Transcription Cofactor LBH is a Direct Target of the Oncogenic WNT Pathway with an Important Role in Breast Cancer

Megan Elizabeth Rieger

University of Miami, hurricanemego@gmail.com

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UNIVERSITY OF MIAMI

TRANSCRIPTION COFACTOR LBH IS A DIRECT TARGET OF THE ONCOGENIC WNT PATHWAY WITH AN IMPORTANT ROLE IN BREAST CANCER

By
Megan E. Rieger

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

August 2010
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

TRANSCRIPTION COFACTOR LBH IS A DIRECT TARGET OF THE
ONCOGENIC WNT PATHWAY WITH AN IMPORTANT ROLE IN BREAST
CANCER

Megan E. Rieger

Approved:

Zafar Nawaz, Ph.D.
Professor of
Biochemistry and Molecular Biology

Terri A. Scandura, Ph.D.
Dean of the Graduate School

Karoline Briegel, Ph.D.
Assistant Professor of
Biochemistry and Molecular Biology

Joyce Slingerland, M.D.,
Ph.D., F.R.C.P.(C)
Professor of Medicine,
Director Braman Family
Breast Cancer Institute

Thomas K. Harris, Ph.D.
Associate Professor of
Biochemistry and Molecular Biology

Xiang-Xi (Mike) Xu, Ph.D.
Professor of Medicine
Limb-bud-and-heart (Lbh) was previously identified as a transcriptional regulator of embryonic development that functions to promote self-renewal and to attenuate differentiation of fetal progenitor cells. The expression pattern of Lbh during mouse embryonic development was suggestive of Lbh regulation by Wnt signaling. The Wnt signaling pathway controls cell fate decisions during development and is essential for adult stem cell maintenance and self-renewal. Aberrant constitutive activation of the Wnt pathway leads to oncogenic transformation by promoting an invasive tumor stem cell phenotype. Given the role of Wnt in aggressive breast cancer, the aims of this thesis were to (1) examine if LBH gene expression is directly regulated by the canonical Wnt signaling pathway, (2) determine LBH expression and function in breast cancer, and (3) further characterize the biochemical properties and post-transcriptional regulation of the LBH protein.

We showed that Lbh is a direct transcriptional target of the canonical Wnt/β-catenin signaling pathway. This was evident by identification of four conserved TCF/Lef binding elements (TBE) within the Lbh genomic locus, which were efficiently bound by the transcriptional mediators of Wnt signaling, TCF4
and β-catenin, in in vitro electrophoretic mobility shift and in vivo chromatin immunoprecipitation assays. Functionality of the TBE sites was confirmed by cell based reporter assays, in which co-transfection of luciferase constructs containing Lbh TBEs fused upstream of a minimal promoter with a plasmid vector expressing constitutively active β-catenin resulted in increased Lbh promoter activity in mammalian cells. Additionally, endogenous LBH mRNA expression in human and mouse epithelial cells was quickly activated in response to Wnt3a ligand in a β-catenin dependent manner, whereas non-canonical Wnt ligands Wnt5a and Wnt7a had no such effect. Instead, Wnt7a, but not Wnt5a, blocked Wnt3a induced LBH expression, suggesting that non-canonical Wnt7a can inhibit canonical Wnt signaling.

To investigate if Lbh acts downstream of Wnt signaling during tumorigenesis, we examined mammary tumors from MMTV-Wnt1 transgenic mice, and found that Lbh expression was elevated in all Wnt-induced mammary tumors analyzed relative to normal mammary epithelial cells. More importantly, LBH was found to be overexpressed in the most clinically aggressive human breast cancer, which is the hormone-independent basal-like tumor subtype, for which there is currently no cure. Deregulation of LBH in human basal breast cancer appears to be Wnt/β-catenin dependent as DKK1 and Wnt7a inhibit LBH expression in breast tumor cells.

Detection of LBH primarily in basal breast cancers led us to investigate if, similar to its role in development, LBH maintains the undifferentiated stem-like phenotype that is characteristic of this aggressive breast tumor subtype. In
support of this theory, we identified a correlation between LBH expression and CD44\textsuperscript{high}/CD24\textsuperscript{low} surface marker expression, which has been associated with stem cell-like characteristics. RNAi mediated knockdown of LBH in basal breast cancer cell lines resulted in loss of CD44\textsuperscript{high}/CD24\textsuperscript{low} tumor cells, luminal differentiation, reduced cell growth, reduced colony forming ability, and increased apoptosis, suggesting a novel pro-survival and stem cell maintenance function of LBH in breast cancer. Reciprocal overexpression studies in the basal breast carcinoma line BT549 resulted in increased tumorigenicity \textit{in vitro}, suggesting that LBH overexpression is indeed oncogenic.

Biophysical characterization revealed that LBH belongs to the family of intrinsically disordered proteins, which have the potential to take on multiple conformations and protein interactions. Additionally, cellular localization of endogenous LBH, which was identified as a phosphoprotein, was observed for the first time in both development and tissue culture cells by immunofluorescence. Finally, \textit{in silico} analysis identified several new potential mechanisms of post-transcriptional regulation of LBH that will be important for future studies. Collectively, this thesis demonstrates that \textit{LBH} is a direct Wnt target gene in both development and basal breast cancer, that promotes the undifferentiated phenotype and survival of basal breast tumor cells.
I dedicate this work to my Aunt Peggy and Aunt Cheri: two amazing women that have fought and survived breast cancer. I did this work for you and all families affected by breast cancer.
Acknowledgements

I would like to thank my mentor, Dr. Karoline Briegel, for all of her hard work and patience with this project over the last four years. I could not have asked for a more understanding, caring, and supportive mentor. I look forward to seeing great things from the Briegel laboratory. I would also like to thank my committee members Dr. T.K. Harris, Dr. Zafar Nawaz, and Dr. Joyce Slinglerand, as well as my external examiner Dr. Mike Xu, for giving their time to help me become a better scientist. I also have to thank the Department of Biochemistry and Molecular Biology. The students, faculty, and staff have provided an environment that has allowed me to develop as a scientist. I appreciate everyone that has shared their time and provided me with materials over the past five years. Finally, I want to thank my family, for their continuous support and encouragement, including everyone that came down to Miami for my commencement ceremony. Mom, you have always been there to listen and calm me down, and you have never stopped telling me how proud you are. Jeremy, our sibling rivalry has always pushed me to do my best, and I’m glad you have found a career that you love. And, last but not least, Alfonso you are the love of my life and you have always believed in me. I look forward to our many years ahead together.
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### List of Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AER</td>
<td>Apical ectodermal ridge</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>aCGH</td>
<td>Comparative genomic hybridization array</td>
</tr>
<tr>
<td>CHD</td>
<td>Congenital heart disease</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CIP</td>
<td>Calf intestinal phosphatase</td>
</tr>
<tr>
<td>CK I/II</td>
<td>Casein kinase 1 or II</td>
</tr>
<tr>
<td>CK (KRT)</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen-receptor</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence associated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>IDP</td>
<td>Intrinsically disordered protein</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LBH</td>
<td>Limb-bud and heart</td>
</tr>
<tr>
<td>MEC</td>
<td>Mammary epithelial cell</td>
</tr>
<tr>
<td>MiR</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus.</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>PFA</td>
<td>Para formaldehyde</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SAN</td>
<td>Sinoatrial node</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBE</td>
<td>TCF/Lef binding element</td>
</tr>
<tr>
<td>TIC</td>
<td>Tumor initiating cells</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal end bud</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
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Chapter 1: Introduction
1.1 LBH

1.1.1 Identification and molecular characterization of Lbh as a transcriptional regulatory protein

Limb-bud and heart (Lbh) was identified in a yeast two-hybrid screen to identify interacting transcription co-factors of the homeodomain protein Engrailed 1 (En1; (Briegel and Joyner, 2001)). Although subsequent analysis failed to demonstrate a direct interaction between Lbh and En1, the unique expression pattern of Lbh during mouse embryonic development prompted further characterization of this gene (Briegel and Joyner, 2001). The LBH gene maps to chromosomal region 2p23 in humans, and chromosome 17 band E2 in mice (Briegel et al., 2005). The Lbh protein product consists of 105 amino acids with a predicted molecular weight of 12.3 kDa and an isoelectric point of 4.04 (Figure 1.1; Briegel and Joyner, 2001). Lbh is encoded by 3 exons spanning nearly 24kb of the mouse genome (28.5 kb in humans), with a transcript length of 3,064 bp (2,933 bp in humans). The first 8 amino acids (AA) are encoded by exon 1, followed by 2.6 kb intron 1. Amino acid 9 is formed by correct splicing to exon

![Figure 1.1](image_url): Schematic of the LBH protein, which contains 105 amino acids with a predicted molecular weight of 12.3 kDa. The N-terminal hydrophobic stretch is shown in green, the putative nuclear localization signal (NLS) is shown in blue, and the glutamate-rich acidic domain at the C-terminus is depicted in red (Briegel and Joyner, 2001).
2, which encodes the next 34 AA, followed by 17.9 kb intron 2, and exon 3 encoding the last 62 AA. The mouse 5’ and 3’ UTRs are, 248 and 2,501 bp respectively (208 and 2,410 bp in human). Only a single protein coding transcript has been identified in mice, whereas human LBH now has 11 predicted splice variants, seven of which may encode for protein products (www.ensembl.org). However, Northern blot analysis using a probe specific for the last 42 bp and entire 3’UTR or a cDNA probe, have only detected a single Lbh transcript in both mouse and human, respectively (Ai et al., 2008; Briegel and Joyner, 2001). Thus, it is likely that the other predicted Lbh transcripts may be the result of in silico analysis error. Primary amino acid sequence analysis revealed an N-terminal hydrophobic region, a putative nuclear localization signal (NLS) and an acidic glutamate-rich domain at the carboxy terminus, but no DNA binding domain (Figure 1.1; Briegel and Joyner, 2001). The acidic carboxy domain, a feature commonly found in unstructured transcriptional activation domains (Ptashne and Gann, 1997), also contains a predicted alpha-helix at positions 85-101 indicating a likely protein-protein interaction site (Al-Ali et al., 2009; Briegel and Joyner, 2001). The unstructured nature of LBH is further supported by SDS-PAGE analysis where the Lbh protein migrates at 16-17 kDa, several kDa larger than its predicted molecular weight (Briegel and Joyner, 2001; Chapters 3, 4, 5). Further biophysical and structural analysis will be described in Chapter 5. Lbh is only present in vertebrates and is highly conserved among 37 putative orthologs (www.ensembl.org), yet it has no known homology to any other protein families (Al-Ali et al., 2009; Briegel and Joyner, 2001).
Several features of the primary protein structure such as the NLS and acidic carboxy terminus indicated that Lbh may be a nuclear protein with transcriptional activity (Briegel and Joyner, 2001). In support of this theory, transient expression of Flag-tagged Lbh in mammalian cells resulted in mostly nuclear localization of Lbh as assessed by immunofluorescence analysis (Briegel and Joyner, 2001). The transcriptional activity of Lbh was examined by GAL4 reporter assays (Briegel and Joyner, 2001). C-terminal fusion of the entire Lbh Open Reading Frame to the Gal4 DNA binding domain (DBD) stimulated luciferase reporter activity 10 to 20 fold over basal levels (Briegel and Joyner, 2001). Additionally, transient transfection of LBH alone into HeLa cells has been demonstrated to activate SRF and AP-1 luciferase reporter constructs (Ai et al., 2008). Although these initial findings suggested a role for Lbh in transcriptional co-activation, all in vivo studies have subsequently demonstrated that Lbh acts as a transcriptional co-repressor (Briegel et al., 2005; Conen et al., 2009). The molecular details of Lbh co-repression will be described in the next section and later in Chapter 4. Future identification of transcriptional targets of Lbh will be an important breakthrough in understanding the molecular function of Lbh in both development and disease.

1.1.2 Expression and functional role of Lbh in embryonic development

The unique expression pattern of Lbh during early embryonic development was extensively studied by RNA in situ hybridization, (Briegel and Joyner, 2001) and led to the initiation of the studies described in this thesis.
Expression of *Lbh* begins shortly after gastrulation at embryonic day 7.5 (E7.5) in the presumptive cardiac mesoderm and primitive gut endoderm (Briegel and Joyner, 2001). By E9.5 *Lbh* expression is strongly apparent in the limb-buds and heart, hence its name, with restriction to the right ventricle of the heart and ventral ectodermal domains, which include the apical ectodermal ridge (AER) in the limb-bud (Figure 1.2; Briegel and Joyner, 2001). The dynamic expression pattern of *Lbh* overlaps with expression of other marker genes of early heart development, suggesting a role for *Lbh* in proper heart formation (Briegel and Joyner, 2001). Additionally, the spatio-temporal expression pattern in the limb suggested that *Lhb* may be regulated by morphogenic signaling pathways. Later in development, *Lhb* is more broadly expressed in several nervous system components such as the dorsal root and trigeminal ganglia, as well as many epithelial compartments including oral epithelium and otic vesicles (Briegel and Joyner, 2001). In later stages of heart development, *Lhb* expression is restricted

**Figure 1.2:** *Lhb* expression is primarily restricted to the developing heart and limb-buds at E9.5 as shown by whole mount *in situ* hybridization (left panel). Cross sections of the heart (a) and limb-bud (b) demonstrate a right-left or dorsal-ventral asymmetry, respectively, suggestive of regulation by morphogenic signaling pathways (Briegel and Joyner, 2001, modified).
to the sinoatrial node (SAN) and excluded from adjacent atrial tissue (E17.5) (Hoogaars et al., 2007). This expression pattern is downstream of Tbx3 expression, as \( Lbh \) expression is lost in the SAN in Tbx3 deficient mice (E13.5; (Hoogaars et al., 2007). Examination of Lbh protein expression during mouse development will described in Chapter 5. Interestingly, the Xenopus ortholog, \( XLCl2 \), shown to be a maternal RNA polyadenylated, and thus translated, early after fertilization (Paris et al., 1988; Paris and Philippe, 1990), shares a similarly high expression pattern in the heart during Xenopus development (Gawanntka et al., 1998). The parallels between \( Lbh \) expression in mammals and frog suggest that Lbh function is conserved between species.

Transgenic mis-expression of Lbh in the heart was performed to study Lbh function in heart development using a gain of function approach. Transgenic mice were engineered to express \( Lbh \) uniformly in the developing myocardium using the heart-specific promoter of the Cardiac Ankyrin Repeat Protein (\( Carp \)) (Briegel et al., 2005). In general, transgenic mice were not healthy, had enlarged hearts, and possessed multiple cardiac defects including anomalies of the outflow tract (OFT) and septation (Briegel et al., 2005). Mis-expression of Lbh also prolonged cardiomyocyte proliferation in the post-natal heart, providing the first functional evidence the Lbh may have pro-mitogenic activity in cardiac progenitor cells (Briegel et al., 2005). Because homozygous and hemizygous littermates had similar phenotypes despite differences in the level of transgene expression, it was suggested that in addition to increased gene dosage, abnormal distribution of Lbh played an important role in the observed cardiac defects.
(Briegel et al., 2005). Interestingly, these mice phenocopied congenital heart
disease symptoms of human patients with partial trisomy 2p syndrome (Briegel et
al., 2005). These patients have a triplication of chromosomal region 2p23
harboring LBH. These results suggested that Lbh plays an important role in heart
development and that gain of function of Lbh in the heart causes congenital heart
disease (Briegel et al., 2005). The observed cardiac defects appear to be due to
functional repression of key cardiac transcription factors Nkx2.5 and Tbx5 by
Lbh, leading to down regulation of a common target gene, Nppa/ANF, and
blocking proper cardiomyocyte differentiation (Briegel et al., 2005). Recently,
ectopic Lbh expression in developing chick limbs was shown to prolong
proliferation of immature chondrocytes and markedly delay bone formation and
vasculogenesis (Conen et al., 2009). These effects were proposed to be the result
of Lbh-mediated downregulation of bone transcription factor Runx2 and the pro-
angiogenic factor Vegf (Conen et al., 2009). Taken together, these results suggest
that Lbh functions to promote proliferation and to delay differentiation of
progenitor cells. Moreover, these data suggest that in vivo, Lbh functions
primarily as a transcriptional co-repressor.

1.1.3 LBH expression in adult tissues

Northern blot analysis has demonstrated highly elevated expression levels
of LBH in the mouse, frog, and human adult heart (Ai et al., 2008; Briegel and
Joyner, 2001; Paris and Philippe, 1990). LBH expression has also been detected
in the lung, and to a lesser extent spleen, kidney, and brain, but not in skeletal
muscle or testis (Briegel and Joyner, 2001; Paris and Philippe, 1990). Microarray analysis generally corroborates with these findings, and, in addition, highlights elevated levels of \textit{LBH} expression in many, but not all, hematopoietic lineages, the thymus, and lymph nodes (Figure 1.3; Su et al., 2004). \textit{LBH} was also highly expressed in endothelial cells, which our lab has verified on the protein level by immunofluorescent staining of mouse tissue (data not shown). \textit{LBH} expression in mouse adult mammary glands will be discussed in Chapter 3. The role of Lbh in adult organisms is not known, but will be addressed in regards to breast cancer in Chapters 3 and 4.

\textbf{Figure 1.3:} Expression of LBH in both human and mouse tissues, from (Su et al., 2004). LBH is highly expressed in several lineages of the hematopoietic system. According to this data it is also highly expressed in the heart, lung, Thymus and lymph node. These findings have not all been validated.
1.2 Wnt signaling

1.2.1 Overview of the Wnt signaling pathway

Wnt signaling is essential during embryonic development as it regulates pattern formation, cell proliferation, differentiation and migration (Logan and Nusse, 2004). Secreted lipid-modified Wnt ligands are morphogenic glycoproteins that elicit different cell behaviors depending on whether receptor interaction activates a canonical β-catenin-dependent transduction pathway, or other β-catenin-independent noncanonical pathways (Komiya and Habas, 2008; van Amerongen et al., 2008). Canonical Wnt signaling is required for normal tissue homeostasis in adults and promotes the self-renewal and maintenance of adult stem cells (Nusse et al., 2008), a function that becomes oncogenic when this pathway is deregulated (Clevers, 2006).

Canonical Wnt signaling is primarily mediated through its downstream effector, β-catenin; a multifunctional protein involved in epithelial cell adhesion, signaling and gene regulation (Logan and Nusse, 2004). In the absence of secreted Wnt ligands, cytoplasmic β-catenin is sequestered in a degradation complex consisting of Adenomatous Polyposis Coli (APC), Axin, and Glycogen Synthase Kinase 3 beta (GSK3β). In this complex, β-catenin is targeted for proteasomal degradation via phosphorylation by GSK3β (Figure 1.4A; Logan and Nusse, 2004). Binding of Wnt ligands, such as Wnts 1 or 3, to co-receptors Low density lipoprotein 5 or 6 (LRP5/6) and Frizzled (Fzd) results in recruitment of Dishevelled (Dsh) to the cell membrane. Dsh aggregates, forming a scaffold that
recruits casein kinase I (CKI), which in turn phosphorylates LRP5/6 co-receptors, signaling for the recruitment of the APC/Axin/GSK3β destruction complex (Bilic et al., 2007; Davidson et al., 2005). Recruitment of this complex to the membrane inactivates GSK3β, leading to accumulation of cytoplasmic β-catenin (Figure 1.4B; Bilic et al., 2007).

Excess cytoplasmic β-catenin leads to its nuclear localization, where β-catenin forms a transcriptionally active complex with T-cell factor (TCF)/Lymphoid enhancing factor (LEF) DNA binding proteins at TCF/LEF
binding elements (TBE) within regulatory regions of DNA (Figure 1.4B). TCF/LEF factors, constantly bound to DNA, act as transcriptional repressors when in complex with HDAC and Groucho; activation of canonical Wnt target genes occur in part by displacement of HDAC and Groucho by β-catenin (Figure 1.4A, B; Logan and Nusse, 2004). Numerous target genes have been identified, including TCF/LEF and genes involved negative feedback loops regulating Wnt signaling such as Dickkopf 1 (DKK1; Chamorro et al., 2005; Gonzalez-Sancho et al., 2005; Niida et al., 2004). For a comprehensive list of Wnt signaling components and target genes be referred to http://www.stanford.edu/~rnusse/wntwindow.html.

In addition, there are several different non-canonical pathways. The Wnt/planar cell polarity (PCP) pathway is involved in providing positional and vectoral information across tissues by modulating cytoskeletal dynamics (Jessen, 2009). A second non-canonical pathway, termed the Wnt/Ca\(^{2+}\) pathway, involves G-protein signaling resulting in an increase of cytosolic levels of calcium and recruitment of calcium dependent enzymes such as Protein Kinase C (PKC) to the membrane (Jessen, 2009; Sheldahl et al., 1999). Numerous additional non-canonical pathways have also been described (Semenov et al., 2007).

Non-canonical Wnt5A signaling has been demonstrated to inhibit canonical Wnt1 signaling in Xenopus potentially though a decrease in Ca\(^{2+}\) dependent cell adhesion (Torres et al., 1996). Receptor context, however, likely determines the signaling pathways that Wnt ligands activate. For example, Wnt5a inhibits canonical Wnt signaling by binding Ror2, but can stimulate canonical
Wnt signaling in the presence of Fzd4 (Mikels and Nusse, 2006). Similarly, Wnt7a has been demonstrated to signal through both canonical and non-canonical pathways depending on whether it binds to Fzd5 or Fzd10 receptors, respectively (Carmon and Loose, 2008). Additional mechanisms of canonical Wnt pathway control include a series of secreted Wnt antagonists. The Dickkopf family of secreted Wnt antagonists (Kawano and Kypta, 2003) blocks canonical Wnt signaling by binding LRP5/6 and Kremen, which induces LRP endocytosis, thereby preventing complex formation with Wnt ligands (Gonzalez-Sancho et al., 2005). Interestingly, DKK1 is a known target gene of the canonical Wnt pathway, and thus is involved in negative feedback regulation of canonical Wnt signaling (Chamorro et al., 2005; Gonzalez-Sancho et al., 2005; Niida et al., 2004). A second class of secreted antagonists, including Secreted Frizzled-related proteins (sFRP) and Wnt Inhibitory Factor 1 (WIF1) inhibit Wnt signaling via direct interaction with Wnt ligands (Hsieh et al., 1999; Kawano and Kypta, 2003).

