiquitously expressed except in the liver and its localization is dependent on cellular copper levels [131]. While levels of cellular copper are low, ATP7A is localized to the trans-golgi network, allowing for protein incorporation of molecular copper delivered by ATOX1 [132]. When cellular copper levels are high, ATP7A translocates to the plasma membrane allowing for the export of excess copper [132]. ATP7B, however, is expressed predominantly in the liver where it makes copper available for incorporation in holo-ceruloplasmin or mediates its excretion in the bile [124, 131].

Two well described disease states result in altered copper metabolism: Menkes and Wilson disease. These diseases result from mutations in the Cu-ATPases where a multitude of different mutations are described [133]. Menkes disease results from mutations in ATP7A and is typically lethal by age 3 [131]. These mutations result in copper deficiency due to defective copper export from the intestine [128, 131]. Wilson disease, on the other hand, is caused by mutations in ATP7B [124]. Patients with this disease are unable to excrete copper or synthesize apo-ceruloplasmin [124]. These patients suffer from hepatic copper overload, which leads to liver damage as a result of oxidative stress [124]. Both of these diseases are rare, however, there have been reports of cancer associated with Wilson disease [134]. Cancer incidence in Wilson disease patients is low, likely due to the latency of hepatic cancers and the use of copper chelators to manage the disease.

## **b. Copper and cancer**

Copper levels are elevated in cancer patients [119] and copper levels have been shown to be associated with progression and recurrence of cancer [103]. Copper is a known cofactor for matrix metalloproteinase-2, fibroblast growth factor-1 and angiogenin, proteins involved in angiogenesis [135-137]. The importance of angiogenesis in cancer progression is underscored by the implementation of copper chelators, clinically used for treatment of Wilson disease, as potential cancer therapy [138]. One such chelator, tetrathiomolybdate (TM), has been

demonstrated to suppress angiogenesis and reduce tumor volume of orthotopically injected SUM149 breast cancer cells in nude mice [139]. *In vitro* treatment of SUM149 breast cancer cells also reduced expression of the proangiogenic factors: VEGF, IL1 and IL6 through repression of NF- $\kappa$ B-dependent transcription [139]. Another chelator, trientine, was demonstrated to reduce tumor volume of HuH-7 hepatoma transplanted into nude mice [140]. Copper chelation in this context suppressed IL8 induced angiogenesis [140].

Cancerous cells are capable of developing resistance to therapy through elimination of cytotoxic drugs through efflux or sequestration, termed multidrug resistance (MDR). MDR is a large concern for cancer patients and physicians as it is often the cause of failed chemotherapy [141]. MDR was first characterized by expression of MDR1/P-glycoprotein (ABCB1) [141], but other proteins have since been identified as participants in MDR. The copper ATPases, ATP7A and ATP7B, have been found to be involved with MRD [142, 143]. Early research focused on copper transporter dependent resistance to cis-diaminedichloroplatinum (II) (CDDP), however, resistance to Taxol, doxorubicin and other drugs have also been found to be related to expression of these ATPases [143, 144]. ATP7A sequesters cytotoxic drugs in the golgi [145]. The intrinsic red fluorescence of doxorubicin was localized to the golgi, rather than the nucleus, of CHO cells and human fibroblasts in the presence of ATP7A [144], thus inhibiting toxicity. Expression of ATP7B results in reduced accumulation and decreased sensitivity of cisplatin in human epidermoid KB carcinoma cells due to increased efflux of the drug [146].

### **c. Cellular response to copper in the presence of DCYTB**

DCYTB has been shown to be a cupric reductase [59]. Due to the relevance of copper in multiple aspects of cancer development and progression and the ability of DCYTB to reduce copper for cellular import, we investigated the effects of DCYTB expression on the cellular response to copper. We used four genes described by Song et al. [147] as copper responsive: metallothionein (MTA2), HSP70 (HSPA1A1), heme oxygenase (HMOX1) and cytochrome p450

(CYP1A1) [147]. Metallothioneins, such as MTA2, are copper storage proteins, sequestering excess copper until it is needed by the cell [148]. HSP70 is one of the heat shock proteins responsible for proper protein folding and aids in the cellular stress response [149]. HMOX1 is the oxygenase responsible for the first step in heme catabolism [150]. CYP1A1 is a phase I monooxygenase involved in drug metabolism and sterol synthesis [151].

To determine whether DCYTB was affecting cells in a copper-dependent manner, MCF7 cells expressing empty vector or DCYTB were treated with cuprous or cupric chloride and the expression of the copper responsive genes MTA2, HSPA1A1, HMOX1 and CYP1A1 [147] was evaluated by qRT-PCR. Cupric copper will require reduction prior to import via CTR1. We would expect that reduction would be increased in cells expressing DCYTB. On the other hand, cuprous copper would not require reduction prior to import and therefore should not be influenced by the expression of DCYTB.

The original study that identified these genes as copper responsive found that gene expression increased for all genes with copper treatment [147]. Our results recapitulate these findings (Figures 3-1a-d). There was no difference in HMOX1 expression at the basal level and an increase was not appreciable until higher copper treatment concentrations were used (Figure 3-1a). At these higher concentrations, DCYTB expression resulted in an increase of gene expression without respect to the oxidation state of the copper treatment (Figure 3-1a). HSPA1A1 displayed a pattern of increase similar to HMOX1 in that only higher concentrations had an effect (Figure 3-1b). DCYTB expression in this case also resulted in an increase of gene expression, but only when the cells were treated with cupric copper (Figure 3-1b). MTA1 showed no difference in basal expression with or without DCYTB expression (Figure 3-1c). Expression increased with copper treatment regardless of the oxidation state of copper and expression of MTA2 was significantly increased in DCYTB expressing cells at all levels of copper treatment (Figure 3-1c). Expression of CYP1A1 was decreased in DCYTB expressing cells when untreated and when treated with either oxidation state of copper (Figure 3-1d). It was

not until cells were treated with 200  $\mu$ M cupric copper that expression of CYP1A1 in DCYTB expressing cells began to approach the expression seen in cells expressing the empty vector (Figure 3-1d).