1.2.2 Wnt signaling in normal mammary gland development

Wnt signaling is essential for both embryonic and adult mammary gland development; the three main stages of mouse mammary gland formation are shown in Figure 1.5A. Canonical Wnt activity has been detected in the mammary line of E10.5 mouse embryos in BAT-GAL and TOPGAL reporter mice (Chu et al., 2004; Howard and Ashworth, 2006), coinciding with expression of Wnt10b (Veltmaat et al., 2004), suggesting that Wnt10b regulates early mammary gland formation. Expression of multiple Wnt, Fzd, Tcf, and Lef mRNA in dissected
mammary buds and the developing embryo have been determined at E12.5 and E15.5 developmental stages (Chu et al., 2004). Interestingly, in vitro culture of embryos in the presence of Wnt3a conditioned medium leads to accelerated mammary placode formation (Chu et al., 2004). Wnt signaling is required for proper mammary bud and placode formation, as loss of Lef1 or transgenic overexpression of Dkk1 results in the loss of mammary bud formation (Andl et al., 2002; Boras-Granic et al., 2006). In later stages of embryonic development in
TOPGAL reporter mice, Wnt activity is restricted to mesenchymal and epithelial cells near the nipple region (Boras-Granic et al., 2006).

In post natal mouse mammary gland development, the mammary gland undergoes large morphological changes at puberty, during pregnancy, lactation, and involution after pregnancy. During puberty, ductal structures expand to fill the mammary fat pad, with growth occurring at highly proliferative terminal end buds (TEB, Figure 1.5A; Brennan and Brown, 2004). During puberty, there is little direct evidence for Wnt regulation, but it has been suggested that Wnt may play a role in hormone independent ductal growth (Brennan and Brown, 2004).

At pregnancy, the ductal tree undergoes extensive secondary branching and alveolar units form to create the secretory apparatus of the mammary gland (Figure 1.5A, left panel; Brennan and Brown, 2004). Ectopic expression of Wnt1 or Wnt10b induces ductal hyperbranching and alveolar hyperplasia in virgin mammary glands that mimics hormonal induced morphological changes during pregnancy (Brennan and Brown, 2004). Expression of several Wnt ligands is elevated during early pregnancy and appears to be downregulated during late pregnancy and at the onset of lactation (Gavin and McMahon, 1992).

Transforming Wnts, such as Wnt1 or Wnt3, are normally not expressed in the adult mammary gland (Gavin and McMahon, 1992), partially explaining why abnormal expression of these Wnts leads to cancer. Wnt4, however, which in some cases can transform normal mammary epithelial cells, has been demonstrated to be a mediator of progesterone function in mammary gland morphogenesis (Brisken et al., 2000).
1.2.3 Wnt signaling in breast cancer

Wnt1 was identified as the first mammary oncogene, as it was commonly
downstream of viral insertions of mouse mammary tumor virus (MMTV; Nusse
and Varmus, 1982). Not all Wnt ligands have equal transforming abilities, as
canonical Wnt1, Wnt2, Wnt3, Wnt3a, and Wnt11 strongly transform normal
mouse mammary epithelial cell line C57MG (Christiansen et al., 1996; Shimizu et
al., 1997; Wong et al., 1994), Wnt6 and Wnt7a have weak transformation abilities
(Shimizu et al., 1997), and Wnt4, and non-canonical Wnt5a were unable to
morphologically transform cells (Shimizu et al., 1997; Wong et al., 1994). The
transforming ability of the Wnts depended upon their ability to increase cytosolic
β-catenin levels (Shimizu et al., 1997). Cells transformed by Wnt acquire a
spindle like morphology and are no longer susceptible to contact inhibition
(Shimizu et al., 1997). Moreover, HC11 non-transformed mammary epithelial
cells stably expressing Wnt1 lose their differentiation capabilities (Howe et al.,
2003; Humphreys and Rosen, 1997). In contrast to their parental counterparts,
they fail to form spherical structures during prolonged tissue culture, and cannot
form normal ductal structures in mammary gland reconstitution assays
(Humphreys and Rosen, 1997). Interestingly, similar to overexpression of Wnt1
or Twist in HC11 cells, we observed a block in differentiation to milk producing
cells upon overexpression of Lbh in HC11 cells (Chapter 3; Howe et al., 2003;
Rieger et al., 2010).

Several mouse models have been established for Wnt driven breast cancer.
Please refer to (Mohinta et al., 2007) for a listing of the current models. Of
particular interest to our lab, *MMTV-Wnt1* transgenic mice progress from early mammary hyperplasia to aggressive breast adenocarcinoma with full penetrance within 6–9 months (Tsukamoto et al., 1988). Similarly, *MMTV-ΔN89β-catenin* transgenic mice display precocious lobuloalveolar development and differentiation, as well as the formation of multiple aggressive adenocarcinomas early in life (Imbert et al., 2001). These mice express a truncated form of β-catenin in the mammary gland that is protected from GSK3β mediated degradation, leading to nuclear localization of β-catenin and thus Wnt pathway activation (Imbert et al., 2001).

Unlike other cancers, mutations in Wnt pathway genes are uncommon in breast cancer (Brennan and Brown, 2004), yet β-catenin has been found to be stabilized in approximately 50% of breast cancers and correlates with a poor outcome (Lin et al., 2000). Canonical Wnt proteins are also frequently overexpressed in breast cancer or breast cancer cell lines as compared to normal tissues, whereas non-canonical Wnts are generally downregulated (Turashvili et al., 2006). In terms of prognosis, reduced membrane expression of β-catenin has been associated with a poorer prognosis (Dolled-Filhart et al., 2006). Recently, both increased cytoplasmic and nuclear β-catenin localization associated with a poor prognosis in African American women, and was enriched for in basal subtype breast cancers (Khramtsov et al., 2010). Expression of Wnt component LRP6 has also been shown to be overexpressed in triple negative breast cancers (Liu et al., 2010). Other studies have also implicated a role for canonical Wnt
signaling in basal subtype breast cancer (Dimeo et al., 2009; Smid et al., 2008). Additionally, antagonists of Wnt signaling, such as WIF-1 and sFRP, have been demonstrated to be epigenetically silenced and thus downregulated in breast cancer as compared to normal tissues (Ai et al., 2006; Suzuki et al., 2008). Thus, the majority of aberrant Wnt signaling in breast cancer is due to upstream regulatory events.

The functional role of Wnt in breast cancer varies depending upon the pathway and transcriptional target gene activation. Cell cycle progression regulator Cyclin D1, a well known target of canonical Wnt, has been shown to be overexpressed and amplified in breast cancers (Gillett et al., 1994) and is associated with a poor prognosis (Lin et al., 2000). Despite evidence linking Cyclin D1 to breast cancer, it has been demonstrated that Wnt driven mouse mammary tumors to not require Cyclin D1 for tumor formation (Rowlands et al., 2003). C-Myc, another cell cycle regulator and Wnt target, may be able to overcome this Cyclin D1 deficiency in Wnt driven mouse models (Hatsell et al., 2003). Aside from the role of Wnt in increase cellular proliferation, increased Wnt may lead to an increase in epithelial to mesenchymal transition (EMT), resulting in more motile cells with mesenchymal characteristics (Mohinta et al., 2007). Increasing evidence also suggests a role for Wnt in both normal and cancer mammary stem cell maintenance (Lindvall et al., 2007). In mouse models, Wnt signaling increases the pool of cells with progenitor or “stem-like” properties, herein referred to as breast tumor initiating cells (TIC; Li et al., 2003;
Liu et al., 2004). New evidence suggests that the LRP5 co-receptor is required for maintaining breast cells in the basal lineage, and is expression enriched in TIC (Badders et al., 2009).

1.3 Breast Cancer

1.3.1 Overview

One in eight American women will develop breast cancer over their lifetime. Breast cancer is the seventh largest cause of death of women, and the second largest cause of cancer related deaths in women behind lung cancer (American Cancer Society). Women with germline mutations in BRCA1 or BRCA2 have a 20-30 fold increased risk for developing breast cancer over their lifetime (Antoniou et al., 2003). This section of the chapter will outline the different methods of breast tumor classification, and will focus on basal subtype breast cancers, as they are most relevant to the work in this thesis.

1.3.2 Clinical parameters and molecular subtypes

Malignant tumors derived from mammary epithelial cells are termed adenocarcinomas; 17 distinct histological “special types” are currently recognized (Weigelt and Reis-Filho, 2009). Approximately 50-80% of invasive ductal carcinomas lack distinguishing features to classify them into one of the special types (Weigelt and Reis-Filho, 2009). Histological types have been demonstrated to be useful as prognostic factors in outcome analysis of patients (Ellis et al.,
Tumor grade, a measure of the degree of differentiation and proliferation, also gives insight into the tumor’s aggressiveness (Elston and Ellis, 1991). For a detailed review of the clinical histopathological and molecular subtypes of breast cancer please see (Weigelt and Reis-Filho, 2009).

There are currently three prognostic markers used regularly in the clinic to identify the proper therapeutic course of treatment. Estrogen receptor (ER) and Progesterone receptor (PR) status are predictive for response to Tamoxifen and endocrine therapy treatments (Fisher et al., 1983; Stierer et al., 1993). The third marker, human epidermal growth factor receptor (HER2/ERBB2/NEU) (Slamon et al., 1987), is also used to determine effectiveness of HER2 targeted therapies such as Trastuzumab (Piccart-Gebhart et al., 2005). These markers have traditionally been assessed in the clinic by immunohistochemical staining (Harvey et al., 1999), but it has recently been demonstrated that microarray-based determination of marker expression is highly concordant with immunohistochemical results (Roepman et al., 2009). Survival analysis using these three prognostic markers has demonstrated that ER/PR/HER2 negative (triple negative) breast tumors have the poorest survival in comparison to the most common subtype of ER/PR positive, HER2 negative breast cancers (Onitilo et al., 2009). Breast cancers are highly heterogeneous, however, and further molecular profiling has been performed to enhance prognosis and treatment predictions.
1.3.3 Gene expression profiling of breast cancer subtypes

Hierarchical clustering of molecular profiles of breast cancer samples revealed five ‘intrinsic’ molecular tumor subtypes including Luminal A, Luminal B, Normal-like, HER2/ERBB2, and Basal (Perou et al., 2000; Sorlie et al., 2001). Additional subtypes have branched from the basal subtype including Claudin-low and metaplastic subtypes, but sample size is still relatively small (Hennessy et al., 2009; Herschkowitz et al., 2007). Each intrinsic breast tumor subtype has a different clinical course and outcome. See Table 1.1 for a summary of these features. Luminal A tumors are ER positive (ER+), well differentiated, have the best prognosis, and are treatable with Tamoxifen and aromatase inhibitors (Sims et al., 2007). Luminal B tumors have a poorer prognosis as they tend to be more undifferentiated and ER negative. However, Luminal B cases which are ER+ may still be treated with endocrine therapies (Sims et al., 2007; Sorlie et al., 2001; Sorlie et al., 2003). Normal-like breast tumors are generally ER negative but are poorly understood, and their gene signature may be the byproduct of adjacent

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Prognosis</th>
<th>Clinical Markers</th>
<th>Additional Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal-like</td>
<td>Mixed</td>
<td>ER+/-, PR+/-, HER2-</td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>Good</td>
<td>ER+, PR+, HER2-</td>
<td>CK8/18+, GATA3+</td>
</tr>
<tr>
<td>Luminal B</td>
<td>Poor</td>
<td>ER+/-, PR+/-, HER2/-</td>
<td></td>
</tr>
<tr>
<td>ERBB2</td>
<td>Good</td>
<td>ER+/-, PR+/-, HER2+</td>
<td>GRB7</td>
</tr>
<tr>
<td>Basal</td>
<td>Worst</td>
<td>ER-, PR-, HER2-</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1: Breast cancer subtypes including prognosis, clinical markers and additional immunohistochemical markers. Additional markers only include those specific to each subtype. Adapted and modified from (Matos et al., 2005; Ribeiro-Silva et al., 2005; Sims et al., 2007; Weigelt et al., 2010).
normal mammary tissue during tumor removal (Parker et al., 2009). ERBB2 tumors are both ER/PR positive and negative, but are unique in that the introduction of Trastuzumab has dramatically improved the outcome of ERBB2 positive patients to better than patients with ERBB2 negative tumors (Dawood et al., 2010). Basal subtype breast cancers are ER/PR/HER2 negative (triple negative) and have the worst clinical outcome. Gene expression profiling has also determined a 70 gene prognostic set that can be used to predict outcome (van ’t Veer et al., 2002), which has been further expanded by (Sotiriou et al., 2003).

1.3.4 Basal subtype breast cancer

Basal subtype breast cancer is a highly heterogeneous and clinically aggressive form of breast cancer that comprises 15-20% of all breast cancer cases (Carey et al., 2006; Millikan et al., 2008). Over 70% of triple negative breast cancers (ER/PR/HER2 negative) are classified as basal subtype (Bertucci et al., 2008), and hence the two will not be differentiated herein. Basal breast cancer is disproportionally associated with African American women and a young age of onset (Carey et al., 2006; Millikan et al., 2008), has the poorest prognosis (Parker et al., 2009), and accounts for a disproportionate number of breast cancer related deaths (Gluz et al., 2009). The aggressive nature of basal breast cancer is reflected by the fact that recurrence, both local and regional, tends to occur within 3 years of initial treatment (Dent et al., 2007; Voduc et al., 2010). Basal breast cancers do not correlate with typical clinical parameters including tumor size or node status (Carey et al., 2006), but tend to present as grade III tumors (Matos et
al., 2005; Ribeiro-Silva et al., 2005). These tumors are poorly differentiated, have a high mitotic index, are highly invasive, include zones of necrosis, and contain lymphocytic infiltrates (Fulford et al., 2006; Lakhani et al., 2005; Livasy et al., 2005; Winter, 2008). Additionally, basal breast cancers have a higher incidence of metastasis (Gluz et al., 2009). Breast tumors from BRCA1/2 germline mutation carriers share similar clinical, gene expression, and pathologic profiles to basal breast cancers (Lakhani et al., 2005). Recently, a lung metastasis signature was derived that links Wnt and cancer cell self-renewal with basal subtype breast cancer (Dimeo et al., 2009). Chemotherapy is currently the only treatment option available to these patients, as no targeted therapies have been developed (Gluz et al., 2009). Potential future targeted therapies include targeting EGFR, PARP inhibitors for BRCA1 null tumors, kinase inhibitors, and anti-angiogenic therapies (Gluz et al., 2009).

Basal breast cancers are also enriched for CD44\textsuperscript{high}/CD24\textsuperscript{low} breast tumor initiating cells (TIC; Honeth et al., 2008). Breast TIC are characterized by an increased ability to self-renew and differentiate into distinct lineages recapitulating all the cells of a tumor (Fillmore and Kuperwasser, 2008). Functional assays to study TIC include 3D suspension tumorsphere assays, mammary gland reconstitution assays, as well as serial passaging \textit{in vitro} and in xenografts (Charafe-Jauffret et al., 2009). TIC are highly tumorigenic in \textit{in vivo} xenograft models (Al-Hajj et al., 2003), are enriched for and resistant to chemotherapy and radiation (Fillmore and Kuperwasser, 2008; Li et al., 2008; Phillips et al., 2006), and have an increased metastatic potential due to a
mesenchymal cell morphology and molecular profile suggestive of an epithelial to mesenchymal transition (EMT; Fillmore and Kuperwasser, 2007; Fillmore and Kuperwasser, 2008; Sheridan et al., 2006; Shipitsin et al., 2007). The increased resistance of TIC to multiple conventional treatments has been proposed to be the cause for tumor recurrence (Creighton et al. 2010; Jones et al., 2004). Additionally, both endocrine and chemotherapy have been shown to increase the mesenchymal and TIC characteristics of breast tumors (Creighton et al., 2009), further supporting the need for new therapies that target TIC and EMT characteristics.

Originally, it was proposed that basal subtype breast cancers arose from the basal myoepithelial layer in the mammary gland (Figure 1.5B) because of a shared gene signature with basal myoepithelial cells (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003). However, the exact origin of basal breast cancer is still debated. One possibility is that these tumors are derived from the most primitive mammary stem cells, as basal breast cancer cells share a similar gene signature with embryonic stem cells (Ben-Porath et al., 2008). Recently, luminal progenitors were proposed as the cell of origin for basal breast cancers that arise due to BRCA1 loss of function mutations (Lim et al., 2009). Due to the heterogeneity in breast cancer in general, it is unlikely that all cancer types or even all tumors of each subtype arise from a single type of cell.

The goal of this thesis was to elucidate the regulation of Lbh by the Wnt signaling pathway. Moreover, because 50% of breast cancers exhibit aberrant Wnt pathway activation (Lin et al., 2000), we sought to identify the expression
patterns of LBH in Wnt driven mouse mammary tumor models, human breast cancer cell lines, and primary human tumors. Association of LBH overexpression with the most aggressive and difficult-to-treat form of breast cancer led us to further investigate the functional role of LBH in breast carcinogenesis. These studies uncovered that LBH is required for survival and maintenance of basal breast cancer tumor cell lines that are enriched in ‘stem-like’ tumor initiating cells.
Chapter 2: Materials and Methods
2.1 Materials

2.1.1 Commonly used lab materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
<th>Source</th>
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<tr>
<td>Radioisotope</td>
<td>$^{32}$P γATP</td>
<td></td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td>PCR primers, EMSA etc.</td>
<td>Sigma Genosys</td>
</tr>
<tr>
<td>Media</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial</td>
<td>Luria broth and Agar</td>
<td>Difco</td>
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<tr>
<td>Mammalian</td>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM), Roswell Park Memorial Institute -1640 (RPMI 1640), Improved MEM (IMEM)</td>
<td>Mediatech</td>
</tr>
<tr>
<td></td>
<td>FBS</td>
<td>Sigma, Invitrogen</td>
</tr>
<tr>
<td></td>
<td>Non-essential amino acids (NEAA), Penicillin-streptomycin (Pen/strep), Sodium Pyruvate, Glutamine, G418</td>
<td>Invitrogen</td>
</tr>
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<td>Protein Work</td>
<td>Protein A/G Fast Flow Sepharose Beads</td>
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<td>Ni-NTA His bind reagent</td>
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<tr>
<td></td>
<td>Nitrocellulose membrane</td>
<td>Whatman</td>
</tr>
<tr>
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<td>West Femto Super Signal Western Blot Detection Kit</td>
<td>Pierce</td>
</tr>
<tr>
<td></td>
<td>8 x 5 inch film</td>
<td>Denville, Midwest Scientific</td>
</tr>
<tr>
<td></td>
<td>Hoechst Dye</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td>SlowFade mounting media</td>
<td>Molecular Probes</td>
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<td>Invitrogen</td>
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<td>Dharmacon</td>
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<td>Nucleofection Kits V and HMEC</td>
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<td>DNA work</td>
<td>ON-Target smart pool siRNA</td>
<td>Dharmacon</td>
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<td></td>
<td>Mini-elute PCR purification kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td></td>
<td>QuickChange Site-Directed mutagenesis kit</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

Table 2.1: General laboratory materials used in this work.

2.1.2 Mammalian cell lines

All cell lines were obtained from ATCC with the exception of HMEC, which were obtained from Cambrex. Cells were cultured in an atmosphere of 5% CO$_2$ at 37°C. Cell lines with appropriate culture conditions are listed in Table 2.2.
<table>
<thead>
<tr>
<th>Name</th>
<th>Culture Conditions</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>293T/17</td>
<td>DMEM, 10% FBS, 1% pen/strep</td>
<td>Human embryonic kidney epithelial cells</td>
</tr>
<tr>
<td>HC11</td>
<td>RPM1 1640, 10% FBS, 10 ng/ml EGF, 5 µg/ml insulin, 1% pen/strep</td>
<td>Mouse non-transformed mammary epithelial cells</td>
</tr>
<tr>
<td>HMEC</td>
<td>HuMEC basal serum free media and supplement kit</td>
<td>Human primary mammary epithelial cells</td>
</tr>
<tr>
<td>MCF10A</td>
<td>HuMEC basal serum free media and supplement kit</td>
<td>Human non-transformed mammary epithelial cells</td>
</tr>
<tr>
<td>L-Wnt3A</td>
<td>DMEM, 10% FBS, 1% pen/strep (0.4 mg/ml G418 for passaging)</td>
<td>Mouse fibroblasts stably expressing Wnt3A</td>
</tr>
<tr>
<td>MCF7</td>
<td>IMEM, 10% FBS, 10µg insulin, 1% pen/strep</td>
<td>Human breast adenocarcinoma, pleural effusion derived cell line</td>
</tr>
<tr>
<td>T47D</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast ductal carcinoma cell line</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast ductal carcinoma cell line</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast adenocarcinoma, acites derived cell line</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast adenocarcinoma, pleural effusion derived cell line</td>
</tr>
<tr>
<td>BT-20</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast adenocarcinoma cell line</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast adenocarcinoma cell line</td>
</tr>
<tr>
<td>HCC1937</td>
<td>RPM1 1640, 10% FBS, 25 µg/ml glucose, 1mM Sodium Pyruvate, 1 mM HEPES, 1% NEAA, 1% pen/strep</td>
<td>Human breast ductal carcinoma cell line</td>
</tr>
<tr>
<td>BT-549</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast ductal carcinoma cell line</td>
</tr>
<tr>
<td>MDA-MB-435S</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast ductal carcinoma cell line</td>
</tr>
<tr>
<td>HCC1395</td>
<td>RPM1 1640, 10% FBS, 25 µg/ml glucose, 1mM Sodium Pyruvate, 1 mM HEPES, 1% NEAA, 1% pen/strep</td>
<td>Human breast ductal carcinoma cell line</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast adenocarcinoma, pleural effusion derived cell line</td>
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<tr>
<td>HCC1187</td>
<td>RPM1 1640, 10% FBS, 1% pen/strep</td>
<td>Human breast ductal carcinoma cell line</td>
</tr>
</tbody>
</table>

Table 2.2: Mammalian cell lines
2.1.3 Plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Additional Source</th>
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</thead>
<tbody>
<tr>
<td>pGL3</td>
<td>Promega</td>
</tr>
<tr>
<td>pRL-TK</td>
<td>Promega</td>
</tr>
<tr>
<td>pGL3-OT (TOPFlash)</td>
<td>Bert Volgelstein</td>
</tr>
<tr>
<td>pGL3-OF (FOPFlash)</td>
<td>Bert Volgestein</td>
</tr>
<tr>
<td>pGL3-LbhΔSX (Pwt)</td>
<td></td>
</tr>
<tr>
<td>pGL3-LbhΔSX172/174 (E1wt)</td>
<td></td>
</tr>
<tr>
<td>pGL3-LbhΔSXInt1 (E2wt)</td>
<td></td>
</tr>
<tr>
<td>pCDNA3</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCDNA3 β-cateninS37Y</td>
<td>Antonio Garcia de Herreros</td>
</tr>
<tr>
<td>pCDNA3 TCF4-VP16</td>
<td>Antonio Garcia de Herreros</td>
</tr>
<tr>
<td>pCDNA3 NFlag-Lbh</td>
<td></td>
</tr>
<tr>
<td>pEGFP</td>
<td>Amaxa</td>
</tr>
<tr>
<td>pET28a-TCF4 HMG</td>
<td>Atsushi Niida</td>
</tr>
<tr>
<td>pBluescript</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3: List of plasmids used in this work. All plasmids were constructed by our laboratory, unless otherwise noted.

2.1.4 Bacterial Strains

We used *E. coli* strains HMS147 for protein expression, XL1 gold supercompetent cells (Stratagene) for site-directed mutagenesis transformation, and WM1/F' cells for plasmid propagation.

2.1.5 Antibodies and recombinant protein

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Source</th>
<th>Use</th>
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</thead>
<tbody>
<tr>
<td>Rabbit polyclonal 6th bleed Lbh</td>
<td></td>
<td>WB, IF, IP</td>
</tr>
<tr>
<td>Mouse monoclonal β-catenin</td>
<td>BD</td>
<td>IF</td>
</tr>
<tr>
<td>Rabbit polyclonal β-catenin</td>
<td>Upstate</td>
<td>ChIP</td>
</tr>
<tr>
<td>Rabbit polyclonal β-catenin (H-102)</td>
<td>Santa Cruz</td>
<td>ChIP</td>
</tr>
<tr>
<td>Normal rabbit IgG</td>
<td>Upstate</td>
<td>ChIP</td>
</tr>
<tr>
<td>Rabbit polyclonal TCF4 (N-20X)</td>
<td>Santa Cruz</td>
<td>ChIP</td>
</tr>
<tr>
<td>Rabbit polyclonal Acetyl-histone 3</td>
<td>Upstate</td>
<td>ChIP</td>
</tr>
<tr>
<td>Rabbit polyclonal Keratin 5/6</td>
<td>Covance</td>
<td>WB</td>
</tr>
<tr>
<td>Guinea pig polyclonal Keratin 8/18</td>
<td>Progen</td>
<td>WB</td>
</tr>
<tr>
<td>Rabbit polyclonal ERα (HC-20X)</td>
<td>Santa Cruz</td>
<td>WB</td>
</tr>
<tr>
<td>Rabbit polyclonal CD24 (FL-80)</td>
<td>Santa Cruz</td>
<td>WB</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------</td>
<td>----</td>
</tr>
<tr>
<td>Mouse monoclonal β-actin (AC-15)</td>
<td>Sigma</td>
<td>WB</td>
</tr>
<tr>
<td>Mouse monoclonal phosphoserine (PSR-45) ascites fluid</td>
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<td>WB</td>
</tr>
<tr>
<td>Mouse monoclonal phosphothreonine (PTR-8)</td>
<td>Sigma</td>
<td>WB</td>
</tr>
<tr>
<td>Mouse monoclonal [HIS.H8] 6X His tag</td>
<td>Abcam</td>
<td>EMSA</td>
</tr>
<tr>
<td>PE-mouse anti-human CD24</td>
<td>BD Pharmigen</td>
<td>FACS</td>
</tr>
<tr>
<td>APC-mouse anti-human CD44</td>
<td>BD Pharmigen</td>
<td>FACS</td>
</tr>
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</table>

**Secondary Antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Format</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-rabbit HRP</td>
<td>Upstate, Invitrogen</td>
<td>WB</td>
</tr>
<tr>
<td>Anti-mouse HRP</td>
<td>Amersham</td>
<td>WB</td>
</tr>
<tr>
<td>Anti-guinea pig HRP</td>
<td>Sigma</td>
<td>WB</td>
</tr>
<tr>
<td>Anti-mouse Cy3</td>
<td>Jackson</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-rabbit FITC</td>
<td>Sigma</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-rabbit or mouse Alexafluor 488</td>
<td>Molecular Probes</td>
<td>IF</td>
</tr>
<tr>
<td>Anti-rabbit or mouse Alexafluor 594</td>
<td>Molecular Probes</td>
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</tr>
<tr>
<td>Anti-guinea pig Alexafluor 568</td>
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**Recombinant Proteins**

<table>
<thead>
<tr>
<th>Protein</th>
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<th>Induction/inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCF4-HMG</td>
<td>In house</td>
<td>EMSA</td>
</tr>
<tr>
<td>Human Dkk1</td>
<td>R&amp;D Systems</td>
<td>Induction/inhibition</td>
</tr>
<tr>
<td>Mouse Wnt5A</td>
<td>R&amp;D Systems</td>
<td>Induction/inhibition</td>
</tr>
<tr>
<td>Human Wnt7a</td>
<td>R&amp;D Systems</td>
<td>Induction/inhibition</td>
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</table>

**Table 2.4:** Antibodies and recombinant proteins used in this work.

### 2.1.6 Oligonucleotide sequences

<table>
<thead>
<tr>
<th><strong>qPCR Primers</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name</strong></td>
</tr>
<tr>
<td>Human GAPDH forward</td>
</tr>
<tr>
<td>Human GAPDH reverse</td>
</tr>
<tr>
<td>Human LBH forward</td>
</tr>
<tr>
<td>Human LBH reverse</td>
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<tr>
<td>Human DKK1 forward</td>
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<tr>
<td>Human DKK1 reverse</td>
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<tr>
<td>Human CTNNB1 forward</td>
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<tr>
<td>Mouse Gapdh forward</td>
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<tr>
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</tr>
<tr>
<td>Mouse Lbh forward</td>
</tr>
<tr>
<td>Mouse Lbh reverse</td>
</tr>
<tr>
<td>Mouse β-casein forward</td>
</tr>
<tr>
<td>Mouse β-casein reverse</td>
</tr>
<tr>
<td>Human CD24 forward</td>
</tr>
<tr>
<td>Human CD24 reverse</td>
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### Site Directed Mutagenesis

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (-6245) mut (s)</td>
<td>GAAAAAATCCCTTTGAGCACAAGGCCCTTGATAG</td>
</tr>
<tr>
<td>T1 (-6245) mut (as)</td>
<td>CTATCAAGGGGCTTTGCTCAAGGGATTFTTTTTTCC</td>
</tr>
<tr>
<td>T2 (-6195) mut (s)</td>
<td>CAGGCTGAGTCTTTTGGCATTTGGGAAGAGCAG</td>
</tr>
<tr>
<td>T2 (-6195) mut (as)</td>
<td>CTGGCTTCTCAGAGGCTCCCTACCCCATATG</td>
</tr>
<tr>
<td>T3 (+1558) mut (s)</td>
<td>CATATGATGAGGTCCTGGAT</td>
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<tr>
<td>T3 (+1558) mut (as)</td>
<td>GGCCTCCACACTCTCTAGGGCTCCCATATG</td>
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<tr>
<td>T4 (+2145) mut (s)</td>
<td>GTACCTGCTTGGAGCTCTTCAGTGACATG</td>
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<td>T4 (+2145) mut (as)</td>
<td>CCAATGCGCTGACATGAAAGTAGTCTTCAGGAGG</td>
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### EMSA Oligonucleotides

<table>
<thead>
<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>T1 (-6245) mut</td>
<td>GGGATCCCTTTGACGCAGAGGCCCTTGAT</td>
</tr>
<tr>
<td>T2 (-6195)</td>
<td>GGGGCCTGAGTCTCTTTGATATTTGGGAAG</td>
</tr>
<tr>
<td>T2 (+1558) mut</td>
<td>GGGGCCTGAGTCTCTCGAGGCTCGAAG</td>
</tr>
<tr>
<td>T3 (+1558)</td>
<td>GGGATGCTGGTGAGGCTTTGAAAGTGGTGGA</td>
</tr>
<tr>
<td>T3 (+1558) mut</td>
<td>GGGATGCTGGTGAGGCTACAGAGGATG</td>
</tr>
<tr>
<td>T4 (+2145)</td>
<td>GGGCTGCTTGGAGCTTGAGAGGCT</td>
</tr>
<tr>
<td>T4 (+2145) mut</td>
<td>GGGCTGCTTGGAGCTTGAGAGGCT</td>
</tr>
</tbody>
</table>

### ChIP Primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh promoter forward</td>
<td>TACTAGCGGTTTTACGGGCG</td>
</tr>
<tr>
<td>Gapdh promoter reverse</td>
<td>TCGAACAGGAGAGACAGAGCAGCA</td>
</tr>
<tr>
<td>T1/T2 (-6245/-6195) forward</td>
<td>CCTGGTCCCTCAAGATTCTG</td>
</tr>
<tr>
<td>T1/T2 (-6245/-6195) reverse</td>
<td>TAAAAGAGGCGCTTGGGAACCT</td>
</tr>
<tr>
<td>T4 (+2145) forward</td>
<td>TAACTGGACCCTTGCTGTGTC</td>
</tr>
<tr>
<td>T4 (+2145) reverse</td>
<td>CAGGCTACTAACTCCAGCA</td>
</tr>
</tbody>
</table>

Table 2.5: Oligonucleotide sequences separated by application.

### 2.2 Methods

#### 2.2.1 Purification of recombinant TCF4

**Bacterial transformation**

For large-scale protein preparation, cells were freshly transformed with 0.5 µl of pET28a-TCF4 HMG, generated by Ebony coats using GST-TCF4 (generously provided by Atsushi Niida) as a template, in 10 µl *E. coli* HMS147
cells by heat shock at 42°C for 15 seconds after initial 1-minute incubation on ice. Immediately following, 100 µl of LB was added to the cells, and incubated for 1 hour at 37°C. 50 µl of bacteria was plated on LB Kanamycin plates and grown overnight at 37°C to select for transformants.

Protein expression and purification

10 ml of 100 ml overnight starter culture inoculated with a single transformed colony was used to further inoculate two 500 ml cultures of LB plus Kanamycin. Cells were grown at 37°C with shaking at 250 rpm until mid-log (OD = 0.5). After removal of an aliquot for whole cell extract, 500 µl of 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to each of the cultures which were then grown for 4 hours. A whole cell extract plus IPTG aliquot was removed and the cells were collected by centrifugation at 5000 rpm at 4°C. Cells were lysed and recombinant TCF4 was purified using Nickel beads (Novagen) according to the manufacturer’s protocol.

2.2.2 Electrophoretic Mobility Shift Assays (EMSA)

Double-stranded DNA oligonucleotides (30 mers, Table 2.5) were 5’ end-labeled with 32P and 5000 cpm/µl of labeled probe was incubated with 1 µg of recombinant His-TCF4 protein in a total volume of 15 µl binding buffer (Giese et al., 1991). For competition and supershift experiments, His-TCF4 was pre-incubated with unlabelled oligonucleotides at 400-fold excess or with 1 – 5 µg of anti-6X His tag antibody (Abcam) for 10 minutes prior to addition of labeled probe. Samples were separated on 5% non-denaturing polyacrylamide gels, that had been poured and allowed to polymerize overnight in the cold room, for 1 hour
at 400V. Gels were dried and exposed for 1.5 days in a phosphoimaging cassette. Phosphoimaging was performed on a Storm 840 Scanner (Molecular Dynamics).

2.2.3 Luciferase reporter assays

One day prior to transfection, 2.0 x 10^5 HC11 mouse mammary epithelial cells were plated per well on 12-well plates. Cells were co-transfected with 100 ng different reporter plasmids (Pwt, E1wt, E2wt generated by Dr. Karoline Briegel, or TOPFlash, FOPFlash) and 300 ng each of pCDNA/β-catenin^{S37Y} expression plasmids using Lipofectamine 2000 reagent (Invitrogen). 25 ng of pRL-CMV (Promega) was used to normalize for transfection efficiency and pBluescript was added to equalize total DNA to 1.6 µg per transfection. Forty-eight hours post transfection, cells were harvested in passive lysis buffer and luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was normalized to both Renilla luciferase activity and protein concentration. The fold transactivation of each Lbh-luciferase construct represents the ratio between normalized luciferase values of β-catenin^{S37Y} co-transfected cells and cells transfected with the respective Lbh-luciferase constructs alone. For TOPFlash reporter assays, fold activation represents the ratio between normalized TOPFlash and FOPFlash activities. All transfections were performed in duplicates, and representative results of at least three independent experiments were statistically analyzed using a paired Student’s t-test.
2.2.4 Cell culture

*Wnt3a conditioned media*

L-Wnt3a cells were maintained in DMEM plus 400 µg/ml G481 in a T75 flask and were split 1:10 on T75 flask in media free of G418. Four days after splitting, when the cells had approximately reached confluence, conditioned media was collected and fresh media was applied to the cells. Three days later, a second round of conditioned media was collected. The two conditioned media collected were combined and filtered for use in induction experiments.

*Induction and inhibition studies*

For time course experiments, 293T cells were co-cultured with Wnt3a-conditioned medium for 0, 4, 8, 16 and 24 hours. Inhibition experiments used 100 ng/ml of recombinant human DKK1, Wnt5a, or Wnt7a (R&D Systems), which were added 8 hours prior to an 8-hour treatment of cells with Wnt3a-conditioned media unless noted otherwise. For all other experiments, cells were co-cultured with Wnt3a-conditioned medium for 16 hours unless noted otherwise.

*Knockdown by RNAi*

For RNAi studies, cells were transfected with 100 nM of synthetic siRNA specific for CTNNB1/β-catenin, LBH, or a scrambled control sequence using Dharmafect 1 reagent (Dharmacon). For the β-catenin studies, approximately 65 hours after siRNA transfection, 293T cells were trypsinized and transferred to a well with twice the surface area to allow for growth. 72 hours post-transfection, Wnt3a conditioned media was added for an additional 16 hours. For all other RNAi studies, the cells were incubated with the siRNA containing media for 72
hours prior to splitting for other studies. A portion of cells was always collected for checking knockdown efficiency. After harvesting the cells, total RNA was isolated using TRIzol® Reagent (Invitrogen) and treated with Turbo DNase (Ambion).

**shRNA lentiviral knockdown**

MDA-MB-231 cells were seeded at $1.6 \times 10^5$ cells per well on 12 well plates, and allowed to grow to a density of approximately 70% confluency. At the time of transduction, the estimated cell number was approximately $3.5 \times 10^5$ cells per well. Based on the titer of each shRNA clone, the equivalent of an MOI of 1 was applied to the cells after the addition of 8 $\mu$g/ml hexadimethrine bromide to increase transduction efficiency. The media was changed approximately 20 hours post-transduction, and the cells were collected for RNA and protein analysis or subject to selection 48 hours post-transduction. Optimal selective media conditions were determined by kill curve analysis, where the lowest dose of puromycin killed all cells within 5 days. Optimal concentrations: HCC1395 - 5 mg/ml, MDA-MB-231 and HCC1187 – 500 $\mu$g/ml.

**Generation of stable cell lines by nucleofection**

2x$10^6$ BT-549 or BT-20 cells were nucleofected with 2 $\mu$g linearized pCDNA3 or pCDNA3+NFlag-Lbh and 1 $\mu$g pEGFP (Lonza) in Solution V (Lonza), using program A-023 on an Amaxa nucleofector. pEGFP served as a transfection control and EGFP expression was monitored 20-36 hours post nucleofection on a Leica MZ 16 FA UV binocular microscope. 48 hours post nucleofection, growth media containing 350 $\mu$g/ml G518 was applied to the cells
to select for stably integrated transfectants overexpressing functionally active N-terminal FLAG tagged Lbh (N-Lbh) or vector alone. Three polyclonal sets of each stable line were established for BT-549, and one set was established for BT-20.

2.2.5 Quantitative Real-Time PCR (qPCR)

cDNA was synthesized from 1 µg DNase-treated total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) or MLV-RT (Promega). qPCR reactions were carried out in 20 µl using SYBR Green Master Mix (NEB) containing 10 nM of 6-carboxyfluorescein (Sigma) as a reference dye, 50 – 100 ng of cDNA and 2 µM primers. The reactions were performed in triplicates on a Biorad iCycler and quantified using the iCycler iQ software. Later experiments were performed on a BioRad CFX96 instrument. The relative quantities of the different mRNA were determined for each sample based on the $C_\text{t}$ value and normalized to the corresponding values of the housekeeping gene $GAPDH$.

2.2.6 Immunofluorescence

HC11 cells were grown overnight on BD Bioscience Culture Slides seeded at 2x10^5 cells per well and induced with Wnt3a conditioned medium for 6 hours. Other cell lines were seeded so as not to be greater than 70% confluent the next day. Cells were fixed with 2% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature, followed by cell permeabilization in 0.3% Triton X-100 in PBS. For fresh frozen embryo or tissue sections, tissues were fixed in 4% PFA in
PBS for 10 minutes, red blood cells were lysed with 0.2% H₂O₂ in PBS for 30 minutes, and slides were washed in PBT (0.2% TritonX-100, PBS) three times for five minutes each at room temperature. Cells were blocked for 1.5 hours at room temperature in PBS (or PBT at all steps for tissues) plus 10% Normal goat serum (NGS) in a humidified chamber. Antibodies were incubated for 1 hour at room temperature (β-catenin antibody (BD) – 1:200, Lbh 6th bleed - 1:1,000, Keratin 5 (Covance) - 1:5,000, Keratin 8/18 – 1:100) in 10% NGS followed by washing and a subsequent one hour incubation in the dark with the appropriate antibodies in 10% NGS (anti-mouse Cy3 - 1:400, anti-rabbit FITC – 1:200, anti-mouse or rabbit Alexafluor 488 or 594 - 1:500, anti-guinea pig Alexafluor 568 – 1:500). After sufficient washing, coverslips were mounted with Slowfade + DAPI (Molecular Probes) according to the manufacturer’s protocol. If no DAPI was included, Hoechst dye (Sigma) was included at a dilution of 1:10,000 in the first wash after the secondary antibody incubation to stain the nuclei. Images were taken on a Lyca Inverted Microscope located at the Diabetes Research Institute.

2.2.7 Chromatin immunoprecipitation

HC11 cells were grown to 70% confluence prior to addition of Wnt3a conditioned medium for 3 h. Cells were fixed in a final concentration of 1% formaldehyde for 10 min at room temperature followed by a quenching of fixation with 125 mM Glycine. Cells were incubated for 10 min on ice in swelling buffer (5 mM PIPES pH 8.0, 85 mM KCl, 1% NP-40) at a concentration of 5x10⁷ cells/ml followed by dounce homogenization 15 times. Nuclei were pelleted at
2,500 rpm for 5 min and resuspended in sonication buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) at a concentration of 1x10^8 cells/ml. Sonication of cells for 6 pulses of 15 sec each on ice/water at 50% power on a Misonix sonicator resulted in chromatin fragments of an average length of 1 kb. Lysates were cleared for 10 minutes at top speed. For each IP reaction, 1x10^7 cell equivalents were diluted to 1 ml total volume in dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.1 mM EDTA, 16.7 mM Tris-HCl pH 8, 167 mM NaCl) and pre-cleared for 2 h with 40 µl Protein A/G sepharose beads (GE Healthcare). Cleared lysates were incubated overnight with 5 µg of normal rabbit IgG, anti-acetyl Histone 3 or anti-β-catenin antibodies (Upstate). Thereafter, precipitation of immunocomplexes was performed according to the Upstate EZ ChIP protocol. PCR reactions for 35 cycles were carried out using Phusion polymerase (Finnzymes).

2.2.8 Protein analysis

*Tissue collection and isolation of mammary epithelial cells*

Mammary epithelial cells (MEC) from mammary glands of wild type mice were isolated via proteolytic digestion with 100 units/ml hyluronidase (Sigma) and 2 mg/ml collagenase A (Roche) in 15 ml DMEM for 3 hours at 37°C with gentle agitation followed by washing in DMEM plus 5% FBS. Tumors from *MMTV-Wnt1* transgenic mice were snap frozen in liquid nitrogen and mechanically pulverized. Isolated MEC and ground tumors were lysed in RIPA lysis buffer (20mM Tris pH7.5, 150mM NaCl, 1% NP-40, 0.5% Sodium
Deoxycholate, 1mM EDTA, 0.1% SDS) containing protease inhibitors (Amresco).

**Dephosphorylation analysis**

HCC1187 cells were lysed in modified RIPA lysis buffer (150 mM NaCl, 1% TritonX-100, 0.1% SDS, 10 mM Tris pH 7.5, 1% Sodium Deoxycholate, protease inhibitors (Sigma)). For dephosphorylation, 30 mg of total protein was incubated for 30 minutes at 37°C with 30 units (3 µl) of calf intestinal phosphatase (CIP, NEB) in a total volume of 20 ml of NEB Buffer 1. After the addition of 5 ml of 5x SDS sample buffer, the samples were boiled and separated on 20% SDS-PAGE to fully resolve differentially phosphorylated bands. Gels were transferred to nitrocellulose and analyzed by Western Blotting.