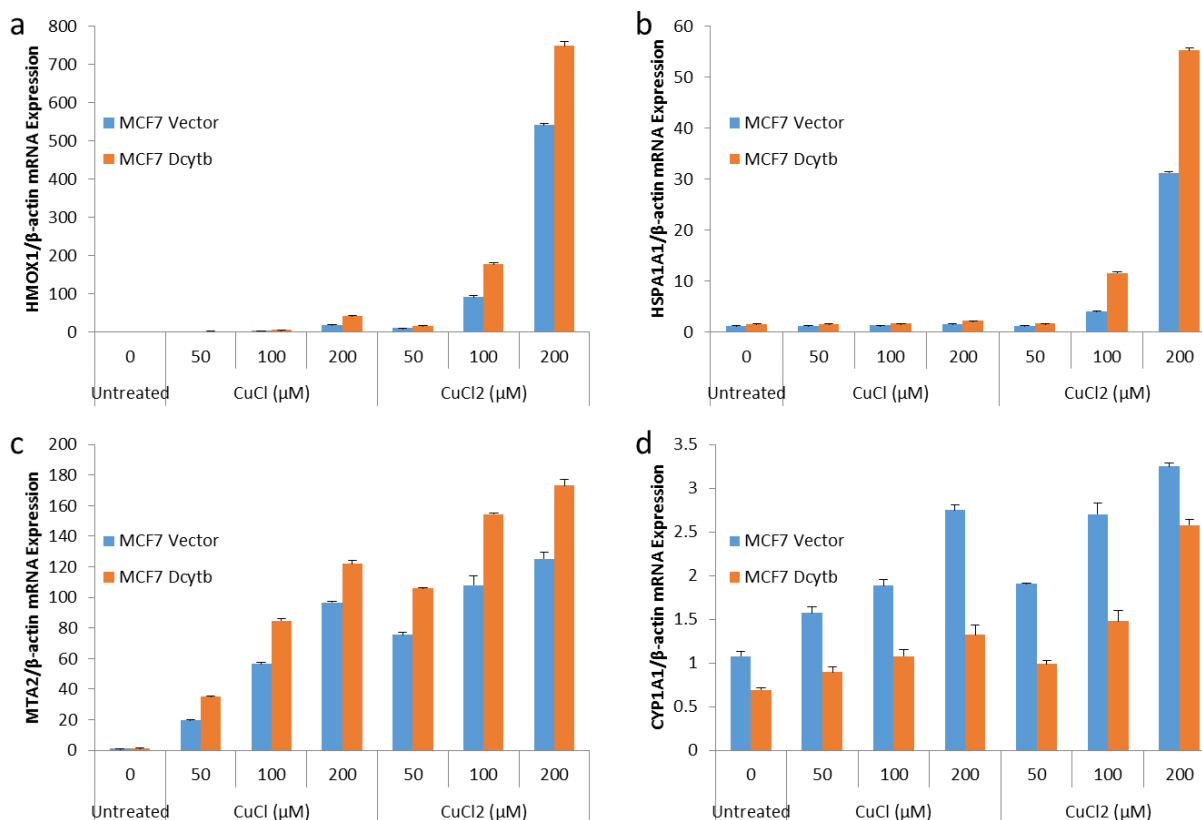
#### **d. Discussion**

Increased expression of metallothionein does not appear to be an effect of DCYTB expression alone as the basal levels of expression are unaffected, while overexpression of DCYTB results in increased MTA2 expression in response to copper treatment (Figure 3-1c). Metallothioneins are capable of binding multiple copper atoms allowing them to be inertly, but reversibly stored [148]. This may indicate that cells expressing DCYTB have enhanced copper uptake since upregulation of metallothioneins is a result of increased cellular copper [147].

Alternatively, copper is capable of participating in Fenton-like reactions [152] and the response of these genes to copper has been shown to be ROS dependent [153, 154]. Induction of HMOX1 and CYP1A1 is a response to ROS and GSH depletion through induction of AP-1 transcription factor [153]. This may suggest that alterations to ROS signaling or GSH levels are inducing CYP1A1 expression in DCYTB expressing cells (Figure 3-1d) indicating that further investigation of the redox state of cells as a result of DCYTB expression is required.

The striking repression of CYP1A1 in DCYTB expressing cells with and without copper treatment also warrants further investigation. In addition to drug metabolism, CYP1A1 has also been shown to be involved in survival and proliferation in breast cancer cell lines [155]. Breast tumors show upregulation of CYP1A1 which positively correlates with grade [156]. It is interesting to note that we see a decrease of DCYTB expression as grade increases (Figure 2-S1), which may suggest that loss of DCYTB is de-repressing CYP1A1 expression. Study of the effect of DCYTB expression on CYP1A1 expression should be undertaken with the Dox-inducible DCYTB expressing breast cells, which would reveal to what extent DCYTB influences CYP1A1.





**Figure 3-1. Copper responsive genes are differentially expressed with copper treatment in DCYTb overexpressing MCF7 cells.**

mRNA expression of a. HMOX1, b. HSPA1A1, c. MTA2 and d. CYP1A1 in MCF7 cells overexpressing DCYTb treated for 24 hours with cuprous or cupric chloride.

## **B. Modulation of ascorbate metabolism and cellular oxidants in response to DCYTB expression**

The initial studies of DCYTB identified it as a cytochrome b561 homolog potentially capable of reducing ascorbate through conserved ascorbate and ascorbate radical (AR) binding domains [9, 51, 157]. Ascorbate is an essential antioxidant that serves a cofactor in many cellular processes [158, 159]. It has been shown to be an effective adjuvant cancer therapy [160-165]. Levels of ascorbate also influence DCYTB-mediated ferrireduction [59], but directed investigations have not been carried out to evaluate the interplay between ascorbate, or its oxidation products, and DCYTB.

### **a. Ascorbate metabolism**

Ascorbate is a potent water-soluble antioxidant. It is possibly the most important antioxidant in extracellular fluid [163, 166]. Many mammals are capable of synthesizing ascorbate from glucose. Humans lack L-gulonolactone oxidase (GULO), which is required for synthesis, and must rely on transplasma membrane electron transport (tPMET) to maintain circulating ascorbate levels [165].

Ascorbic acid (AA, ascorbate, vitamin C) is regenerated at the expense of GSH and NAD(P)H. AA is reversibly converted to ascorbate radical (AR) following one electron oxidation reaction. Two molecules of AR can be converted to one molecule each of AA and DHA or one molecule can be reversibly oxidized to dehydroascorbate (DHA) through donation of a second electron. DHA can be converted to diketogulonic acid through an irreversible hydrolytic ring opening or it can be converted to AA by GSH or NAD(P)H.