**Western Blot analysis**

For cell extracts, cells were either collected or lysed directly on the plate in SDS sample buffer (125 mM Tris-HCl pH6.8, 10% glycerol, 2% SDS, 0.0005% Bromophenol blue). Protein concentration was measured by BCA assay (Pierce). A total of 25 mg protein extract per sample was separated by SDS-PAGE, blotted on nitrocellulose membrane and incubated with the following antibodies in TBST (20 mM Tris HCl pH 7.5; 140 mM NaCl, 0.1% Tween 20) plus 5% non-fat dry milk: Lbh 6th bleed (1:10,000), Keratin 5 (Covance; 1:10,000), Keratin 8/18 (Progen, 1:2,000), CD24 (Santa Cruz, 1:200), ERα (Santa Cruz, 1:1,000), and β-actin (Sigma, 1: 50,000), and anti-rabbit, anti-mouse, or anti-guinea pig HRP-coupled secondary antibodies (1:25,000). Signal was detected using the West Fempto Super Signal kit (Pierce).
2.2.9 Proliferation Assay

2000 cells were seeded in triplicate on 96 well plates. Two hours post-plating, 20 ml of CellTiter 96 AQ
ueous One Cell Proliferation Assay reagent (Promega) was added to the cells and blank media controls. Two hours later, the absorbance was measured at 492nm. Reagent was applied around the same time daily and absorbance was measured two hours later for 5 additional days.

2.2.10 Fluorescence associated cell sorting (FACS) analysis

Cells were collected 6 or 9 days after siRNA transfection. A minimum of $2 \times 10^5$ and a maximum of $1 \times 10^6$ cells were resuspended in 100 ml FACS buffer (PBS, 2% FBS, 0.1% Sodium Azide), and incubated for 20 minutes in the dark with 20 µl CD44-APC and CD24-PE (BD Pharmingen). Cells were rinsed in 3 ml FACS buffer and resuspended in a final volume of 500 µl FACS buffer for analysis. Unstained samples were used to calibrate the analyzer for each experiment. FACS analysis was performed on a FACS Calibur II (BD) flow cytometric analyzer at the University of Miami Sylvester Cancer Center Flow Cytometry Core Facility.

2.2.11 Colony Formation

1 ml of 0.6% Noble Agar (BD) was used as the bottom layer in a 35 cm dish and allowed to solidify at least 30 minutes prior to adding the top layer of 0.3% noble agar mixed with $5 \times 10^4$ cells. Agar was mixed with proper growth media and contained 350 ng/ml G418 for stable cell lines. All lines were plated in triplicate, fed 40 ml growth media every 2 days, and allowed to grow for 21-28
days. Cells were stained with 0.005% crystal violet for at least 2 hours prior to taking images on a light box with a 3.4 or 12 megapixel digital camera. Colonies were counted if they were larger than 8 megapixels at 100% magnification on Adobe Photoshop.

2.2.12 Tumorsphere formation assay

2x10⁴ cells were seeded on 6 well ultra-low adherence plates (Corning) in normal growth media containing 4 µg/ml heparin and 0.48 µg/ml hydrocortisone. Tumorsphere formation was monitored for 7 days, after which, spheres larger than 10 cells were counted. In attempts to generate secondary tumorspheres, primary spheres were dissociated in 50 µl 0.025% trypsin-EDTA for 5 minutes at 37 °C, stopping trypsinization by the additon of 200 µl growth media, and mechanical separation of the spheres by pipetting 100 times. Cells were re-counted and re-plated on ultra-low adherence plates.

2.2.13 Apoptosis assay

Approximately 2x10⁵ cells were stained using the Vybrant Apoptosis Assay Kit #2 (Molecular Probes) according to the manufacturer’s protocol. Samples were analyzed on an LSR-I flow cytometric analyzer (BD) at the Sylvester Cancer Center Flow Cytometry Core Facility.

2.2.14 Scratch Test

Three days post-transfection with siRNA treatment, a single scratch was created across the confluent monolayer of cells. Size of the scratch was
monitored every 4 or 24 hours for MDA-MB-231 or HCC1187, respectively. Images were taken at 3 different parts of the scratch at each time point. The size of the scratch (gap width) was determined by averaging the distance measured in pixels between the two cell fronts on photoshop.

2.2.15 3D collagen differentiation assay

Rat tail collagen I (BD) was neutralized with 1N NaOH in PBS and mixed to a concentration of 2 mg/ml on ice. HC11 cells were trypsinized and re-suspended to a concentration of 5×10⁴ cells/ml in growth media. Quickly, 400 µl of cells were mixed in equal volume with collagen solution yielding a final concentration of 25 cells/µl in 1 mg/ml collagen I. 100 µl of the mixture was quickly added to the center of the well in triplicates on 96 well plates and allowed to solidify at room temperature for 10 minutes. Subsequent incubation at 37°C for 30 minutes further solidified the mixture prior to the addition of 100 µl growth media. Media was changed every two days.

2.2.16 In silico analysis

Multiple alignments were performed using Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/index.html). In silico phosphorylation site prediction was performed by Scansite (Yaffe, 2003), and Disorder Enhanced Phosphorylation Predictor (DEPP) from Molecular Kinetics (Iakoucheva et al., 2004). PONDER disorder prediction was also from Molecular Kinetics (Li et al., 1999; Romero et al., 2001). ELM database and glycosylation predictors were
accessed through ExPASy tools (http://expasy.org/tools/). Micro RNA predictions were performed using TargetScan 5.1 software (http://www.targetscan.org/).
Chapter 3: The Embryonic Transcription Cofactor LBH is a Target of the WNT Signaling Pathway in Epithelial Development and in Aggressive Basal Subtype Breast Cancers.

*A version of this chapter is in press at Molecular and Cellular Biology*
3.1 Background

Increasing evidence suggests that embryonic development and tumorigenesis share some of the same molecular mechanisms. In particular, aberrant reactivation of latent developmental signaling pathways and transcription factors in tumor cells has been associated with, and shown to play causal roles in advanced-stage, invasive cancers (Ben-Porath et al., 2008; Briegel, 2006; Yang and Weinberg, 2008).

Limb-bud and heart (Lbh) is a highly conserved, novel tissue-specific transcription cofactor in vertebrates with important roles in embryonic development (Briegel et al., 2005; Briegel and Joyner, 2001; Conen et al., 2009). We have previously identified Lbh as a novel mouse gene with a unique spatio-temporal expression during early embryogenesis that reflects pattern formation in the developing limb buds and heart (Briegel and Joyner, 2001). Lbh encodes a small acidic protein (MW = 12.3 kDa) that contains a conserved putative nuclear localization signal and a glutamate-rich putative transcriptional activation domain, but lacks a DNA binding domain (Briegel and Joyner, 2001). In mammalian reporter assays, Lbh has both transcriptional activator and co-repressor function (Briegel et al., 2005; Briegel and Joyner, 2001). Recent biophysical analysis has revealed a high degree of structural disorder in Lbh, suggesting that conformational plasticity may play a significant role in modulating Lbh-dependent transcriptional processes (Al-Ali et al., 2009).

Aberrant gain of LBH function is associated with partial trisomy 2p syndrome (Briegel et al., 2005), a rare human autosomal disorder that is characterized by multiple congenital anomalies, including cardiovascular, skeletal
and postaxial limb defects (Lurie et al., 1995). Partial trisomy 2p syndrome patients harbor a triplication of chromosomal region 2p23, where \( LBH \) maps, indicating that increased \( LBH \) gene dosage is pathologic in humans (Briegel et al., 2005). Transgenic misexpression of Lbh during embryonic heart development in mice phenocopies congenital heart disease observed in these patients (Briegel et al., 2005), and indicates that Lbh functions to attenuate cardiac chamber differentiation due to co-repression of key cardiac transcription factors Nkx2.5 and Tbx5 (Briegel et al., 2005). Interestingly, gain-of-function of Lbh during mouse heart development also causes various growth defects, such as ventricular hyperplasia, increased cardiac valve formation, as well as the abnormally sustained self-renewal of cardiomyocytes after birth, suggesting that Lbh has pro-mitogenic activity (Briegel et al., 2005). Consistent with these findings, retroviral overexpression of Lbh in chick embryonic limbs leads to increased cell proliferation of immature chondrocytes and markedly delays bone development (Conen et al., 2009). However, the regulatory pathways acting upstream of Lbh and its role in adult development have remained obscure.

Wnt signaling plays a fundamental role in embryonic development by regulating pattern formation, cell proliferation, differentiation and migration (Logan and Nusse, 2004). Wnt ligands are secreted lipid-modified glycoproteins that act as morphogens and elicit different cell behaviors depending on whether receptor interaction activates a canonical \( \beta \)-catenin-dependent transduction pathway, or other \( \beta \)-catenin-independent noncanonical pathways (Komiya and Habas, 2008; van Amerongen et al., 2008). In adults, canonical Wnt signaling
promotes the self-renewal and maintenance of stem cells required for normal tissue homeostasis (Nusse et al., 2008), a function that becomes oncogenic when this pathway is deregulated (Clevers, 2006). Activation of canonical Wnt signaling leads to the stabilization of cytoplasmic β-catenin and its subsequent translocation into the nucleus, where it forms a heteromeric complex with DNA-binding proteins of the T cell factor (TCF)/Lymphoid enhancer-binding factor (Lef) family to activate target gene transcription (Clevers, 2006). In the absence of Wnt, TCF/Lef factors are bound to target gene promoters, but act as transcriptional repressors by forming a complex with Groucho/Grh/TLE corepressors (Clevers, 2006). The amplitude of canonical Wnt signaling is autoregulated via multiple positive and negative feedback mechanisms that include TCF/Lef factors themselves and the secreted antagonist Dickkopf 1 (Dkk1), respectively (Chamorro et al., 2005; Clevers, 2006; Gonzalez-Sancho et al., 2005; Niida et al., 2004). In addition, Wnt ligands can activate multiple non-canonical signaling pathways, including the planar cell polarity (PCP), Wnt/Ca2⁺, and, in the case of Wnt7a in limb development, a pathway involving the homeodomain transcription factor Lmx1b (Kengaku et al., 1998b; Semenov et al., 2007).

Genetic studies in mice first indicated that canonical Wnt signaling is oncogenic and implicated in breast tumorigenesis (Imbert et al., 2001; Nusse and Varmus, 1982; Teuliere et al., 2005; Tsukamoto et al., 1988). Abnormal activation of the canonical WNT pathway is also associated with human breast cancer (Lin et al., 2000) and is most common in a highly aggressive subtype of
breast cancers known as basal carcinomas (Dimeo et al., 2009; Hayes et al., 2008; Khramtsov et al., 2010; Smid et al., 2008). This tumor subtype accounts for 15-20% of breast cancers and is characterized by early onset and a highly invasive, poorly differentiated tumor phenotype. Basal-like breast cancers have a poor clinical outcome and represent a challenge for therapeutic intervention due to their lack of expression of the therapeutic targets estrogen receptor (ER), progesterone receptor (PR) and the ERBB2 oncogene (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003), thus emphasizing the need for identification of new tumor-specific markers.

The spatio-temporal expression pattern of \( Lbh \) during mouse embryogenesis (Briegel and Joyner, 2001) led us to hypothesize that \( Lbh \) may be controlled by morphogenic signaling pathways that orchestrate cell specification and pattern formation. Using a combination of molecular, mammalian tissue culture, mouse genetics and \textit{in silico} analyses, we set out to identify the molecular pathways operating upstream of \( Lbh \). In doing so, we discovered that \( Lbh \) expression is tightly controlled by an antagonistic relationship between canonical Wnt/\( \beta \)-catenin and non-canonical Wnt7a signaling in epithelial development. Whereas \( Lbh \) is induced by Wnt/\( \beta \)-catenin via four conserved TCF/LEF binding sites in the \( Lbh \) gene locus, this induction is efficiently blocked by Wnt7a signaling. Given the parallels between Wnt signaling in development and cancer, we hypothesized that \( LBH \), as a canonical Wnt target gene, might be deregulated in breast cancer. Indeed, we found that Lbh is aberrantly overexpressed in mammary tumors of \textit{MMTV-Wnt1} transgenic mice as well as in highly aggressive
basal-subtype human breast cancers. Overexpression of Lbh in HC11 cells further demonstrates that Lbh suppresses terminal mammary epithelial cell differentiation, an effect that could contribute to Wnt-induced tumorigenesis. Collectively, our data suggest that Lbh is a direct Wnt target gene that is reactivated in a particularly lethal form of human breast cancer.

3.2 Identification of functional Wnt-responsive elements in the Lbh gene locus

To elucidate the molecular pathways acting upstream of Lbh, we screened murine Lbh genomic sequences for potential transcription factor binding sites. This in silico search identified four conserved putative TCF/LEF-binding elements (TBEs) in the Lbh gene locus. Two TBEs with the consensus motif 5’-CTTTG(A/T)(A/T)-3’ (van de Wetering et al., 1991) were located within an enhancer region (E1) -6245 (T1) and -6195 (T2) base pairs (bp) upstream of the Lbh transcriptional start site (Figure 3.1A). In addition, two consensus TBEs were found in an enhancer (E2) contained within the first intron of the Lbh gene at positions +1558 (T3) and +2145 (T4) bp (Figure 3.1A). To directly assay for TCF binding to these sites, electrophoretic mobility shift analysis (EMSA) was performed. Recombinant TCF4 protein bound with high affinity to all Lbh-specific TBEs (T1 - T4), but not to an unspecific oligonucleotide (Figure 3.1B). TCF4 binding to these sites was efficiently competed by addition of 400-fold excess of unlabeled wild-type (+) oligonucleotide as well as increasing amounts of an antibody against recombinant TCF4 protein, but not by addition of 400-fold excess of mutant (m) oligonucleotide (Figure 3.1B, C).
Subsequently, cell-based reporter assays were performed to test whether the *Lbh* gene-specific TBE sites (T1-T4) were functionally responsive to overexpression of β-catenin, which is the Wnt-inducible component of the TCF/β-catenin transcriptional complex. HC11 mouse mammary epithelial cells were used, because this cell line abundantly expresses TCF4, but has low endogenous Wnt/β-catenin signaling activity (Civenni et al., 2003). The *Lbh* enhancer regions E1 and E2 were cloned by Dr. Briegel individually into a promoter-luciferase construct (Pwt) upstream of approximately 1.5 kb of murine *Lbh* gene promoter sequences that do not contain any apparent consensus binding sites for TCF/β-catenin. The three *Lbh*-Luciferase reporter constructs (Pwt, E1wt and E2wt) were co-transfected with a pCDNA plasmid vector expressing constitutively active β-catenin (β-catenin\textsuperscript{S37Y}). As shown in Figure 3.1D, *Lbh*-luciferase constructs containing wild-type TBE sites (E1wt, E2wt) were induced by β-catenin\textsuperscript{S37Y} approximately 14 – 18 fold. The basal *Lbh* promoter-Luciferase construct (Pwt) also showed transcriptional activation despite the lack of TBEs, indicating that β-catenin may also have indirect effects on *Lbh* promoter activity. Most importantly, however, mutations of both T1 and T2 together (E1t1-2), or of T3 and T4 either individually or in combination (E2t3, E2t4 and E2t3-4) significantly reduced (2.5 - 3 fold; \(p<0.02\)) transcriptional activation of Lbh reporters by β-catenin\textsuperscript{S37Y} (Figure 3.1D). Mutation of either T1 or T2 alone had little effect, suggesting that binding of a β-catenin/TCF4 transcriptional complex
Figure 3.1: Identification of functional TCF-binding elements (TBEs) in the mouse Lbh gene locus. (A) Schematic of genomic Lbh promoter/enhancer sequences in the 5’ upstream region and the first intron between exon 1 and 2 (black boxes). The positions of four putative conserved TBE sites (T1 – T4; grey boxes) predicted by MatInspector (Genomatix) and/or rVista (http://rvista.dcode.org) computer software are shown in relationship to the transcriptional start site (+1). Sequences of T1 – T4 in comparison with the TBE consensus site are indicated. S = sense strand; AS = antisense strand. (B) Electrophoretic mobility shift assay (EMSA) showing high affinity binding of recombinant His-tagged TCF4 protein to T1-T4. In oligonucleotide competition experiments, no competitor oligonucleotide (-), 400-fold excess of unlabeled Wild-type TBE oligonucleotide (+), or mutant TBE oligonucleotide (m) was added to gel shift reactions. Migration of free probe (brackets) and of TCF4 protein-DNA complexes (solid and open arrows) is indicated. (C) Supershift of His-TCF4 binding to the T3 (+1558) site with 1-5 µg of anti-Histidine antibody (α-His). (D) Transient reporter assays in HC11 mouse mammary epithelial cells. Luciferase (Luc) reporter constructs containing different murine Lbh promoter/enhancer sequences (P, E1, E2) with wild-type (wt) and mutant TBEs (t1 - t4) as shown schematically were co-transfected with a pCDNA construct expressing constitutively active β-catenin (β-cateninS37T). Values represent relative fold increase of transcriptional activation for each construct (see Materials and Methods). * P < 0.02.
to only one of these sites is sufficient for activity of this enhancer (E1). These data suggest that \textit{Lbh} is activated by the canonical Wnt pathway at the transcriptional level via high-affinity TCF-binding elements located within upstream and intronic enhancer regions of the \textit{Lbh} gene.

3.3 Expression of endogenous LBH is upregulated by canonical Wnt signaling in 293T cells

To further test whether \textit{LBH} is a \textit{ bona fide } Wnt/\(\beta\)-catenin target gene, we examined whether endogenous \textit{LBH} mRNA expression was responsive to Wnt. Human 293T embryonic kidney epithelial cells were co-cultured with Wnt3a conditioned medium (hereafter referred to as Wnt3a), and mRNA levels of \textit{LBH}, as well as of a known Wnt target gene, \textit{DKK1} (Chamorro et al., 2005; Gonzalez-Sancho et al., 2005; Niida et al., 2004), were assayed over a 24 hour time course using quantitative realtime-PCR (qPCR) analysis. Induction of \textit{LBH} was detectable within 4 h of Wnt3a treatment and reached a maximum at 16 h (> 4 fold increase; Figure 3.2A). \textit{DKK1} was induced to a smaller degree and its induction was delayed as compared to \textit{LBH} (Figure 3.2A). Induction of both \textit{LBH} and \textit{DKK1} mRNA expression by Wnt3a was efficiently blocked by recombinant DKK1 protein (Figure 3.2A, C), a potent inhibitor of canonical Wnt/\(\beta\)-catenin signaling (Niehrs, 2006). Moreover, Wnt3a-mediated induction of \textit{LBH} and \textit{DKK1} was abrogated by depletion of \(\beta\)-catenin expression using RNAi, while scrambled control siRNA had no effect (Figure 3.2B). These results reinforce the notion that \textit{LBH} is a direct transcriptional target of the canonical Wnt signaling pathway.
To investigate whether Wnt ligands that signal through non-canonical pathways could also induce *LBH* gene expression, 293T cells were treated for 16 h with recombinant Wnt5a or Wnt7a (Figure 3.2C). In contrast to Wnt3a, both Wnt5a and Wnt7a treatment alone did not induce *LBH*, but modestly reduced baseline *LBH* and *DKK1* expression (Figure 3.2C). Since Wnt5a has previously been shown to inhibit Wnt3a-induced canonical Wnt signaling (Mikels and...
Nusse, 2006), we also examined LBH gene expression in cells treated with both Wnt3a and the individual non-canonical Wnt ligands. Surprisingly, Wnt7a strongly inhibited LBH and DKK1 induction by Wnt3a, whereas Wnt5a failed to block Wnt3a-mediated induction of these genes (Figure 3.2C). Thus, LBH is specifically induced by canonical Wnt signaling, whereas non-canonical Wnt7a signaling has an antagonistic effect on LBH expression and its induction by Wnt3a.

### 3.4 Lbh is downstream of Wnt signaling in mouse embryonic limb development

As Lbh was identified as a developmental gene (Briegel and Joyner, 2001), we next examined whether Wnt signaling might play a role in regulating Lbh expression during vertebrate embryonic development. We have previously shown that during early mouse limb development Lbh is expressed in the Apical Ectodermal Ridge (AER) and in a ventral pattern in non-AER limb bud ectoderm (Briegel and Joyner, 2001). These ectodermal domains provide the cues for proximo-distal and ventral limb patterning, respectively (Chen and Johnson, 1999; Tickle, 1999), and have recently been shown to be patterned by canonical Wnt/β-catenin signaling in concert with the BMP pathway (Figure 3.3A; Barrow et al., 2003; Soshnikova et al., 2003). Conversely, Wnt7a, which is expressed and secreted from dorsal limb ectoderm, induces dorsal limb differentiation through a non-canonical pathway involving Lmx1b upregulation in the underlying dorsal limb mesenchyme (Figure 3.3A, Bi; Kengaku et al., 1998; Parr and McMahon,
Thus, we reexamined *Lbh* expression in relationship to the different Wnt signaling activities during crucial stages of mouse embryonic limb development.

As shown in Figure 3.3Bi, *Lbh* expression was exclusively confined to the ventral limb and AER ectoderm of embryonic day 10.5 (E10.5) wild type embryos as revealed by whole mount *in situ* RNA hybridization analysis performed by Dr. Karoline Briegel. Similarly, canonical Wnt activity was
detected predominantly in the AER and ventral limb ectoderm, as evident by lacZ expression in TopGal embryonic limbs at the same stage, although some cells in the most distal dorsal ectoderm also expressed lacZ (Figure 3.3Bii). Moreover, genes regulated by canonical Wnt signaling, such as Fgf8 and En1 (Kengaku et al., 1998), were exclusively restricted to the AER and/or ventral limb ectoderm, respectively, and overlapped with Lbh expression in these ectodermal domains (Figure 3.3Bi, iv, v). The pattern of Lbh expression in embryonic limb ectodermal cells is also similar to that described for other Wnt/β-catenin target genes, including Dkk1 and axin2, which are expressed in the AER (Adamska et al., 2004; Soshnikova et al., 2003). In contrast, expression of Lbh, as well as of En1 and FGF8, was mutually exclusive with the Wnt7a expression domain in dorsal limb ectoderm (Figure 3.3Bi, iii-vi).

The complementary expression patterns of Wnt7a and Lbh (Figure 3.3iii and vi), as well as repression of LBH by Wnt7a in tissue culture cells (Figure 3.2C), prompted us to test potential Wnt7a effects on Lbh expression in vivo. Lbh expression was examined in mouse mutants, in which the non-canonical Wnt7a pathway was rendered inactive by gene targeting (Chen et al., 1998; Parr and McMahon, 1995). Remarkably, in Wnt7a−/− mutant mice and animals lacking the Wnt7a downstream transcriptional regulator Lmx1b, Lbh was ectopically expressed in the distal dorsal limb ectoderm (Figure 3.3Cii and iii). In contrast, Lbh exhibited a normal ventral expression pattern in wild-type littermates (Figure 3.3Ci). Taken together, these developmental genetic studies provide the first
functional evidence that Lbh expression in vivo coincides with canonical Wnt signaling activity and that it is repressed by non-canonical Wnt7a-Lmx1b signaling during embryonic limb development.

3.5 Lbh is expressed in postnatal mammary gland development and levels are elevated in mammary tumors of MMTV-Wnt1 transgenic mice

To test our hypothesis that LBH might be implicated in Wnt-induced tumorigenesis, we examined Lbh expression in MMTV-Wnt1 transgenic mice, a mouse model for Wnt-induced breast cancer (Figure 3.4; Tsukamoto et al., 1988)). Moreover, since the expression pattern of Lbh in normal adult breast tissue was not known, we analyzed Lbh expression during postnatal mouse mammary gland development using RNA in situ hybridization and Western blot analyses.

In post-pubertal (7 weeks) virgin female mammary glands, expression of Lbh was restricted to stromal, basal-myoeipithelial, and terminal end bud (TEB) mammary epithelial cells (Figure 3.4A; data not shown). In contrast, Lbh was absent from ductal luminal mammary epithelial cells at all postnatal development stages analyzed (Figure 3.4A). During pregnancy, Lbh levels drastically increased and Lbh transcripts were primarily detected in the proliferating lobuloalveolar compartment, a pattern that was maintained during early involution (Figure 3.4A, B). Notably, Lbh expression was virtually absent in lactating mammary glands, suggesting that Lbh is not expressed in terminally differentiated secretory mammary epithelial cells (Figure 3.4A, B). Most remarkably, Lbh expression levels were significantly elevated (2.8 - 4.2 fold) in 9 out of 10 mammary tumors
from different *MMTV-Wnt1* transgenic mice as compared to non-pregnant mammary glands, HC11 cells, and mammary epithelial cells isolated from equiparous wild-type littermates (Figure 3.4A-D; and data not shown). Moreover, in *MMTV-Wnt1* tumors, Lbh expression correlated with expression of basal
myoepithelial marker Keratin 5, whereas it inversely correlated with expression of the luminal markers Keratin 8/18 (Figure 3.4C). Thus, Lbh is expressed at normal levels in basal and proliferative alveolar mammary epithelial cells during normal mammary gland development, whereas it is overexpressed in Wnt-induced breast epithelial tumors.

3.6 Lbh overexpression suppresses the differentiation of HC11 mammary epithelial cells

As we found Lbh expression specifically in cellular targets of canonical Wnt signaling during normal mammary gland tissue homeostasis (Brisken et al., 2000; Teuliere et al., 2005; Turashvili et al., 2006) and that Lbh is upregulated in Wnt-induced mammary tumors (Figure 3.4), we further investigated the functional relationship between Wnt/β-catenin signaling and Lbh in a cell culture system for mammary epithelial development. HC11 was chosen, because it is one of few existing non-transformed mammary epithelial cell lines that can be induced to differentiate in vitro upon induction with lactogenic hormones (Ball et al., 1988). Moreover, overexpression of different Wnt ligands has been shown to lead to cellular transformation of these cells (Howe et al., 2003; Humphreys and Rosen, 1997). To test whether Lbh could be downstream of canonical Wnt signaling in mammary epithelial cells, we treated HC11 cells, which do not express Lbh (Figure 3.4), with Wnt3a. As shown in Figure 3.5, Wnt3a treatment resulted in nuclear localization of β-catenin as well as a rapid increase in Lbh mRNA levels (Figure 3.5A, B). In addition, ChIP analysis showed that the Lbh gene regulatory sequences T1-T4 (Figure 3.1A) were occupied by endogenous β-
catenin in Wnt3a-treated cells, but not in untreated control cells (Figure 3.5C; data not shown).

Having demonstrated that Lbh is a direct transcriptional target of Wnt/β-catenin in HC11 cells, we asked whether overexpression of Lbh elicits some of the same effects that have been reported for overexpression of Wnt ligands in this cell line. Several polyclonal HC11 cell lines stably expressing Lbh (Lbh c1 and c2) were generated by transfection with a pCDNA3-Lbh plasmid, and Lbh
overexpression was confirmed by qPCR and Western Blot analysis (Figure 3.5D). No Lbh expression was detectable in vector control transfected, or in the parental HC11 cells (Figure 3.4D, 3.5D). Although ectopic Lbh expression did not result in cell transformation as determined by soft agar assays (data not shown), the growth rates of Lbh-expressing HC11 cells were significantly increased as compared to vector control cells (Figure 3.5E). Moreover, whereas differentiation induction with prolactin and dexamethasone induced mRNA expression of the milk protein β-casein in parental and vector control cells, induction of β-casein in response to these lactogenic hormones was completely absent in HC11-Lbh cells (Figure 3.5F). Thus, overexpression of Lbh promotes cell proliferation and blocks terminal differentiation of HC11 mammary epithelial cells.

3.7 LBH is over-expressed in highly invasive ER-negative, basal subtype human breast cancers

To further examine whether LBH might be deregulated in human breast cancer, in collaboration with Dr. Andrew Sims we performed meta-analysis of six Affymetrix gene expression datasets comprising 1107 primary human breast cancers was performed as previously described (Sims et al., 2008). These data represent the five ‘intrinsic’ breast tumor subtypes Normal-like, Luminal A, Luminal B, ERBB2-positive and Basal-like, which can be distinguished by specific gene signatures and differences in clinical outcome, with Basal-like breast cancers having the worst prognosis (Figure 3.6; Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003). Strikingly, LBH expression was significantly
Figure 3.6: LBH gene expression in human breast cancer correlates with ER-negative, basal-like tumor subtype. (A) Meta-analysis of 1107 human primary breast carcinoma samples from six published Affymetrix datasets (Sims et al., 2008) showing a strong positive correlation of LBH with basal tumor type, as well as expression of Keratin 5 (KRT5) and Wnt pathway genes SFRP1, TCF7, TCF4, and DKK3. In contrast, the LBH signature inversely correlates with Estrogen Receptor (ESR1) expression. Clustering of tumor subtypes: basal (red), ERBB2 (purple), Luminal A (dark blue), Luminal B (light blue) and Normal-like (green) was according to (Perou et al., 2000). Red = high expression, green = low expression. (B) Table showing the proportion of breast cancer specimen with high level (overall upper quartile) of LBH mRNA expression in individual tumor subtypes. Chi squared test $p=0.0000014$. (C) Dot plots demonstrating correlations between LBH expression and clinical markers (Top panel) and Wnt pathway genes (Lower panel). Pearson correlation coefficients (R values) between LBH expression and other genes are listed. LBH inversely correlates with estrogen receptor (ESR1) gene expression, has no correlation with ERBB2, and positively correlates with expression of basal Keratin 5 (KRT5). LBH expression also positively correlates with expression of Wnt pathway genes that are also targets of canonical Wnt signaling, including Secreted Frizzled-Related Protein 1 (SFRP1), TCF7, TCF4, and Dickkopf 3 (DKK3). All R values are statistically significant ($p<0.0001$) with the exception of ERBB2. These data were generated by Dr. Andrew Sims.
associated with aggressive, poorly differentiated basal-type carcinomas. Almost half (45%) of the basal breast tumors showed high LBH expression levels (Figure 3.6B). In contrast, elevated LBH was observed in far smaller proportions of Normal-like (24%), Luminal A (16%), Luminal B (23%) and ERBB2+ (27%) breast carcinomas (Figure 3.6B). Moreover, a strong inverse correlation between LBH expression and expression of the estrogen receptor alpha (ESR1) gene was observed in all five probe sets (Figure 3.6A, Figure S1; R = -0.14 to -0.29, p<0.0001), whereas no significant correlation existed with ERBB2 status (Figure 3.6C; R = -0.01). Most remarkably, however, LBH expression in breast tumors was strongly correlated with basal Keratin 5 and canonical Wnt pathway genes, such as SFRP1, TCF4, TCF7 and DKK3 (Figure 3.6A, C; p<0.0001). These data highlight LBH as a novel molecular marker for difficult-to-treat ER-negative basal-type breast cancer and suggest that LBH deregulation in breast cancer could be a consequence of oncogenic Wnt signaling.

The lack of suitable antibodies currently precludes analysis of LBH protein expression in clinical specimens. Therefore, we analyzed human breast carcinoma cell lines to validate our findings. We first queried published microarray gene expression data from 51 different human breast cancer cell lines (Neve et al., 2006) to confirm the existence of a relationship between LBH expression and breast cancer subtype in these tumor cell lines. As shown in Figure 3.7A, LBH expression was significantly higher in both Basal A and Basal B breast carcinoma compared to Luminal tumor cell lines (p=0.009; Figure 3.7A). Specifically, 50% of Basal A (n=12) and 29% of Basal B (n=14) breast
cancer, whereas only 12% of Luminal tumor cell lines (Luminal A and Luminal B combined; n = 25) expressed LBH levels in the upper quartile (Figure 3.7A; data not shown).

We next examined LBH expression in a panel of 13 established human breast cancer cell lines using qPCR and Western Blot analysis. High levels of LBH expression were only detected in the ER-negative basal subtype breast tumor cell lines HCC1395, MDA-MB-231 and HCC1187 (Figure 3.7B, D). In contrast, none of the ER-positive lines (MCF7, T47D, ZR-75-1, MDA-MB-361) or ER-negative (SK-BR-3) luminal cell lines, expressed LBH at detectable levels (Figure 7B, D). Furthermore, LBH protein was not detected in finite lifespan human mammary epithelial cells (HMEC) or in non-malignant MCF10A cells (Figure 3.7B, D). Thus, consistent with our gene expression analysis in primary breast tumors, LBH expression in breast cancer-derived cell lines correlated with invasive basal carcinoma phenotype and inversely correlated with expression of the good prognostic marker ER.

3.8 LBH deregulation in breast cancer may be due to aberrant Wnt/β-catenin pathway activation

To begin to investigate the mechanisms underlying LBH deregulation in breast cancer, we queried comparative genomic hybridization array (aCGH) data that were available for these breast tumor cell lines. Only one of three LBH-overexpressing basal tumor cell lines (HCC1395) had a modest increase in LBH copy number (Figure 3.7C). Moreover, aCGH analysis of primary breast tumor
Figure 3.7: Validation of LBH expression in human breast tumor cell lines. (A) LBH mRNA expression is significantly higher in basal rather than luminal breast carcinoma cell lines classified according to tumor subtype. Values represent the mean and error bars the standard error. (n) = number of samples per tumor subtype. (B) qPCR analysis of relative LBH mRNA expression in a panel of human breast tumor cell lines showing overexpression of LBH in HCC1395, MDA-MB-231 and HCC1187 tumor cells. Cell lines are arranged by tumor subtype. All measurements were performed in triplicate and expression levels were normalized to mRNA levels of GAPDH. (C) Comparative genomic hybridization array (aCGH) analysis of the same breast tumor cell lines as in (B). (D) Western blot analysis detecting expression of LBH protein exclusively in invasive ER-negative basal-type breast cancer lines, but not in two non-transformed (normal) mammary epithelial cell lines or in low-invasive breast tumor cell lines. β-actin was used as a loading control. (E) TOPFlash reporter assay detects Wnt signaling activity in LBH-expressing HCC1395 and HCC1187 cells, but not in MDA-MB-231 cells. HC11 and HC11 transiently transfected with pCDNA3/β-cateninE373K were used as negative and positive controls, respectively. Values represent the mean ratio of TOPFlash over FOPFlash activity ± SD (F) Administration of recombinant DKK1 and Wnt7a (100 µg/ml) for the indicated time points strongly inhibits LBH and DKK1 mRNA expression in HCC1395 cells as revealed by qPCR analysis. Values represent mean ± SEM (n=3). Data shown in (A) and (C) were generated by Dr. Andrew Sims.
data sets did not show a significant correlation between increased \textit{LBH} copy number and LBH overexpression in basal subtype tumors (data not shown), suggesting that changes in \textit{LBH} gene dosage play a minor role in LBH dysregulation in basal breast carcinomas. To further test whether LBH overexpression may be a consequence of aberrant Wnt signaling, we measured endogenous Wnt signaling activity in LBH-positive breast tumor cell lines using TOPFlash reporter assays. Strikingly, 2 out of 3 of these cell lines (HCC1187 and HCC1395) displayed increased Wnt/\(\beta\)-catenin signaling activity similar to HC11 cells transfected with pCDNA/\(\beta\)-catenin\(^{S37Y}\) (Figure 3.7E). In contrast, no detectable Wnt activity was measured in MDA-MB-231 cells, or in HC11 cells, which served as a negative control. Furthermore, treatment of HCC1395 cells with DKK1 inhibitor blocked \textit{LBH} expression, indicating that expression of \textit{LBH} in this breast tumor cell line is dependent on Wnt/\(\beta\)-catenin signaling (Figure 3.7F). Finally, we explored whether Wnt7a could serve as a means to inhibit \textit{LBH} expression in basal breast tumor cells. Remarkably, treatment of HCC1395 cells with Wnt7a efficiently suppressed mRNA expression of \textit{LBH} as well as of \textit{DKK1} (Figure 3.7F). Thus, aberrant canonical Wnt signaling, at least in part, is responsible for LBH overexpression in basal subtype breast carcinoma cells.

\textbf{3.9 Discussion}

Previous overexpression studies in mice and chick have clearly defined a pivotal role of the novel vertebrate transcriptional regulator Lbh in embryonic development and congenital disease (Briegel et al., 2005; Briegel and Joyner,
2001; Conen et al., 2009). However, the molecular mechanisms upstream of Lbh and its role in adult development have remained obscure. The present study links Lbh for the first time to the Wnt signaling pathway, an essential developmental and oncogenic signaling network, as well as implicates LBH overexpression in breast cancer.

We demonstrate through cell-based induction, RNAi-knockdown of β-catenin, and reporter assays, as well as through molecular in vitro EMSA and in vivo ChIP analysis that Lbh is a direct transcriptional target of the canonical Wnt/β-catenin pathway. The placement of LBH downstream of Wnt appears to be ‘universal’ in mammalian epithelial development, as it was observed in both embryonic (293T, limb ectoderm) and adult epithelial systems (postnatal mammary gland, HC11 cells), as well as in epithelial neoplasia (MMTV-Wnt1 mammary tumors, human breast cancer cell lines). Such an intimate relationship between Wnt/β-catenin and other target embryonic transcription factors does not always exist. For instance, TWIST, a transcriptional regulator of mesenchymal cell fate and inducer of epithelial-mesenchymal transition (EMT) during breast metastasis, is upregulated in mouse Wnt-induced breast tumors (Dimeo et al., 2009; Howe et al., 2003; Yang et al., 2004), but unlike Lbh is not expressed in normal epithelial development. Interestingly, the Lbh expression pattern not only overlapped with Wnt/β-catenin signaling activity during embryonic limb development, but also with the expression patterns of several Wnt ligands (Brisken et al., 2000; Buhler et al., 1993; Gavin and McMahon, 1992) during postnatal mammary gland development. Wnt has been shown to stimulate the
growth of TEB cells during mammary gland branching morphogenesis, the expansion of the lobuloalveolar compartment during pregnancy and the self-renewal of mammary epithelial stem cells, which are interspersed in the basal myoepithelial layer (Brisken et al., 2000; Teuliere et al., 2005; Turashvili et al., 2006). Thus, specific expression of \textit{Lbh} in these cellular targets of canonical Wnt signaling during normal mammary gland development, as well as the pro-mitogenic and differentiation-inhibiting effects of Lbh overexpression in HC11 cells suggests a potential role of Lbh in adult mammary gland tissue homeostasis downstream of Wnt. Finally, a positive relationship between canonical Wnt signaling and \textit{LBH} expression may also have relevance to embryonic heart development. This is suggested by a recent microarray study showing that \textit{Lbh} induction during cardiac differentiation of mouse embryonic stem (ES) cells is abolished by inhibition of canonical Wnt signaling (Liu et al., 2007). Thus, LBH may act downstream of the canonical Wnt pathway in multiple aspects of embryonic and adult vertebrate development.

By studying the effects of different Wnt ligands on \textit{LBH} expression in tissue culture, we discovered that Wnt7a efficiently blocks Wnt3a-mediated induction of \textit{LBH} as well as of the known \(\beta\)-catenin target gene \textit{DKK1} in 293T cells. Moreover, Wnt7a, which is a tumor suppressor in lung cancer (Winn et al., 2005), strongly repressed \textit{LBH} and \textit{DKK1} expression in human HCC1395 breast cancer cells. To our knowledge, this is the first evidence that Wnt7a can inhibit canonical Wnt signaling. One possible mechanism for the observed inhibitory effect could be that Wnt7a activates the non-canonical planar cell polarity (PCP)
pathway in tissue culture cells (Carmon and Loose, 2008; Le Grand et al., 2009). However, given our developmental genetics studies and the fact that Wnt7a and Wnt5a, which can also activate the PCP pathway, exerted different effects on Wnt3-induced gene expression, we propose that the inhibitory effect of Wnt7a on canonical Wnt3a signaling in tissue culture cells could be mediated by a poorly understood non-canonical pathway involving Lmx1b (Kengaku et al., 1998b).

The antagonistic relationship between canonical Wnt/β-catenin and non-canonical Wnt7a signaling may have important implications for dorso-ventral limb pattern formation during embryonic development. It has remained enigmatic how expression of canonical Wnt target genes gets restricted to ventral limb and AER ectoderm, especially since Wnt3 and its downstream signaling components are expressed throughout the entire limb ectoderm (Barrow et al., 2003; Oosterwegel et al., 1993; Soshnikova et al., 2003). Furthermore, canonical Wnt signaling activity is detected in some dorsal ectodermal cells as evident by our TopGal reporter assays. Thus, ectopic expression of \textit{Lbh} in distal dorsal limb ectoderm of mouse mutants lacking Wnt7a pathway activity (\textit{Wnt7a}^{-/-} and \textit{Lmx1b}^{-/-}) together with the repressive effects of Wnt7a on canonical Wnt signaling in tissue culture suggest that Wnt7a-Lmx1b signaling may be an important repressive mechanism that blocks Wnt/β-catenin target gene expression, and thus ventral differentiation, in dorsal limb ectoderm.

Most importantly, aberrant overexpression of \textit{Lbh} in MMTV-\textit{Wnt1} mammary tumors, as well as in human breast tumors and cell lines, provides the first indication that somatic gain of LBH function occurs in cancer. Notably, \textit{LBH}
is specifically deregulated in ER-negative breast tumors and correlates most strongly with the most clinically aggressive basal-like tumor phenotype (Sorlie et al., 2003). Elevated expression of \textit{LBH} mRNA is observed in approximately half of basal-like tumors, but is present only in 16 - 23\% of luminal breast tumors, which have a good prognosis. Since only few distinct molecular markers have been identified to date that are uniquely associated with basal-like breast cancers (Mani et al., 2007; Rodriguez-Pinilla et al., 2007), LBH may prove to be a valuable diagnostic marker for this difficult-to-treat clinical subtype.

The strong correlation between expression of \textit{LBH} and canonical Wnt pathway genes in basal breast tumors, as well as TopFlash reporter and inhibition assays in breast tumor cell lines, furthermore suggest that dysregulation of LBH in breast cancer is due, at least in part, to aberrant Wnt/\(\beta\)-catenin signaling. Additional meta-analysis also showed a clinical association of LBH expression with Wnt activation in colon cancer (A. Sims unpublished; data not shown). Although deregulation of LBH in congenital heart disease is associated with increased LBH gene dosage, aCGH analysis does not suggest that increased LBH gene copy number is the underlying cause for LBH deregulation in basal-like breast cancers. However, we noted that luminal tumor cell lines frequently displayed a decrease in \textit{LBH} copy number, suggesting that a decrease in LBH gene dosage may play a role in inactivation of \textit{LBH} in ER-positive luminal tumors. Nevertheless, we cannot rule out the possibility that genomic instability inherent to tumor cells may lead to chromosomal abnormalities that could result in \textit{LBH} overexpression. Karyotypic anomalies involving chromosomal region
2p23 are frequent in a variety of other human cancers, including hematopoietic malignancies, testicular cancers and neuroblastoma (Crockford et al., 2006; Griffin et al., 1999; Kansal et al., 2005; Satge et al., 2003). Thus, deregulation of \textit{LBH} may be a more general event in human cancer.

In summary, our findings raise the intriguing possibility that LBH may act as a downstream effector of canonical Wnt/\(\beta\)-catenin signaling in both normal and neoplastic epithelial development, which is under the tight control of antagonistic non-canonical Wnt7a signaling.
Chapter 4: Functional Role of LBH in Breast Carcinogenesis
4.1 Background

Basal breast cancers are undifferentiated tumors that are enriched in CD44\textsuperscript{high}/CD24\textsuperscript{low} cells (Honeth et al., 2008; Winter, 2008). CD44\textsuperscript{high}/CD24\textsuperscript{low} cells, in turn, share properties with stem cells, and have the ability to differentiate into different cell types (Ponti et al., 2005). While CD44\textsuperscript{high}/CD24\textsuperscript{low} cells may account in part for basal breast tumor heterogeneity, the molecular mechanisms driving the undifferentiated phenotype of basal breast cancer cells, however, are poorly understood.

Treatment of basal breast cancer has also proven difficult, as CD44\textsuperscript{high}/CD24\textsuperscript{low} TIC and cells with more mesenchymal morphology are enriched for after conventional chemotherapy and endocrine treatments (Creighton et al., 2009). Therapeutically resistant tumor cells are primed for invasiveness due to an EMT-like signature, and may seed future tumors and metastases (Creighton et al., 2010; Jones et al., 2004; Sheridan et al., 2006). New treatments will need to be developed that specifically target TICs or induce differentiation of TICs to make them more susceptible to conventional therapy. The current lack of understanding of the molecular regulation of this resistance precludes the development of new therapies.