### **b. Ascorbate in cancer**

Typical serum levels of ascorbate range from 0.6 to 2.0 mg/dL, with levels of 0.3 mg/dL

indicative of deficiency [163]. Cancer patients are AA depleted with serum levels of  $0.06 \pm 0.01$  mM ( $\sim 0.001$  mg/dL) [167]. This can result in collagen destabilization (similar to that seen in scurvy) and aberrant activation of hypoxia inducible factors [159, 163, 168-171].

*In vitro* experiments have demonstrated selective dose-dependent cytotoxic and growth inhibitory effects of ascorbate in multiple cancer cell lines, including: lymphoma, breast and pancreatic, compared to normal counterparts [162, 163, 172]. Administration of ascorbate (4 g/kg) to mice with ovarian or pancreatic tumor xenografts decreased tumor growth [172]. Evidence suggests that the therapeutic benefit of ascorbate is derived from its pro-oxidant activities. Ascorbate generates the ascorbate radical through autoxidation, which in turn reacts with oxygen to form peroxide [165]. Autoxidation of ascorbate occurs slowly at physiological pH [173, 174]. However, reduction of ferric iron by ascorbate occurs rapidly, resulting in the formation of bioreactive ferrous iron and the potential for Fenton chemistry to occur. Both of these reactions result in the generation of superoxide anion and have the ability to cause cellular damage [162, 164, 175-177].

The utility of ascorbate in cancer therapy has been somewhat controversial. Several published studies found that oral administration of ascorbate to cancer patients had benefit though these studies were not properly controlled [178-180]. In two double-blind studies, another group found that oral ascorbate had no benefit in the treatment of cancer [181, 182]. Oral administration only achieves 220  $\mu$ M (3874 mg/dL) plasma concentrations [183]. Plasma and tissue levels of ascorbate are tightly regulated following oral administration [162, 183, 184].

To achieve the desired plasma concentrations of ascorbate, doses must be given intravenously (I.V.) to bypass saturable gastrointestinal absorption [163]. I.V. administration of as little as 10 g/day achieved plasma concentrations from 1 to 5 mM (17.6 to 88.06 mg/dL) [165], more than equivalent to the 1000  $\mu$ M doses shown to have cytotoxicity *in vitro* [183]. A review by Lamson et al. [185], summarizes the benefits of combining vitamin C with other therapies. There are no reported cases where addition of vitamin C has been shown to

decrease effectiveness of the primary therapy, while benefits include improved quality of life, increased therapeutic effect and decreased toxicity [185].

### **c. Cytochrome b561 ascorbate modulation**

Cytochromes b561 mediate transplasma membrane electron transport (tPMET). The prototypical cytochrome b561, chromaffin granule cytochrome b561 (CYB561A1), is a transplasma membrane protein in neuroendocrine secretory glands vesicles. This protein transfers electrons from extravesicular (intracellular) AA to intravesicular AR for the ultimate generation of dopamine in chromaffin granules [54, 186]. Other members of this protein family, such as the putative tumor suppressor 101F6 (CYB561D2), have been shown to accept electrons from extracellular AA. These electrons are transferred to the intracellular space resulting in the accumulation of intracellular AA. Intracellular AA accumulation has been linked to peroxide formation and selective growth inhibition of non-small cell lung cancer *in vitro* and *in vivo* [187].

DCYTB (CYB561A2) acts as a ferriredutase in the duodenum [9]. However, iron reduction kinetics suggest that iron may not be the primary substrate [59]. In fact, the putative AA and AR binding sites are partially conserved [9]. This suggests that DCYTB is donating an electron to extracellular AR, forming AA, prior to iron reduction. This function has been demonstrated in erythrocytes wherein DCYTB transfers electrons from the intracellular space to extracellular AFR, thus maintaining extracellular levels of AA [55].

### **d. Influence of DCYTB expression on ascorbate metabolism in breast cancer cells**

Cytochromes b561 have been implicated in trans-membrane electron transport and this likely underlies the mechanism of iron reduction by DCYTB [52]. The ascorbate radical may also act as an extracellular electron acceptor while ascorbate is the primary intracellular electron

donor [9, 55, 59]. In this manner, it is possible that expression, or loss of expression, of DCYTB will result in alterations to the levels of cellular ascorbate and potentially disrupt intracellular antioxidant mechanisms. The goal of these experiments was to identify DCYTB-dependent alterations to ascorbate and cellular reactive oxygen species.

We first used HPLC to directly determine the influence of DCYTB expression on cellular ascorbate levels. Cell lysates from MCF7 cells expressing vector or DCYTB, T47D cells transfected with siDCYTB or siScramble and Tet-off DCYTB-EGFP MDCK cells were collected in a solution of 0.05% trifluoroacetic acid (TFA) to stabilize ascorbate. DCYTB knockdown appeared to increase cellular ascorbate while overexpression had a minimal effect (Figure 4-1). However, there was no change of intracellular ascorbate in MDCK cells with or without doxycycline treatment (Figure 4-1). These results indicate that there are technical issues with the experiment. The trend of increased ascorbate in both of the breast cancer cell lines with higher DCYTB expression may indicate that the issue is with the MDCK cells or that the assay requires further refinement to identify the effects of DCYTB expression.

Ascorbate is the major antioxidant species in cells and its regeneration at the expense of GSH and NAD(P)H may prevent cellular levels from being detectably altered. Depletion of GSH and NAD(P)H would result in an increase of reactive oxygen species. To determine how DCYTB expression would affect peroxide generation, we treated DCYTB overexpressing MCF7 cells with Amplex UltraRed to determine the levels of extracellular peroxide as a surrogate for levels of superoxide [60]. Extracellular peroxides are higher in cells expressing DCYTB (Figure 4-2), suggesting the levels of superoxide would also be increased.

To further investigate the effect of DCYTB expression of ROS and peroxides generation, we did the converse knockdown experiment. In this system we were able to interrogate intracellular ROS as well as extracellular peroxides using 6-carboxy-2',7'-dichlorofluorescein diacetate (DCFH-DA) and Amplex UltraRed. Comparison of siDCYTB and siScramble treated T47D cells showed no difference in either intracellular or extracellular peroxide, despite a

successful knockdown of DCYTB (Figure 4-3).

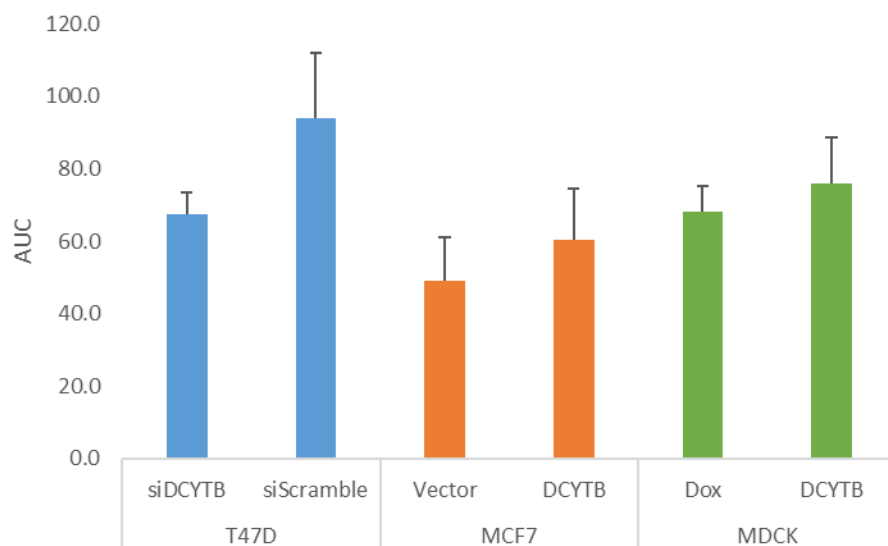
#### **e. Discussion**

Current results show that extracellular peroxide increases (Figure 4-2) while intracellular ascorbate may be increasing (Figure 4-1) with exogenous DCYTB expression in MCF7 cells. Peroxide has been shown to diffuse through the plasma membrane [188] and this has been proposed as one of the mechanisms by which ascorbate mediates cell death [175]. We do not see alterations to cell growth regardless of DCYTB expression (Figure 2-10) it is possible that this is not detectable in this *in vitro* system due to the buffering capacity of the media. We were unable to evaluate intracellular peroxide levels due to the interference of GFP expression in the system with the measurement of DCFH-DA fluorescence. Utilization of doxycycline-inducible DCYTB cells, lacking GFP expression, may reveal increased intracellular peroxide, providing an avenue for further investigation.