Previous studies have demonstrated that LBH plays a role in maintaining a self-renewing progenitor phenotype during embryonic cardiogenesis and osteogenesis (Briegel et al., 2005; Conen et al., 2009). Additionally, in adult mammary gland development, LBH expression is restricted to cells with a greater regenerative potential within the basal-myopithelial cell layer and TEB cells. Lbh is also a direct target of canonical Wnt/β-catenin signaling (Rieger et al.,
2010); a key pathway involved in the maintenance and self renewal of stem cells (Nusse et al., 2008). Furthermore, ectopic overexpression of Lbh HC11 non-transformed mammary epithelial cells, a cell line with stem cell-like characteristics (Williams et al., 2009), resulted in blocking expression of \( \beta \)-casein transcripts upon exposure to lactogenic hormones and, thus, prevented terminal differentiation (Rieger et al., 2010).

We previously found that LBH is overexpressed in 45% of basal breast tumors (Rieger et al., 2010), which have a characteristically undifferentiated tumor phenotype (Winter, 2008). We therefore wanted to identify whether LBH functions, similar to embryonic and adult development, to maintain an undifferentiated breast tumor cell phenotype, contributing to the inherent resistance of basal breast cancer cells to conventional treatment. Our approach to identify the functional role of LBH in breast carcinogenesis was to modulate LBH levels in human basal breast cancer cell lines by RNAi knockdown or overexpression and to monitor phenotypic changes in cell surface marker expression, cell growth, survival, and tumorigenicity. The findings in this chapter further support that LBH functions to maintain cells in an undifferentiated state and revealed a novel role of LBH in survival of CD44\(^{\text{high}}\)/CD24\(^{\text{low}}\) TIC and suppression of luminal tumor cell differentiation.

4.2 LBH expression correlates with a CD44\(^{\text{high}}\)/CD24\(^{\text{low}}\) TIC phenotype in breast cancer cell lines

In collaboration with Diana Azzam in Dr. Slingerland’s laboratory, we performed FACS analysis on several LBH-positive breast cancer cell lines to
identify the proportion of cells with a CD44^{high}/CD24^{low} and ALDH+ cell surface marker TIC phenotype (Table 4.1). ALDH+ cells are a second population of TIC cells that have similar characteristics to CD44^{high}/CD24^{low} cells (Croker et al., 2008; Ginestier et al., 2007; Tanei et al., 2009), and have been associated with BRCA1 deficiency (Liu et al., 2008) as well as inflammatory breast cancer (Charafe-Jauffret et al., 2010). As shown in Table 4.1, HCC1395 and MDA-MB-231 basal breast cancer cell lines, which express similarly high levels of LBH, comprised a large proportion (> 90%) of CD44^{high}/CD24^{low} cells. In contrast, HCC1187, despite the highest levels of LBH expression, was virtually devoid of a

<table>
<thead>
<tr>
<th>Cell line</th>
<th>LBH mRNA expression</th>
<th>CD44^{high}/CD24^{low}</th>
<th>ALDH+</th>
<th>Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>-</td>
<td>0.70 ± 0.1</td>
<td>2.37 ± 0.02</td>
<td>Luminal</td>
</tr>
<tr>
<td>T47D</td>
<td>-</td>
<td>0</td>
<td>0.52</td>
<td>Luminal</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>-</td>
<td>0</td>
<td>6</td>
<td>Luminal</td>
</tr>
</tbody>
</table>

**Table 4.1:** LBH expression correlates with a breast TIC phenotype. Selected human breast cancer cell lines with indication of LBH expression as measured by qPCR represented as + or -, as well as the percentage of CD44^{high}/CD24^{low} or ALDH+ cells as measured by FACS analysis (performed by Diana Azzam). Subtypes according to (Kao et al., 2009; Neve et al., 2006).
CD44^high^/CD24^low^ subpopulation, but had an increased proportion of ALDH+ cells (8.5%, Table 4.1). Low expression levels of LBH in basal cell lines, such as BT549 and BT20, correlated with an intermediate proportion of CD44^high^/CD24^low^ TIC, while none of the ER positive tumor cell lines contained a proportion of CD44^high^/CD24^low^ cells, consistent with published data (Fillmore and Kuperwasser, 2008; Sheridan et al., 2006). Identification of additional breast cancer cell lines with LBH overexpression will aid in strengthening the correlation between high LBH expression and a TIC phenotype. Nonetheless, these findings suggest that within ER negative basal breast cancer, LBH expression correlates with a TIC cell surface marker phenotype.

4.3 Efficient transient knockdown of LBH in different basal subtype breast cancer cell lines by RNAi

Knockdown of LBH in three basal breast cancer cell lines that overexpress LBH (HCC1395, HCC1187, and HCC1395) was performed using synthetic SMARTpool siRNA specific for human LBH (Dharmacon). Knockdown efficiencies were slightly different in each cell line, whereby the highest efficiency of >90% was achieved in HCC1395 cells, as measured by qPCR and Western Blot analysis (Figure 4.1A, B). Knockdown of LBH in MDA-MB-231 was also highly efficient (>80%), whereas LBH siRNA treatment in HCC1187 generally resulted in only 50% knockdown efficiency (Figure 4.1A) most likely due to vast overexpression of LBH in this cell line. Remarkably, efficient transient LBH knockdown persisted over nine days. Thus, we were able to
deplete LBH levels in basal breast cancer cell lines over long periods of time in a manner suitable for most *in vitro* assays.

4.4 LBH knockdown in basal HCC1395 breast carcinoma cells reduces the CD44\textsuperscript{high}/CD24\textsuperscript{low} population

To investigate the effects of transient knockdown of LBH on the CD44\textsuperscript{high}/CD24\textsuperscript{low} population in basal breast cancer cell lines we performed FACS analysis for cell surface marker expression 6 or 9 days post-transfection. Cells were split three days post-transfection to a larger surface area to avoid contact inhibition. We found that knockdown of LBH in HCC1395 consistently reduced (n>3 experiments) the proportion of CD44\textsuperscript{high}/CD24\textsuperscript{low} cells significantly both 6 and 9 days post-transfection (Figure 4.1C, Table 4.2). In a representative LBH knockdown experiment in this cell line, a shift from 83% to 58% CD44\textsuperscript{high}/CD24\textsuperscript{low} cells upon transient knockdown of LBH for 9 days was observed (Figure 4.1C, red boxes). We consistently observed a significant decrease in the proportion of CD44\textsuperscript{high}/CD24\textsuperscript{low} cells by approximately 25% with a reciprocal increase to the more differentiated phenotype of CD44\textsuperscript{high}/CD24\textsuperscript{high} (p<0.04, Table 4.2, data not shown, (Al-Hajj et al., 2003; Nieoullon et al., 2007; Shipitsin et al., 2007). We did not examine changes in the ALDH+ population in HCC1395 cells due to the very small proportion in the parental cell line. Taken together, these results suggest that LBH plays a role in the maintenance of TIC with a CD44\textsuperscript{high}/CD24\textsuperscript{low} marker phenotype in HCC1395 cells.

We attempted to identify a similar reduction in CD44\textsuperscript{high}/CD24\textsuperscript{low} proportions in MDA-MB-231 and HCC1187 cells upon knockdown of LBH.
Although knockdown was consistently efficient in MDA-MB-231 (Figure 4.1A), there was no significant decrease in the proportion of CD44<sup>high</sup>/CD24<sup>low</sup> TIC (Table 4.2). In this regard, it is worthwhile noting that despite containing >80% CD44<sup>high</sup>/CD24<sup>low</sup> cells, MDA-MB-231 do not form mammospheres (Grimshaw et al., 2008), one of the functional assays for TIC, suggesting that this cell line is a poor model to study TIC function. In contrast, HCC1187 cells, which harbor a...
small proportion of CD44<sup>high</sup>/CD24<sup>low</sup> cells (<6%) and an elevated level of ALDH+ cells (8.5%, Table 4.1), exhibited a 50% reduction in CD44<sup>high</sup>/CD24<sup>low</sup> cells upon knockdown of LBH but showed little change in ALDH population (Table 4.2). This result, however, may not be significant because of the low starting percentage of CD44<sup>high</sup>/CD24<sup>low</sup> cells. We also note, that unlike HCC1395, there was no reciprocal increase in the more differentiated CD44<sup>high</sup>/CD24<sup>high</sup> proportion of cells (data not shown). Nonetheless, our observation that reduction of LBH expression decreased the proportion of CD44<sup>high</sup>/CD24<sup>low</sup> TIC in HCC1395 cells supports that LBH aids in maintaining the undifferentiated phenotype in basal breast cancer cells.

### Table 4.2: Table listing the average knockdown efficiencies measured by qPCR and proportion of cells with TIC cell surface marker expression. Markers CD44 and CD24 or ALDH were measured by FACS analysis. CD44<sup>high</sup>/CD24<sup>low</sup> values represent the average percentage of cells with that phenotype ± SD. (HCC1395 n=2, MDA-MB-231 n=2, HCC1187 n=1). Student’s t-test was used to determine statistical significance.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Knockdown Efficiency</th>
<th>CD44&lt;sup&gt;high&lt;/sup&gt;/CD24&lt;sup&gt;low&lt;/sup&gt;</th>
<th>ALDH+</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC1395 scrambled siRNA</td>
<td>85-95%</td>
<td>83±0.47</td>
<td>ND</td>
<td>P&lt;0.04</td>
</tr>
<tr>
<td>HCC1395 LBH siRNA</td>
<td></td>
<td>58.28±5.66</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231 scrambled siRNA</td>
<td>75-85%</td>
<td>97.69±1.04</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231 LBH siRNA</td>
<td></td>
<td>96.85±1.38</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>HCC1187 scrambled siRNA</td>
<td>50-55%</td>
<td>5.27</td>
<td>7.43</td>
<td></td>
</tr>
<tr>
<td>HCC1187 LBH siRNA</td>
<td></td>
<td>2.73</td>
<td>6.36</td>
<td></td>
</tr>
</tbody>
</table>

*Table 4.2: Table listing the average knockdown efficiencies measured by qPCR and proportion of cells with TIC cell surface marker expression. Markers CD44 and CD24 or ALDH were measured by FACS analysis. CD44<sup>high</sup>/CD24<sup>low</sup> values represent the average percentage of cells with that phenotype ± SD. (HCC1395 n=2, MDA-MB-231 n=2, HCC1187 n=1). Student’s t-test was used to determine statistical significance.*
4.5 Transient knockdown of LBH reduces cell viability and increases cell death

The reduction in CD44\textsuperscript{high}/CD24\textsuperscript{low} TIC upon knockdown of LBH in HCC1395 cells could be due to reduced cellular growth, decreased survival, or differentiation from CD44\textsuperscript{high}/CD24\textsuperscript{low} cells to more luminal CD44\textsuperscript{high}/CD24\textsuperscript{high} cells. To discriminate between these possibilities we measured cell number, viability, apoptosis rates, and expression of luminal differentiation markers (see section 4.12 for luminal differentiation markers). We observed a significant reduction in total cell number of HCC1395, MDA-MB-231 and HCC1187 cells treated with LBH siRNA for 6 or 9 days, ranging from 50-70\% of the total number of scrambled siRNA treated cells (Figure 4.2A). To examine whether the reduction in total cell number was due to decreased viability, we performed an MTT assay (Promega). This assay measures the metabolic conversion of MTS tetrazolium to a colorimetric formazan product at a wavelength of 492 nm, and thus the relative number of viable cells. Cells were seeded 3 days post-transfection, and cell viability was monitored for 5 additional days. We observed a consistent decrease in LBH siRNA treated HCC1395 cell viability as early as 3 days, which became statistically significant after 4 days (p<0.005, Figure 4.2B). On average, no significant decrease in cell viability was observed in MDA-MB-231 and HCC1187 cells treated with LBH siRNA (data not shown), despite consistent knockdown efficiencies and significantly decreased total cell numbers (Figure 4.1A). These results, although informative, do not examine proliferation. Cell cycle analysis still needs to be performed in order to draw any conclusions on the effect of LBH and proliferation in breast cancer cells.
Alternative to reduced cell growth, loss of LBH in basal-breast cancer cell lines may result in increased apoptosis. We checked apoptosis levels by FACS analysis after staining with FITC labeled Annexin V and propidium iodide (PI). Annexin V binds tightly to phosphatidylserine, which translocates to the outer membrane of cells during early apoptosis. In concert with DNA labeling using PI, we can differentiate between apoptosing and necrosing/dying cells (Vermes et al., 1995). After 3 days of transfection with scrambled or LBH siRNA, the cells were split; a portion was assessed for apoptosis that day and a portion was seeded for apoptosis analysis after 6 days. Each cell line behaved slightly different (Figure 4.2C, D), but at 3 days there was a slight, but clear, increase in the percentage of apoptosing cells in HCC1395, MDA-MB-231, and HCC1187 lines treated with LBH siRNA versus scrambled siRNA. Additionally, HCC1187 cells treated with LBH siRNA had an increased percentage of necrotic cells (Figure 4.2D). Analysis 6 days post-transfection showed a general increase in the percentage of cells undergoing apoptosis in all cells, where the gap between control and LBH siRNA treated cells widened (Figure 4.2C). These findings suggest that LBH increases tumor cell survival, an LBH function that has not been previously described. Since basal and/or TIC are resistant to chemotherapy it would be interesting to explore if LBH knockdown makes HCC1395 and other basal tumor cells more susceptible to apoptosis inducing agents. We will also need to validate LBH effects on apoptosis rates by examining expression of apoptosis markers such as cleaved caspase 3 or cleaved PARP. In summary, this
novel finding suggests that LBH may play a role in the maintenance of a CD44<sub>high</sub>/CD24<sub>low</sub> TIC phenotype by enhancing in cell survival.

4.6 Transient knockdown of LBH reduces anchorage independent growth in HCC1395 cells

Changes in survival of HCC1395 cells upon knockdown of LBH could also affect self-renewal and in vitro tumorigenicity. To assess for changes in the self-renewal capacity of HCC1395, MDA-MB-231, and HCC1187 cells treated with LBH siRNA, we plated a single cell suspension at a density of 2x10<sup>4</sup> cells/well on 6 well ultra-low adherence plates (Corning) in normal growth media containing 4 µg/ml heparin and 0.48 µg/ml hydrocortisone, modified from (Dontu et al., 2003). Tumorsphere formation was monitored for 7 days, after which, spheres larger than 10 cells were counted. We attempted to generate secondary tumorspheres in both MDA-MB-231 and HCC1395, but no HCC1395 secondary spheres formed after replating, as perhaps the mechanical separation was too

Figure 4.2: Knockdown of LBH in HCC1395, MDA-MB-231, and HCC1187 human basal breast cancer cell lines affects tumor cell viability and anchorage independent growth. (A) Cell counts performed at collection points before FACS analysis revealed significantly decreased cell numbers in all three cell lines tested, bars represent the mean values, error bars represent the SD (HCC1395 n=7, MDA-MB-231 n=3, HCC1187 n=2), *p<0.01, **p<0.0001. (B) Cell viability assay demonstrating reduced cell viability in HCC1395 cells upon knockdown of LBH, *p<0.005. (C) Representative FACS analysis apoptosis profile for HCC1395 cells treated with scrambled or LBH siRNA for 6 days. Quadrants and percentage within the quadrant are noted. (D) Percentage of alive (black), apoptosing (white), and necrosing/dead (grey) cells from the indicated cell lines as measured by FACS analysis for apoptosis marker Annexin V and propidium iodide. Cells were treated with scrambled or LBH siRNA for 3 days, where a portion were split for analysis at 6 days. We note an increase in the percentage of apoptotic cells in LBH siRNA vs. scrambled siRNA transfected cells in all three basal breast cancer cell lines at both 3 and 6 days post-transfection. (E) Soft agar assays were seeded in triplicate 3 days post siRNA transfection, and colony formation was monitored for 28 days. Cells treated with LBH siRNA had significantly reduced colony forming ability, whereby there were fewer large (>8 pixels) and small colonies. Quantification, right, was performed by photographing plates with a 12 megapixel camera, and counting colonies larger than 8 pixels at 100% magnification on Photoshop, *=p<0.005.
harsh and killed most of the cells. We obtained large primary mammospheres for HCC1395, smaller clumps of MDA-MB-231, and very large strands of HCC1187 cells after 7 days of mammosphere culture (Data not shown). Nevertheless, we observed no difference in primary, or secondary for MDA-MB-231, tumorsphere formation between scrambled and \textit{LBH} siRNA treated cells, with a primary sphere forming efficiency of approximately 0.2% in HCC1395 cells and 2.25% in MDA-MB-231 (data not shown). There was also no noticeable difference in the average size of the spheres/clumps.

Soft agar colony formation assays, which measure anchorage independent growth (a hallmark of transformed cells), revealed a striking difference between scrambled and \textit{LBH} siRNA treated HCC1395 cells after 28 days in culture (Figure 4.2D). Colony formation was quantified on Photoshop, where colonies larger than 8 pixels with images at 100% magnification were counted. \textit{LBH} siRNA treated cells had approximately 3 fold fewer colonies (>8 pixels) and the colonies that formed generally smaller in size (Figure 4.2D). Large colonies that formed in the \textit{LBH} siRNA treated cells may have been derived from HCC1395 cells that were not transfected, and thus still expressed high levels of LBH. This will be tested in the future by isolating large colonies and checking \textit{LBH} mRNA expression.

4.7 Cell motility was not affected by knockdown of LBH in MDA-MB-231 or HCC1187 cell lines

Because CD44\textsuperscript{high}/CD24\textsuperscript{low} cells have been associated with increased motility and metastasis (Mani et al., 2008), we tested the migratory ability of
MDA-MB-231 and HCC1187 cells upon siRNA mediated knockdown of LBH by scratch test. HCC1395 cells were not tested due to their slow growth and inability to be confluent after 3 days of siRNA treatment. Scratches were created across a confluent monolayer of cells that had been transiently transfected with scrambled or LBH siRNA for 3 days using a 200 µl pipette tip. Brightfield images were taken at 3 points on the scratch at the indicated timepoints (Figure 4.3). Gap distance was measured between the cell fronts on photoshop and averaged for the 3 positions at each timepoint. We found that transient knockdown of LBH in both MDA-MB-231 and HCC1187 resulted in no change in migration ability (Figure 4.3). Trendlines (not shown) indicated that the slope, and hence migration into the scratch, were approximately equivalent between scrambled siRNA and LBH siRNA treatments in both cell lines. Interestingly, the scratch was slightly larger in width in cells treated with LBH siRNA, indicating that LBH may have an affect

**Figure 4.3:** Knockdown of LBH in basal breast cancer cell lines does not affect cell motility as assayed by scratch test. Cells were treated with scrambled or LBH siRNA for 3 days at which point cells had reached confluence. A scratch was created with at 200 µl pipette tip, and monitored at the noted timepoints by brightfield microscopy. Gap distance was measured at 3 points on the scratch and averaged. The slopes of both lines were nearly equivalent indicating no change in migration upon knockdown of LBH. We note that the scratch was consistently larger in LBH siRNA treated cells.
on cell attachment. These experiments, however, need to be repeated with the use of serum free medium to further stop proliferation as contact inhibition may not stop the breast tumor cells from dividing. Nevertheless, it appears that LBH is not involved in the motility aspect of basal tumor cells.

4.8 Generation of stable LBH knockdown in breast cancer cell lines

To perform long-term *in vitro* and *in vivo* assays with knockdown of LBH, we needed to generate cells lines stably expressing LBH shRNA. We tested 5 individual pre-packaged LBH specific MISSION shRNA lentiviral clones (Sigma) by infecting MDA-MB-231 cells at an approximate multiplicity of infection (MOI) of 1, where each cell should be infected once by the lentivirus. Approximately 48 hours post-transduction, cells were harvested for RNA and protein for qPCR and Western blot analysis, respectively. As shown in Figure 4.4A, LBH mRNA levels were significantly reduced in cells transduced with LBH shRNA clones 1 and 5, which target the 3’ UTR and exon 2 of LBH, respectively. As clone 1 targets the 3’UTR, it may be ideal for future studies where re-expression of LBH cDNA is needed. Protein levels were also reduced by these clones albeit at less dramatic levels as 48 hours was probably not enough time for sufficient protein depletion. Subsequent transduction clones 1 and 5 in combination further reduced LBH expression at the mRNA level, although still not to 100% (data not shown).

We first attempted to establish a stable knockdown of LBH in HCC1395 cells because of the slow growing nature of these cells. HCC1395 cells were transduced with LBH shRNA clone 1 or non-targeting shRNA at an approximate
MOI of 1, followed by application of selective media containing 5 mg/ml puromycin after 48 hours. During selection, HCC1395 cells transduced with \textit{LBH} siRNA underwent massive cell death, whereas control shRNA treated cells

\textbf{Figure 4.4}: Stable knockdown of LBH in HCC1395 was not possible due to excessive cell death. (A) qPCR (top) and Western blot analysis (bottom) demonstrated that LBH shRNA clones 1 and 5 sufficiently reduced LBH expression in MDA-MB-231 cells after 48 hours of treatment. Values represent the mean expression ± SEM. (B) Light microscope images taken at 20x magnification of HCC1395 cells with non-targeting or LBH shRNA clone 1 after two weeks of selection in 5 µg/ml puromycin. In contrast to cells transduced with non-targeting shRNA, the majority of the LBH shRNA cells were dead.
eventually began to proliferate (Figure 4.4B). Two weeks into selection, less than 1% of the LBH shRNA treated cells remained and had stopped proliferating, whereas the non-targeting transduced cells appeared to be growing normally (Figure 4.4B). We have not yet attempted to create stable knockdown of LBH in MDA-MB-231 cells as we are now considering using an inducible system to avoid complete cell death. Nonetheless, the inability to create a stable knockdown in HCC1395 cells supports the hypothesis that LBH promotes survival and maintenance of TIC.

4.9 Generation of polyclonal LBH overexpression lines in BT549 cells

To compliment our knockdown studies, we overexpressed LBH in triple-negative breast cancer cell lines that express no or low levels of LBH and harbor median/low proportions of CD44^{high}/CD24^{low} TIC: BT549 and BT20 (Figure 4.5A, Table 4.1, data not shown). Three independent polyclonal sets of BT549 + N-terminally Flag tagged Lbh (N-Lbh) or pCDNA3 empty vector were established by Amaxa nucleofection with linearized plasmids followed by selection in media containing 350 µg/ml G418. Protein levels were monitored every few passages. LBH protein expression was undetectable in polyclonal pCDNA3 extracts, whereas LBH protein was highly elevated in all LBH polyclonal lines (Figure 4.5A). LBH protein expression was estimated to be slightly lower than expression in HCC1187 cells, based on Western Blotting comparison (Figure 4.5A, data not shown). Clonal derivatives of these cell lines were not created to avoid unwanted selective pressure to preserve the original proportion of CD44^{high}/CD24^{low} cells in the population. We also attempted to
create polyclonal BT20 +/- LBH cell lines, but these lines were unusually slow to recover from selection, and further studies were not pursued further.

4.10 The proportion of CD44$^{\text{high}}$/CD24$^{\text{low}}$ cells does not change upon stable overexpression of LBH in BT549 cells

We hypothesized that overexpression of LBH in BT549 would increase the proportion of CD44$^{\text{high}}$/CD24$^{\text{low}}$ TIC in the population. We screened cell surface marker expression on all three polyclonal sets of BT549 cells expressing pCDNA3 or N-Lbh by FACS analysis, but found no significant change in the proportion of CD44$^{\text{high}}$/CD24$^{\text{low}}$ TIC in any of the lines overexpressing LBH (Table 4.3). We postulate that the lack of phenotype may be due to the fact that we overexpressed LBH in cells that have other mutations that determine this particular tumor cell phenotype. LBH expression alone may be insufficient to elicit an effect on cells that are already highly tumorigenic.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Overexpression</th>
<th>CD44$^{\text{high}}$/CD24$^{\text{low}}$</th>
<th>ALDH+</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT549+pCDNA3</td>
<td>High overexpression (See Figure 4.5)</td>
<td>9.23±1.40</td>
<td>2.51±1.47</td>
</tr>
<tr>
<td>BT549+Lbh</td>
<td></td>
<td>8.93±1.26</td>
<td>2.41±0.51</td>
</tr>
</tbody>
</table>

Table 4.3: FACS analysis data as in table 4.2 for BT549 stable cell lines. Values represent the mean percentage of the population with CD44$^{\text{high}}$/CD24$^{\text{low}}$ or ALDH+ phenotype in three polyclonal stable cell lines ± SD.
4.11 Overexpression of LBH in BT549 does not effect 2D cellular growth but increases anchorage independent growth

We next wanted to know whether overexpression of LBH in BT549 would increase cell viability, the reciprocal effect to knockdown of LBH in HCC1395 cells. We subjected BT549 +/- LBH cell lines to the Promega MTT assay, as with HCC1395 cells. After optimization to observe cell growth in the linear range, we detected no significant difference in cell viability over time between stable BT549+pCDNA3 and BT549+N-Lbh cells (Figure 4.5B). We subsequently performed soft agar colony formation assays examine differences in anchorage independent growth. Colony formation was monitored for 28 days. We detected numerous large colonies by eye in two of the three polyclonal BT549+N-Lbh cell lines, while very few colonies were observed in the pCDNA3 polyclonal lines (Figure 4.5C, D). Image analysis performed as previously, revealed an approximate ten fold increase ($p<0.001$) in the number of colonies formed by the BT549 lines expressing high levels of LBH (Figure 4.5D). Thus, in this overexpression model, LBH increases anchorage independent growth in BT549 tumor cells without directly affecting cell viability.

4.12 LBH negatively regulates genes involved in luminal differentiation

As LBH has demonstrated transcriptional activity, we wanted to investigate whether LBH could regulate genes involved in the maintenance of a more undifferentiated phenotype. We therefore examined mRNA and protein extracts from cells that had been treated with scrambled or LBH siRNA for a period of 6 or 9 days. We first monitored the luminal marker CD24, as we had
identified an increase in CD24$^{\text{high}}$ cells upon knockdown of LBH by FACS analysis (Figure 4.1C). CD24 protein was nearly undetectable in extracts from mock and scrambled siRNA treated cells, whereas treatment of HCC1395 cells

**Figure 4.5:** Stable overexpression of LBH in triple-negative BT549 breast cancer cells does not affect cell growth, but increases anchorage independent growth. (A) Three individual polyclonal lines were established with pCDNA3 vector alone or pCDNA3+N-Flag Lbh by nucleofection of BT549 with pCDNA3 expression plasmids followed by selection with G481. LBH expression is undetectable by Western Blot analysis in pCDNA3 polyclonal lines, while there is clearly overexpression in the N-Lbh polyclonal lines. LBH overexpression levels are comparable to HCC1187 breast cancer cells. (B) Cell proliferation rates are not significantly different between BT549 plus pCDNA3 or N-Lbh. (C) Overexpression of N-Lbh in BT549 cells significantly increases colony formation in soft agar assays. (D) Quantification of colonies larger than 8 pixels on 100% magnification on Photoshop. Bars represent the mean of three 35 cm dishes, error bars represent the standard deviation, p<0.001.
with *LBH* siRNA significantly increased CD24 protein expression (Figure 4.6A). We performed subsequent qPCR analysis to identify the effects of LBH knockdown on *CD24* expression at the transcriptional level. Upon knockdown of LBH for 9 days, *CD24* mRNA increased approximately 2.5 and 3.5 fold in HCC1395 and MDA-MB-231 cells, respectively (Figure 4.6B). Additional markers for luminal differentiation including keratin 8, GATA3, and MUC 1 are currently being investigated. These findings suggest that LBH may transcriptionally repress luminal mammary epithelial differentiation genes, such as *CD24*.

We identified a second potential target of LBH regulation in the HC11 overexpression model described in Chapter 3. Western blot analysis demonstrated that Estrogen Receptor alpha (ERα) protein expression was significantly reduced in HC11 cells ectopically expressing LBH (Figure 4.6C), which were generated by Virmeliz Fernandez-Vega in our lab. Surprisingly, there was no corresponding reduction in ERα mRNA levels as measured by qPCR (Figure 4.6D). In this case, LBH may be playing a role in the protein stability of ERα or in post-transcriptional regulation involving micro RNAs. These theories could be tested through the use of protease inhibitors and ER stability assays, as well as identification of microRNA transcriptional targets of LBH. ERα gene expression was not increased in reciprocal knockdown experiments in basal breast cancer cell lines. This was expected as ERα gene expression is often epigentically silenced in basal breast cancer (Roll et al., 2008). Nevertheless,
these preliminary studies are suggestive of a role of LBH in ER-negativity of basal breast cancer cells. Although these findings are preliminary, they provide important insight into the molecular function of LBH in both normal and cancerous mammary gland development.
4.13 Discussion

We have identified a novel role of LBH in human breast cancer. So far, our functional studies suggest that LBH promotes the maintenance and survival of basal breast cancer cells with a CD44\textsuperscript{high}/CD24\textsuperscript{low} phenotype. The fact that LBH has such an effect on CD44\textsuperscript{high}/CD24\textsuperscript{low} cells seems logical as both LBH and CD44\textsuperscript{high}/CD24\textsuperscript{low} TIC have been linked to Wnt signaling (Dimeo et al., 2009; Rieger, 2010), and are enriched in basal subtype breast cancers (Honeth et al., 2008; Rieger, 2010).

Given the cancer stem cell theory (Wicha et al., 2006), LBH could potentially be expressed in basal mammary epithelial stem cells, which have been thought to be the cells of origin for basal-like breast cancers (Gusterson et al., 2005). This theory is supported by restriction of Lbh to myoepithelial cells during mammary gland development (Figure 3.4). Therefore, LBH expression in normal mammary epithelial stem cell populations needs to be addressed. Mouse normal mammary stem cells have been isolated and characterized as CD49\textsuperscript{f}+/CD29\textsuperscript{high}/CD24\textsuperscript{−}/Sca1\textsuperscript{−} (Shackleton et al., 2006; Sleeman et al., 2006; Stingl et al., 2006), and the presumptive human equivalent have been identified as CD49\textsuperscript{f}+/EpCAM\textsuperscript{low}/CD24\textsuperscript{−}/CD133\textsuperscript{−} (Visvader, 2009). This classification, however, is insufficient to differentiate between the bi-potential mammary stem cells and myoepithelial cells in the mouse system, therefore making it crucial to identify additional markers to increase the purity of isolated mammary epithelial stem cells (Stingl et al., 2006; Visvader, 2009). Isolation of the different established mouse mammary epithelial progenitor populations (Visvader, 2009),
followed by LBH mRNA and protein analysis would identify the population of normal mammary epithelial cells that express LBH.

Additional TIC populations have been identified since the commencement of this project. Recently, a new population of human TIC was identified by dye retention studies. Query of their microarray profiling did not indicate upregulation of LBH in this isolated population of cells (Pece et al., 2010), suggesting that LBH may not be expressed in extremely slow growing bi-potential cells. Fittingly, Lin−/CD49f+/EpCAM+ luminal progenitor cells were identified as the population of expanded cells in basal-like breast cancers pre-neoplastic mammary glands from patients heterzygous for BRCA1 (Lim et al., 2009). These cells expressed markers for both myoepithelial and luminal cells, and shared a more similar gene expression profile with basal breast cancers than Lin−/CD49fhigh/EpCAM− termed bi-potential mammary stem cells (Lim et al., 2009). Another TIC population includes the recently identified CD44pos/CD49fhigh/CD133/2high population of cells in ER negative breast tumors (Meyer et al., 2010). LBH mRNA and protein levels should be examined in these and other TIC populations to aid in the determination the properties of breast cancer cells that express LBH.

The shift in HCC1395 breast cancer cells to a more differentiated CD24 positive phenotype upon knockdown of LBH demonstrates a relationship between LBH expression and maintenance of a more undifferentiated tumor phenotype. We have shown that loss of LBH in basal breast cancer cell lines HCC1395, MDA-MB-231 and HCC1187 reduces cell viability. Thus, we cannot rule out
that the increase in luminal marker $CD24$ mRNA was due to a greater percentage of differentiated cells in the observed steady state population as the result of increased cell death of $CD44^{\text{high}}/CD24^{\text{low}}$ HCC1395 cells. MDA-MBA-231, however, had elevated levels $CD24$ mRNA and a higher percentage of cells in apoptosis, yet showed no significant change in the CD44/CD24 FACS profile. In all reality, the increase to a more differentiated phenotype upon LBH knockdown may be a combination of both upregulation of luminal markers such as $CD24$ at the transcriptional level, and an increase in cell death of less differentiated cells. Further, a decrease in ER$\alpha$ protein levels in HC11 cells ectopically expressing Lbh further supports the notion that LBH expression leads to a more undifferentiated phenotype, as mammary glands require ER$\alpha$ expression for proper development and differentiation (Bocchinfuso et al., 2000) These findings are also consistent with the fact that LBH is specifically deregulated in ER negative breast cancer, and suggests that Lbh may function to repress ER.

Self-renewal capacity of TIC within basal breast cancer cell lines, did not appear to be affected by knockdown of LBH by RNAi as determined by tumorsphere assays. Tumorsphere assays, however, still need to be optimized to ensure that sphere formation occurs from a single cell rather than the result of cell clumping. This can be achieved by plating single cells in a 96 well format to monitor sphere formation. An alternative method to tumorsphere formation to assess self-renewal capacity is to perform serial colony forming unit analysis (Dimeo et al., 2009; Fillmore and Kuperwasser, 2008). In non-serial colony formation assays, we observed a decrease in colony formation upon knockdown
of LBH in HCC1395 cells, and a reciprocal increase in colony formation upon exogenous overexpression of LBH in BT549 cells. These results demonstrated that LBH affects anchorage independent growth, but it will be necessary to perform \textit{in vivo} xenograft assays using both stable knockdown and stable overexpression cell lines to assess the effect of LBH on tumorigenicity.

It is significant that we identified an increase in cell death upon transient knockdown of LBH, with a more drastic phenotype upon the attempted generation of stable LBH knockdown HCC1395. Increasing evidence suggests that cancer stem cells have an increased resistance to apoptosis (Kruyt and Schuringa, 2010). As such, breast cancer stem cells are a popular target for new therapies (Morrison et al., 2008). The role of LBH in apoptosis will need to be further examined, as the results in this chapter simply demonstrate that downregulation of LBH in a highly expressing breast cancer cell line leads to increased cell death. The mechanism by which LBH effects apoptosis will need to be worked out at the molecular level, and could easily be achieved by qPCR array screening for changes in apoptosis related genes. One potential mechanism of targeting TIC for more efficient apoptosis with conventional treatment would be to push cells towards differentiation, as has worked in several other cancer types (Morrison et al., 2008). The pro-survival and de-differentiation properties of LBH make it a ideal candidate for targeted therapeutics directed at TIC.

The findings in this chapter strongly support that LBH functions to promote survival and to maintain an undifferentiated phenotype in basal subtype breast cancer. LBH may not only have prognostic value in the clinic, but my
serve as an ideal target for future therapeutics given its functional effects in basal subtype breast cancer.
Chapter 5: Analysis of LBH Protein Expression and Post-Transcriptional Regulation
5.1 Background

Although LBH was discovered over 10 years ago, it remains a poorly characterized protein. Further characterization of its biochemical properties and post-translational regulation are needed for planning proper experiments and designing potential therapeutics directed at LBH, given that LBH may play a substantial role in multiple diseases. The initial identification of LBH demonstrated that this small acidic protein (105 amino acids) is highly conserved among vertebrates, but has no homology to any other known protein family. The predicted size of the full-length murine Lbh protein is 12.3 kDa, but it migrates at a higher molecular weight of 16-17 kDa on SDS-PAGE gels. Functionally, Lbh has transcriptional activity in cell based reporter assays when fused to a Gal4 DNA binding domain (Briegel and Joyner, 2001). A more recent publication mirrored the initial studies by Dr. Briegel utilizing the human form of the protein, and identified a potential conserved ERK-D domain and proline dependent serine/threonine kinase group at amino acids 27–41 (PMEEIGLSPR KDGLS) of the human LBH protein (Ai et al., 2008). Finally, we recently published an initial biophysical characterization of recombinant Lbh, demonstrating that Lbh is intrinsically disordered and has conformational flexibility, which may allow it to interact with a multitude of other proteins in the cell (Al-Ali et al., 2009). Apart from these three studies, very little is known about the LBH protein biochemistry.

Previous cellular localization studies have shown that LBH is primarily localized to the nucleus. These studies, however, have relied solely on overexpression of Flag-tagged or EGFP fusion LBH (Ai et al., 2008; Briegel and Joyner, 2001), respectively. We note, however, that large tags such as GFP or
EGFP, as has been shown by our lab, cause aggregation and mislocalization of Lbh to the cytoplasm, indicating that the analysis by Ai et al., 2008 may not be physiologically relevant. Our lab has since generated a highly specific Lbh polyclonal antibody that enables to perform more comprehensive biochemical and protein expression analysis by Western blot, immunofluorescence, immunohistochemistry, and immunoprecipitation analysis. One of the goals of my thesis project was to determine the endogenous cellular location of LBH both in situ and in different cell lines, as well as to examine the regulatory mechanisms involved in LBH localization. Apart from previously discussed transcriptional regulation of LBH by Wnt signaling, post-transcriptional and post-translational regulation of LBH may play an important role in regulation and function of LBH during development and disease. This chapter will describe further biochemical characterization of Lbh, as well as preliminary studies outlining the phosphorylation status of LBH, potential LBH protein interaction motifs, and putative microRNA binding sites in the 3’ UTR of the LBH transcript.

5.2 LBH is highly conserved among vertebrate species

Due to whole genome sequencing efforts, additional sequence information in different species has become available in the last few years. We thus wanted to expand protein sequence alignments to include additional species that may be of use in the future. Clustal W was used to align the LBH protein sequences from a total of 16 species, 11 of which are shown in Figure 5.1. Additional species including chimpanzee, horse, opossum, and pike were also included, but are not
shown in this alignment. Strikingly, this alignment shows that the nuclear localization signal (NLS) and flanking sequences are almost completely conserved among all species (Figure 5.1). In contrast, the C-terminus varies in a manner consistent with the most closely related species sharing similar sequences (Figure 5.1), as verified by COBALT alignment (data not shown, http://www.ncbi.nlm.nih.gov/tools/cobalt/).
5.3 LBH is an intrinsically disordered protein

Given its small size and solubility, LBH was an ideal candidate for NMR structural analysis. Prior to NMR analysis, the relative order/disorder of the amino acid composition of LBH was predicted by the neural network program PONDR_ VL-XT (Li et al., 1999; Romero et al., 2001), available from Molecular Kinetics Inc. at http://www.pondr.com. As displayed in Figure 5.2, 70 of 105 LBH residues (approximately 67%) were predicted to be disordered and were located primarily in two peptide regions spanning residues 15–38 and 60–105. Interestingly, the predicted NLS (residues 56–63) of LBH overlaps with the most ordered region spanning residues 39–59, whereas the Glutamine-rich putative transcriptional activation domain (residues 67–104) resides in the longest disordered region. For further structural and biophysical analysis and recombinant protein properties, please be referred to Al-Ali et al., 2010. Collectively, the studies described in this manuscript demonstrate that LBH belongs to a family of proteins termed intrinsically disordered proteins (IDP). Intrinsically disordered proteins have the ability to form multiple conformations giving them extreme flexibility in terms of form and function (Dyson and Wright, 2005). Thus, the ability of LBH to both activate and repress transcription (Ai et al., 2008; Briegel et al., 2005; Briegel and Joyner, 2001; Conen et al., 2009) may be due to its inherent lack of structure that allows LBH to acquire multiple conformations and, thus activities, depending on its target interacting partners.
5.4 Cellular localization of endogenous LBH

As the endogenous expression pattern of the LBH protein was unknown, we utilized immunofluorescence to study the endogenous expression patterns of LBH in both tissue culture and during embryonic development. I first determined the optimal dilution of the Lbh specific antibody that our lab had generated and found that a dilution of 1:1000 was optimal for detection of a very strong specific signal of endogenous LBH expression. In contrast, pre-immune serum at the same dilution did not yield any specific signal and very little background. In tissue culture cells, particularly proliferating breast cancer cell lines MDA-MB-231 and HCC1395, as well as B16 mouse melanoma cells and NIH3T3 non-transformed mouse embryonic fibroblasts, we found that endogenous LBH protein expression primarily localized to the nucleus as determined by DAPI or Hoechst staining (Figure 5.3A). In contrast, HC11 non-transformed mammary
epithelial cells did not express LBH, as was previously seen in chapter 3. We noted a LBH specific cytoplasmic staining in some cell lines (HCC1395 vs. MDA-MB-231; Figure 5.3A), but in general, nuclear staining was more intense.

**Figure 5.3:** Immunofluorescence analysis showing differential cellular localization of Lbh protein in human tissue culture cells and mouse embryonic tissues. Immunofluorescence was performed using a rabbit polyclonal Lbh antibody at a dilution of 1:1000, and anti-rabbit Alexafluor488 (Molecular Probes) at a dilution of 1:500 as the secondary antibody. (A) LBH is primarily localized to the nucleus in proliferating B16 mouse melanoma, NIH3T3 non-transformed mouse embryonic fibroblasts, as well as MDA-MB-231 and HCC1395 human breast carcinoma cells. LBH expression was undetectable in HC11 non-transformed mouse mammary epithelial cells (B) Immunofluorescent detection of Lbh in the developing mouse embryo revealed distinct cellular localizations in different tissues. Tissues and stage of development are indicated. DRG is the dorsal root ganglia containing more differentiated neuronal cells. Original magnification for all images was 20X.
In addition, we observed that in both HCC1395 and MDA-MB-231 cells nuclear LBH formed foci with as of yet unknown function (Figure 5.3A).

During embryonic development, however, we identified several different patterns of Lbh cellular localization. Lbh was both nuclear and cytoplasmic in heart, eye, and skin ectodermal cells at embryonic day 10.5 (E10.5) or E14.5, respectively (Figure 5.3B). In stark contrast, Lbh was excluded from the nucleus and exhibited striking cytoplasmic expression in post-mitotic neural cells, including cells that are part of the peripheral nervous system in the dorsal root ganglion (DRG; Figure 5.3B). Based on these results, we hypothesize that in proliferating cells, LBH is localized to the nucleus, whereas it becomes excluded from the nucleus in more differentiated progenitor cells that have stopped dividing. Additionally, LBH may perform distinctive roles in both the nucleus and cytoplasm during the different stages in development through alternative cellular localization. We note that organ specific protein Lbh expression during embryonic development coincides with Lbh mRNA expression as analyzed by in situ hybridization (Briegel and Joyner, 2001; Rieger and Briegel, 2010). In brief, we have elucidated the endogenous Lbh protein expression pattern in both tissue culture cells and mouse embryonic tissues. Moreover, these experiments demonstrated that the Lbh antibody detects both mouse and human LBH proteins.