Changes to cellular peroxide or ROS in response to DCYTB knockdown were not detectable (Figure 4-3). It is possible that normal levels of DCYTB expression only slightly perturb cellular oxidants and measuring such changes may require more sensitive techniques. However, endogenous DCYTB expression in these cells may affect ascorbate metabolism more subtly than that seen with exogenous expression. In this system, peroxide may be appropriately mitigated by GSH forming GSSG. GSSG is then reduced by NAD(P)H, which is reduced by glucose. This may result in insufficient glucose to generate the ATP required for cancer cells growth and apoptosis [175]. Evaluation of GSH/GSSG, NAD(P)H and ATP are all called for in this case. However, it is again possible that the buffering capacity of the cell culture system may interfere with these experiments and modifications would be required.

Due to the minimal changes detected in cellular ascorbate levels (Figure 4-1), other electron donors should be interrogated. The flavonoid quercetin has been identified as a substrate for DCYTB-mediated transmembrane electron transport [56]. Dihydrolipoic acid

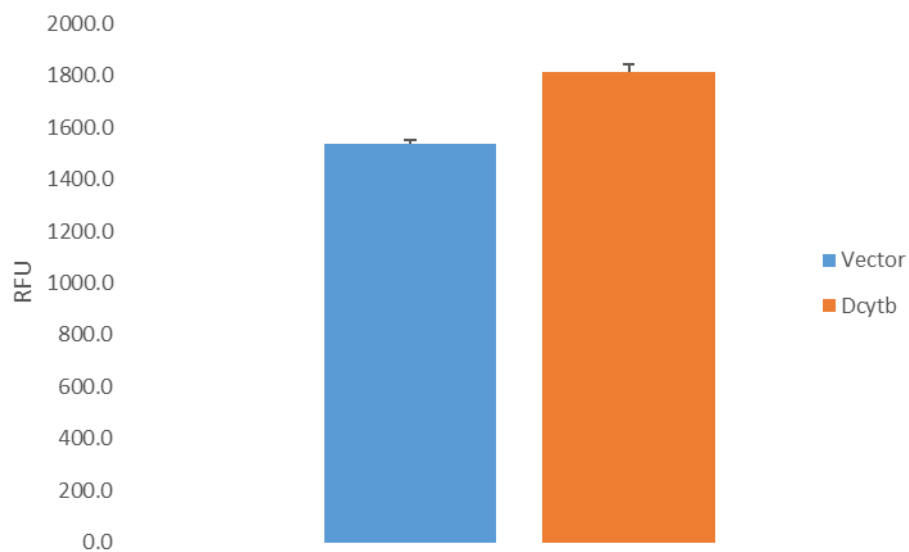
(DHHA) may also serve as a substrate for DCYTB as it has been identified as a substrate for other cytochrome b561 family members [57]. DCYTB can function independently of ascorbate [60], thus it is important to consider these potential electron donors and the potential downstream effects of altered metabolism through loss of DCYTB expression.



**Figure 4-1. Intracellular ascorbate levels are not influenced by DCYTb expression.**

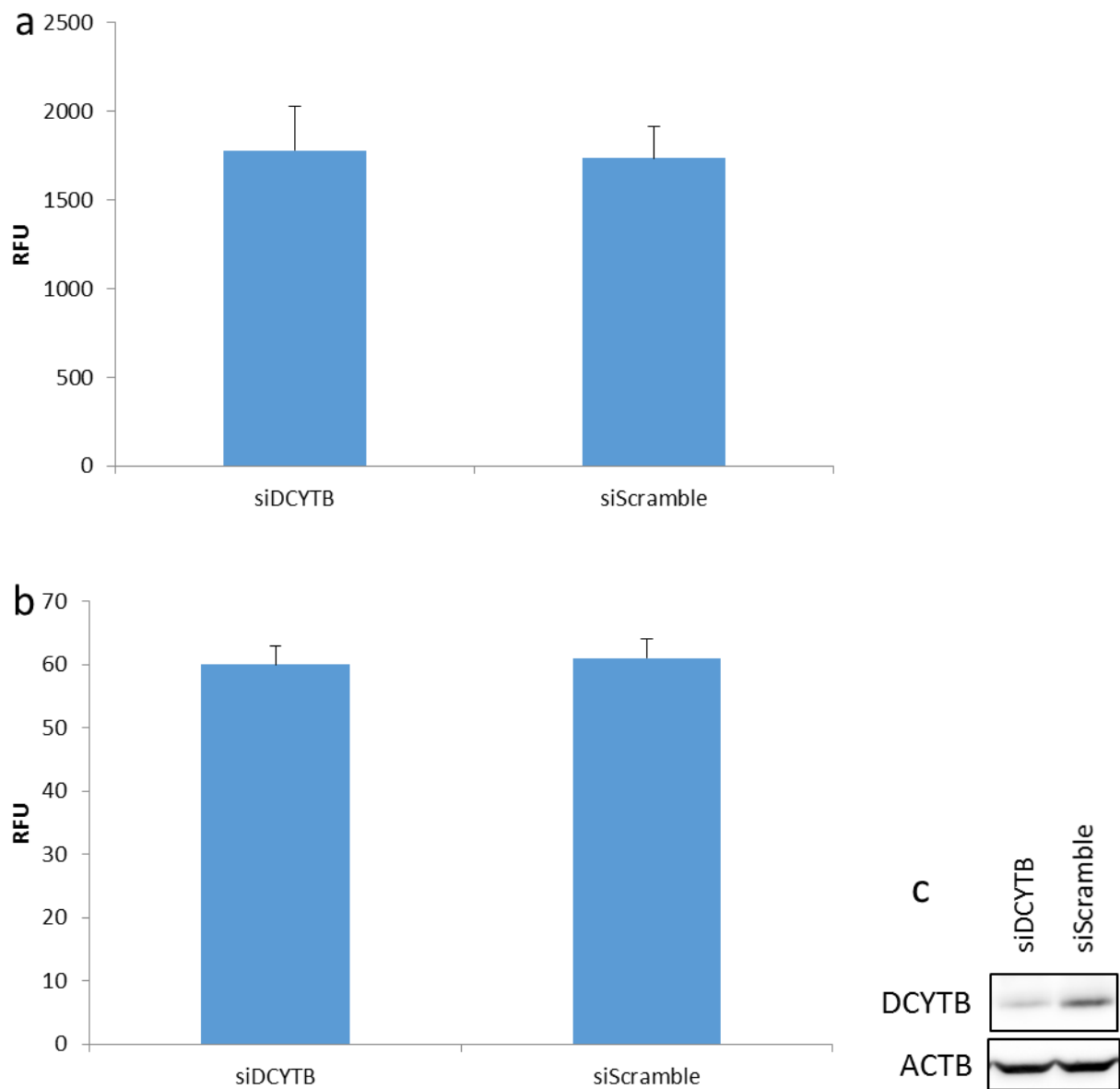
Cells were incubated in phenol-free, serum-free medium for 4 hours, followed by 0.05% TFA wash and harvest. Cells were lysed by sonication and debris removed by centrifugation. Lysate was aliquoted and stored at -20°C until HPLC analysis by Tatiana Shcheglova, Ph.D.





**Figure 4-2. Extracellular peroxides are increased in DCYTB overexpressing cells.**

Extracellular peroxide production determined by Amplex UltraRed. Fluorescence was read with Synergy 2 plate reader.



**Figure 4-3. Knockdown of DCYTB does not affect levels of extracellular peroxide or intracellular reactive oxygen species.**

a. Extracellular peroxide production determined by Amplex UltraRed and b. intracellular reactive oxygen species determined by DCFH-DA in T47D cells transfected with siDCYTB or siScramble, c. Western blot of DCYTB expression following siRNA knockdown

## **Chapter IV**

### **Conclusions & Future Directions**

Our group identified a signature of 16 “iron” genes capable of prediction outcome in breast cancer patients [46]. Among these genes, DCYTB was the most significantly associated with outcome. Our current work further analyzed the prognostic potential of DCYTB expression in breast cancer patients in addition to the functional role of DCYTB in breast cancer. The results of Chapter II demonstrate that DCYTB significantly predicts outcome in multiple patient cohorts and provides additional information beyond that provided by current markers and prognostic signatures. Genetic profiling is currently indicated in cases where the indication for therapy is unclear [4]. Multiple gene panels, such as MammaPrint and Oncotype DX, are currently available for predicting risk of recurrence and response to therapy [189, 190]. These panels do not include DCYTB. However, this research shows that utilization of DCYTB expression as a prognostic marker will provide additional information to physicians. This is expected to aid in treatment decisions which will improve therapeutic response and thereby prolonging time to relapse.

#### **a. Iron Metabolism**

DCYTB functions as an iron uptake protein in the duodenum [9, 47, 59]. We know that increased iron uptake is associated with poor prognosis in a variety of cancers [21, 27, 28, 35, 44, 45, 67-70]. However, we see in Chapter II that DCYTB expression at physiological and supraphysiological levels did not affect cellular iron, suggesting an iron-independent mechanism in the breast.

Cell culture medium lacks ascorbate, an essential electron donor for DCYTB-mediated iron reduction. The lack of iron uptake by DCYTB expressing cells may be severely influenced by this lack of ascorbate, masking the true function of DCYTB in breast cells. This is unlikely, however, as increased cellular iron is associated with poor prognosis in breast cancer patients [35]. The focus on ascorbate as the primary electron donor is borne from its membership in the cytochrome b561 family which primarily accept electrons from ascorbate for transplasma

membrane transport [52, 59, 191]. Previous research also indicates that the cytochrome b561 ascorbate and ascorbate radical binding domains are partially conserved in DCYTB [9] and reduction kinetics suggest that neither iron nor copper are the primary substrate [59]. Other electron donors have been identified for DCYTB. The flavonoid quercetin has been shown to increase iron reduction by DCYTB [56]. Dihydrolipoic acid (DHLA) has also been identified as an electron donor for tonoplast-localized cytochrome b561 (TCYTB) and the putative tumor suppressor cytochrome b561 (TS101F6) [57]. Other cytochrome b561 were not investigated, however this does suggest that they would be capable of accepting electrons from DHLA. Further investigation is warranted into the iron import by DCYTB in the breast in light of these conclusions. It is unlikely that the results will differ, as it is fundamental for cell survival to maintain sufficient ascorbate concentrations and due to the other potential sources of electron donors for DCYTB.

#### **b. Copper-mediated effects**

We investigated 4 genes identified as copper inducible [153, 154], which are in fact responding to ROS signaling induced by Fenton-like reaction due to excessive copper [152]. All copper genes are increased as expected, however in DCYTB expressing cells CYP1A1 was repressed (Figure 3-1a-d). This is interesting because expression of CYP1A1 has been linked to proliferation and survival of breast cancer cells [155]. This provides an excellent direction for further evaluation of the molecular function of DCYTB in breast cancer. Expression of CYP1A1 in response to ROS is induced by AP-1 and inhibited by NF- $\kappa$ B transcription factors [153]. Depletion of GSH induces signaling through p38 MAPK or JNK signaling, inducing AP-1 transcription [153]. Signaling through these pathways and activation of these transcription factors is frequently dysregulated in cancer [192, 193]. Investigation of the redox status with respect to DCYTB expression is a good starting point for evaluating this effect.

### c. In vivo and 3D models

We present many results that suggest DCYTB expression should have an apparent and appreciable effect on breast cancer cells. The most prominent finding in DCYTB expressing cells and tumors is that the cell cycle is expected to be perturbed. The signaling pathway impact analysis of breast cancer patient microarray data specifically indicates that the cell cycle is perturbed in patients with high expression of DCYTB (Figure 2-S4). This analysis also identified alterations to the focal adhesion pathway which has downstream effects on the cell cycle (Figure 2-S4). Additionally, we see that MCF7 cells overexpressing DCYTB have reduced expression of CYP1A1 (Figure 3-1d). The expression of CYP1A1 has been associated with increased proliferation in breast cancer [155]. In spite of the mounting evidence indicating that DCYTB expression perturbs the cell cycle, we were unable to demonstrate any growth difference in knockdown or overexpressing cells (Figure 2-10).

We see in Chapter III, Section B that analysis of cellular ascorbate and detection of ROS are plagued with technical issues. It may be possible to correct these issues, for instance by adjusting the buffering capacity of the medium to more accurately mimic the tumor microenvironment. However, the benefits of DCYTB expression may only be detectable using 3D models or *in vivo* systems. 3D culture involves the culture of cell aggregates grown on or embedded in matrix or grown in scaffold-free suspension [194]. Evidence suggests that two-dimensional cell culture does not accurately mimic cell-cell interactions or the tumor microenvironment and gene expression profiles of 3D models are more similar to tumors than 2D cell culture [195]. The original identification of cell cycle perturbation was identified using data from breast cancer patients, which mounting evidence suggests, are not accurately represented by 2D culture models [194]. Moving these experiments into 3D culture and *in vivo* murine models is the logical next step to understanding the positive association of DCYTB with breast cancer patient prognosis.

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