5.5 The LBH protein is phosphorylated

To enable a better biochemical characterization of Lbh, I next established an immunoprecipitation protocol using our Lbh specific antibody. High resolution SDS-PAGE of LBH immunoprecipitated from cell extracts from B16
mouse melanoma and HCC1187 human breast cancer cells revealed a distinct double banding pattern (Figure 5.4B). The quality of the banding pattern was further improved by performing IgG purification of the Lbh antibody prior to immunoprecipitation (Purified Lbh; Figure 5.4B). In contrast, LBH bands were diffuse on low resolution SDS-PAGE, indicating that LBH may carry post-translational modifications. To test this possibility, we first performed extensive in silico analysis to identify amino acids within the LBH protein sequence that could be phosphorylated. Using Scansite software (Yaffe, 2003, http://scansite.mit.edu), we initially identified four putative phosphorylation sites at positions S34, S49, T69, and T98 (Figures 5.4A), that are conserved among the majority of vertebrate species (Figure 5.1). However, subsequent analysis revealed that S49 was not likely to be phosphorylated due to structural constraints (DEPP; Iakoucheva et al., 2004). Two of the potential phosphorylation sites at T69 and T98 are completely conserved in all mammals, and are predicted to be phosphorylated by Casein Kinase 2 (CK2) and also Protein Kinase C (PKC) in the case of T98. Serine 34 is predicted to be phosphorylated by MAPK or CDK1 depending on the prediction software (ELM, NetPHosK1.0). An additional conserved site at S63, which was not identified by our initial search, was strongly predicted to be phosphorylated by PKA and is not restricted by structural constraints (NetPhosK1.0, DEPP). Interestingly, S63 and T69 are located at the end of or right after the putative nuclear localization signal (Figure 5.4A),
Figure 5.4: Human and mouse LBH are phosphorylated. (A) Schematic of the LBH protein including domains and putative phosphorylation sites as determined by Scansite. Note that T69 is located near the end of the nuclear localization signal. (B) Immunoprecipitation and thus enrichment of LBH from B16 mouse melanoma cells and HCC1187 human breast cancer cells using unpurified or MelonGel IgG purified (Promega) Lbh antibody revealed multiple specific bands detected by the Lbh antibody by Western blot analysis as denoted by the arrows. We note that the multiple forms ran at different molecular weights in the mouse and human cells indicating uniquely modified forms. This banding pattern is indicative of post-translational modification. (C) Treatment of HCC1187 breast cancer cell extracts (30 µg) with 30 units of calf intestinal phosphatase (CIP) for 30 minutes reduces the two highest molecular weight bands of LBH, indicating that LBH is phosphorylated. Three distinct bands were identified in this blot.
suggesting that these sites could potentially play a role in regulating LBH cellular localization (Brunet et al., 2001; Holmberg et al., 2002; Macian et al., 2001).

To identify whether any of the LBH bands identified by high resolution SDS-PAGE were due to phosphorylation, I treated 30 µg HCC1187 breast cancer cell extracts with 30 units of Calf Intestinal Phosphatase (CIP) in a volume of 20 µl for 30 minutes at 37°C prior to gel electrophoresis. We found that treatment of HCC1187 extracts with CIP resulted in a reduction of the two highest molecular weight species of the LBH protein as compared to untreated extracts (Figure 5.4C). We also noted an increase in the intensity of the lowest molecular weight species and a smear in the middle band. Thus, the two highest molecular weight bands appear to be different phosphorylated LBH species. Subsequently, I attempted to identify whether phosphorylation was on serines or threonines by Western blot analysis using mouse monoclonal phosphoserine clone PSR-45 (Sigma; #3430) at a dilution of 1:1,000 or mouse monoclonal phosphothreonine clone PTR-8 (Sigma; #3555) at a dilution of 1:100 in TBST plus 0.5% BSA. Unfortunately, the antibodies did not work very well and optimization was not pursued further. We also tried large-scale immunoprecipitation of endogenous LBH from HCC1187 cells for mass spectrometry analysis to identify phosphopeptides. However, no proteins were successfully identified in the mass spectrometry analysis performed by proteomics facilities at SCRIPPS FL or at Harvard due to technical problems.

Although phosphorylation, at least in part, explained the banding pattern we observed, glycosylation may also alter the molecular weight of LBH. Upon
initial *in silico* analysis, the only predicted glycosylation site was an O-Glycosylation at position T69, but would unlikely to occur *in vivo* due to the lack of glycosylation signal peptides in the LBH sequence (NetO Glyc 3.1 Server http://www.cbs.dtu.dk/services/NetOGlyc/). An additional potential glycation, the addition of a sugar without the presence of an enzyme (Vlassara, 2005), at K58 was detected (NetGlycate 1.0 Server, http://www.cbs.dtu.dk/services/NetGlycate/). These possibilities were not investigated any further, but could be examined with antibodies specific for glycosylation, glycosylation removal treatments, or by mass spectrometry analysis.

**5.6 Mutation of S34 and at least one other putative phosphorylation sites leads to increased nuclear aggregation of Lbh**

As phosphorylation status can change cellular localization and function of proteins (Holmberg et al., 2002), we wanted to identify if mutation of any of the predicted phosphorylation sites would affect the cellular localization of LBH. Although we have yet to verify that these sites are phosphorylated *in vivo*, we created expression plasmids in which the putative phosphorylation sites on murine Lbh were mutated from serine or threonine to alanine. Both individual and combination Lbh phosphomutants were generated, with the help of lab technician Stephanie Duffort, by site-directed mutagenesis. Stephanie transiently transfected the plasmids into COS-7 cells, that do not normally express endogenous Lbh protein. Cellular localization of these Lbh phosphomutants was subsequently visualized by immunofluorescence. In proliferative tissue culture cells, LBH is
Figure 5.5: Transiently transfected Lbh is localized primarily to the nucleus in COS-7 cells. (A) Wild-type Lbh. (B) Lbh harboring mutations S34A and T98A. We note that the majority of the cells with potential phosphorylation site mutations generally looked like the wild-type transfectants. The exception was in cells containing mutations at S34 and at least T69 or T98, where a small percentage of cells had Lbh foci formation in the nucleus as in panel B. Green is Lbh, blue is DAPI. Original magnification was 40X, transfection and imaging performed by Stephanie Duffort.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Cellular Localization</th>
<th>Nuclear Aggregates</th>
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</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>Wild type</td>
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</tr>
<tr>
<td>S34A</td>
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<tr>
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Table 5.1: Listing of phosphomutants (generated by Stephanie Duffort) and cellular localization of Lbh 48 hours post transient transfection of listed constructs into COS-7 cells. Cytoplasmic and nuclear localization were similar for all transfected cells. Constructs that resulted in the aggregation phenotype are noted in bold.
primarily localized to the nucleus with limited cytoplasmic localization (Figure 5.4A, 5.5A). We found no drastic changes in this Lbh expression pattern unless there were two or more mutations including a mutation at S34 (Figure 5.5B, Table 5.1). These Lbh phosphomutants, highlighted in Table 5.1 in bold, were still primarily localized to the nucleus, but a small percentage of the cells accumulated nuclear Lbh foci (Figure 5.5B). The lack of phenotype with S49 mutants further supports that this site may not be phosphorylated in vivo. These findings have yet to be validated in a second cell line, as we need to account for cell specific effects, and we have not yet investigated the nature of the foci.

5.7 Prediction of potential LBH protein partners and interaction motifs

Identification of proteins that interact with LBH is crucial for a better understanding of the transcriptional regulatory function and molecular role of LBH in both development and disease. Initial co-immunoprecipitation studies of endogenous LBH from HCC1187 cells failed to identify any potential interacting partners. We therefore used in silico analysis to identify motifs within the Lbh sequence that could provide clues to protein interactions using the ELM database (Gould et al., 2009). This program searches for known ligand binding motifs located in the protein sequence of interest, citing the exact location and definition of the motif. Motifs identified in the LBH sequence as listed in Table 5.1 are subclassified by cellular localization. Only ligand-binding motifs that are conserved between mouse and human LBH proteins were included in the table. None of these potential interactions have been verified, but may aid in future protein interaction studies.
These predicted motifs provide a glimpse into the regulation and functional role of LBH. Interestingly, LBH contains a PP2B interaction motif, which has previously been demonstrated to lead to nuclear localization of NFAT by dephosphorylating residues near its docking site (Aramburu et al., 1998). Similarly, PP2B may interact with LBH at residues 61-67, resulting in dephosphorylation of residue T69 located adjacent to the NLS and thus, nuclear localization.

<table>
<thead>
<tr>
<th>Name</th>
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<tr>
<td>EVH1 II</td>
<td>PPEEF</td>
<td>81-85</td>
<td>Proline-rich motif binding to signal transduction class II EVH1 domains</td>
</tr>
<tr>
<td>PDZ3</td>
<td>PDYL MEEI KDGL KDRLEEFF</td>
<td>10-13 28-31 37-40 58-61 83-86</td>
<td>Class II PDZ domains binding motif</td>
</tr>
<tr>
<td>SH2 STAT5</td>
<td>YFPI YLRS</td>
<td>4-7 12-15</td>
<td>STAT5 Src Homology 2 (SH2) domain binding motif</td>
</tr>
<tr>
<td>SH3 3</td>
<td>PSIVVEP</td>
<td>62-68</td>
<td>Recognized by SH3 domains with a non-canonical Class I recognition specificity</td>
</tr>
<tr>
<td>TRAF2 1</td>
<td>PMEE PPEE</td>
<td>27-30 81-84</td>
<td>TRAF2 binding consensus motif</td>
</tr>
<tr>
<td>Cytoplasmic and nuclear localization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAPK 1</td>
<td>KDRLPSIVV</td>
<td>58-66</td>
<td>MAPK interacting molecules</td>
</tr>
<tr>
<td>PP2B 1</td>
<td>LPSIVVE</td>
<td>61-67</td>
<td>Calcineruin substrate docking site</td>
</tr>
<tr>
<td>WW 4</td>
<td>IGLSPR</td>
<td>31-36</td>
<td>Class IV WW domains interaction motif, phosphorylation dependent interaction</td>
</tr>
<tr>
<td>Nuclear localization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRCT BRCA1 1</td>
<td>MSIYF</td>
<td>1-5</td>
<td>Phosphopeptide motif directly interacts with BRCT domain of BRCA1 with low affinity</td>
</tr>
<tr>
<td>FHA 2</td>
<td>EPTEGEV EETAKEN</td>
<td>67-73 96-102</td>
<td>Phosphothreonine motif binding a subset of FHA domains that have a preference for an acidic amino acid at the pT+3 position</td>
</tr>
<tr>
<td>RB</td>
<td>IHCPD</td>
<td>7-11</td>
<td>Interacts with the retinoblastoma protein</td>
</tr>
<tr>
<td>USP7 1</td>
<td>PDPSD</td>
<td>46-50</td>
<td>NTD binding motif variant</td>
</tr>
</tbody>
</table>

**Table 5.2:** ELM predicted interaction motifs for human and mouse LBH protein subdivided by cellular localization.
localization of LBH. Additionally, there are several predicted signaling pathway molecules, which may interact and phosphorylate LBH, such as MAPK. Not only does LBH contain a SP motif at S34 signaling for phosphorylation by MAPK, but also contains a docking site which helps to aid in the specificity of the enzyme (Sharrocks et al., 2000). We also identified potential interaction motifs for both an E3 ligase, including a class IV WW domain containing protein NEDD4 (Kamynina et al., 2001), and a protein that has de-ubiquitination abilities, USP7 (Everett et al., 1997). Hence, ubiquitination may play a role in regulating LBH function or degradation, which could help to explain discrepancies between LBH mRNA and protein expression levels in normal mammary epithelial cells such as MCF10A (See Chapter 3).

Post-translational modification of LBH, including phosphorylation, most likely also plays a role in its transcriptional co-activator and repressor function (Holmberg et al., 2002). The putative phosphorylation site at residue T98 is located near the end of the glutamate-rich putative trans-activation domain in the middle of a predicted α-helix. Phosphorylation within this helix could disrupt or enhance binding to other proteins. Fittingly, residues 96-102 represent a putative FHA 2 domain-binding motif. FHA domains were first identified in Forkhead transcription factors (Carlsson and Mahlapuu, 2002), and since then have been identified in numerous proteins involved in cell cycle and checkpoint control (Durocher et al., 2000). LBH also contains a predicted RB interaction motif, whereby RB is a well known tumor suppressor that regulates G1 to S phase cell cycle progression (Weinberg, 1995). Hence, LBH binding could potentially
inhibit RB function, thereby promoting the cell cycle. The above findings further support the results in Chapters 3 and 4 suggesting that LBH may contribute to cell cycle control, or in apoptosis.

An additional observation from in silico motif analysis was that LBH could potentially interact with proteins involved in the cytoskeletal structure, including SH3 and EVH1 II domain containing proteins. High magnification immunofluorescence analysis revealed a defined pattern of LBH expression in the cytoplasm, where it appears to be attached to the structural elements of the cell, and not located freely in the cytoplasm (data not shown). To identify the exact cytoplasmic localization of LBH we would need to perform immunofluorescent co-localization studies. We must note that a few of the predicted proteins interaction motifs are highly unspecific, including PDZ3 and SH2 STAT5, and are therefore poor predictors of interactions (Gould et al., 2009). Finally, LBH is predicted to interact with BRCA through the BRCT domain, which would provide another link between LBH expression and basal subtype breast cancer. This interaction, however, is unlikely to occur in vivo as it requires phosphorylation at S2; this site is not predicted to be phosphorylated due to structural constraints. Nevertheless, the identified motifs will provide a guide in future LBH protein interaction studies.

5.8 The LBH 3’ UTR contains multiple predicted microRNA binding sites

To identify other possible regulatory mechanisms of LBH, we scanned the 3’ UTR of human LBH for potential microRNA (miR) binding sites using TargetScan 5.1 (http://www.targetscan.org/, Friedman et al., 2009; Grimson et al.,
MiRs function to degrade or block translation of their target mRNAs and have been shown to play an important role in gene regulation in many diseases including cancer (Shenouda and Alahari, 2009). We identified several potential miR-binding sites as listed in Table 5.2. The TargetScan prediction indicates that the majority of the sites are projected to be conserved, while the miR-143 binding site is less likely to be conserved (data not shown). The table indicates the position, sequences, and the type of seed region of the miRNA, which has been shown to be one of the most important factors in the functionality of the miRNAs (Brennecke et al., 2005; Doench and Sharp, 2004). Also included is the context score percentile. This number measures the percent of miRNA binding sites with less favorable context as established by Grimson et al., 2007. Thus, a higher percentage means a highly favorable context. Interestingly, the miRs located towards the ends of the 3’ UTR have a more favorable context, which plays part in the favorability prediction (Grimson et al., 2007). Additionally, the cluster of miRs located at the 3’ end of the 3’ UTR may also act in concert increased LBH repression (Grimson et al., 2007). These miR binding sites have not yet been validated, but may be useful in future regulatory studies. MiR binding sites can be validated by the use of luciferase reporter assays incorporating the 3’ UTR of LBH upstream of a luciferase reporter with appropriate mutations in the seed region binding sites.
<table>
<thead>
<tr>
<th>Name</th>
<th>Position (3'UTR)</th>
<th>Sequence</th>
<th>Seed type</th>
<th>Context score percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR19</td>
<td></td>
<td>5’ AAACGAGGCUUCUGUUUGCACC &lt;br&gt;3’ AGUCAAAACGUACUAAACGUGU &lt;br&gt;7mer-m8</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-19a</td>
<td>124-130</td>
<td>5’ AAACGAGGCUUCUGUUUGCACC &lt;br&gt;3’ AGUCAAAACGUACUAAACGUGU &lt;br&gt;7mer-m8</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>miR19</td>
<td></td>
<td>5’ AAACGAGGCUUCUGUUUGCACC &lt;br&gt;3’ AGUCAAAACGUACUAAACGUGU &lt;br&gt;7mer-m8</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-19b</td>
<td>124-130</td>
<td>5’ AAACGAGGCUUCUGUUUGCACC &lt;br&gt;3’ AGUCAAAACGUACUAAACGUGU &lt;br&gt;7mer-m8</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>miR143</td>
<td></td>
<td>5’ CAGAAAACAAAGUCUUCUACUCU &lt;br&gt;3’ CUCGAUGUCACGA-AGUAGAG &lt;br&gt;7mer-m8</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-143</td>
<td>527-533</td>
<td>5’ CAGAAAACAAAGUCUUCUACUCU &lt;br&gt;3’ CUCGAUGUCACGA-AGUAGAG &lt;br&gt;7mer-m8</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>let7/98</td>
<td></td>
<td>5’ UAUAGCUGUAUUUAUACCUCAA &lt;br&gt;3’ UUGAUAUGUUGGAUGAGAG &lt;br&gt;7mer-1A</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>hsa-let7a</td>
<td>1580-1586</td>
<td>5’ UAUAGCUGUAUUUAUACCUCAA &lt;br&gt;3’ UUGAUAUGUUGGAUGAGAG &lt;br&gt;7mer-1A</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>hsa-let7b</td>
<td>1580-1586</td>
<td>5’ UAUAGCUGUAUUUAUACCUCAA &lt;br&gt;3’ UUGAUAUGUUGGAUGAGAG &lt;br&gt;7mer-1A</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>hsa-let7c</td>
<td>1580-1586</td>
<td>5’ UAUAGCUGUAUUUAUACCUCAA &lt;br&gt;3’ UUGAUAUGUUGGAUGAGAG &lt;br&gt;7mer-1A</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>hsa-let7d</td>
<td>1580-1586</td>
<td>5’ UAUAGCUGUAUUUAUACCUCAA &lt;br&gt;3’ UUGAUAUGUUGGAUGAGAG &lt;br&gt;7mer-1A</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>hsa-let7e</td>
<td>1580-1586</td>
<td>5’ UAUAGCUGUAUUUAUACCUCAA &lt;br&gt;3’ UUGAUAUGUUGGAUGAGAG &lt;br&gt;7mer-1A</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>hsa-let7f</td>
<td>1580-1586</td>
<td>5’ UAUAGCUGUAUUUAUACCUCAA &lt;br&gt;3’ UUGAUAUGUUGGAUGAGAG &lt;br&gt;7mer-1A</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>hsa-let7g</td>
<td>1580-1586</td>
<td>5’ UAUAGCUGUAUUUAUACCUCAA &lt;br&gt;3’ UUGAUAUGUUGGAUGAGAG &lt;br&gt;7mer-1A</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>hsa-let7i</td>
<td>1580-1586</td>
<td>5’ UAUAGCUGUAUUUAUACCUCAA &lt;br&gt;3’ UUGAUAUGUUGGAUGAGAG &lt;br&gt;7mer-1A</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-98</td>
<td>1580-1586</td>
<td>5’ UAUAGCUGUAUUUAUACCUCAA &lt;br&gt;3’ UUGAUAUGUUGGAUGAGAG &lt;br&gt;7mer-1A</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>miR137</td>
<td></td>
<td>5’ UGACACACACAUAAGGCAAUAAC &lt;br&gt;3’ GAUGCGCAUAAGAAUCGUUAAU &lt;br&gt;7mer-1A</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-137</td>
<td>1737-1743</td>
<td>5’ UGACACACACAUAAGGCAAUAAC &lt;br&gt;3’ GAUGCGCAUAAGAAUCGUUAAU &lt;br&gt;7mer-1A</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>miR125</td>
<td></td>
<td>5’ UGUGUUUUUUUGGCGUCAGCAG &lt;br&gt;3’ AGUGUCCAUUUCCAGAGGUCCU &lt;br&gt;8mer</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-125a-5p</td>
<td>2323-2329</td>
<td>5’ UGUGUUUUUUUGGCGUCAGCAG &lt;br&gt;3’ AGUGUCCAUUUCCAGAGGUCCU &lt;br&gt;8mer</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>hsa-miR-125b</td>
<td>2323-2329</td>
<td>5’ UGUGUUUUUUUGGCGUCAGCAG &lt;br&gt;3’ AGUGUCCAUUUCCAGAGGUCCU &lt;br&gt;8mer</td>
<td>97</td>
<td></td>
</tr>
</tbody>
</table>
miR128

| miR128     | hsa-miR-128 | 2368-2374 | 5’TCAAACUACUUUUUGUCACUGUGA
|           |             |           | 3’UUUCUCUGGCCAAUGACACU
|           |             |           | 8mer | 99 |

miR127

| miR127     | hsa-miR-27a | 2369-2375 | 5’TAAACUACUUUUUGUCACUGUGAC
|           |             |           | 3’CGCCUUGAAUCCG--UGACACUU
|           |             |           | 7mer-8 | 86 |
|           | hsa-miR-27b | 2369-2375 | 5’TAAACUACUUUUUGUCACUGUGAC
|           |             |           | 3’CGUCUUGAAUCGG--UGACACUU
|           |             |           | 7mer-8 | 86 |

Table 5.3: List of miR binding sites organized from 5’ to 3’ in the 3’UTR sequence. Context score percentile indicates the percent of miR binding sites that are less favorable, thus a high number indicates a favorable position.

It is quite likely, that miRNAs play a significant role in the regulation of LBH expression, and thus function in cells. Notably, in breast cancer, it has been recently demonstrated that miR-19 is reduced in metastatic breast cancer cell lines including MDA-MB-231 that expresses LBH, whereas miR-19 is abundantly expressed in low-invasive MCF7 cells that lack LBH expression (Zhang et al., 2010). Thus, expression of miR-19 in ER positive less aggressive tumors may be leading to the downregulation of LBH expression. Additionally, miR let-7 has been extensively researched, and is downregulated in ER negative breast cancer cells (Iorio et al., 2005). Reduced let-7 levels are also associated with increased self-renewal, as well as maintenance of proliferation and block of differentiation in breast tumor initiating cells (Yu et al., 2007). Although in a different system, we observed a similar phenotype in the block of differentiation upon the overexpression of LBH in HC11 non-transformed mouse mammary epithelial cells (See Chapter 3), further suggesting that LBH may be a target of let-7 in vivo. Although not demonstrated in a mammary epithelial cell system, high levels of miR-137 expression have been shown to induce proliferation and differentiation.
of brain tumor stem cells (Silber et al., 2008). Recently, miR-128 downregulation in breast cancer cells was found to enhance expression of hTERT and was associated with breast tumor initiating cells (Yu et al., 2009). miR-143 has not been examined in breast cancer as of yet, however, it is be depleted in less differentiated cardiac progenitors, and is a direct transcriptional target of Nkx2.5 (Cordes et al., 2009). Expression patterns of miR-125 and miR-27 do not particularly fit with LBH expression patterns in human breast cancer (Iorio et al., 2005; Mertens-Talcott et al., 2007; Yu et al., 2007), suggesting that these miR may not have a particular role in LBH expression in breast cancer. However, we cannot rule out the possibility that these miRs are of importance for Lbh regulation during development. The known association of several of the predicted miRs with “stemness” properties strongly supports the previous findings in Chapter 4 that LBH may have a functional role in stem or progenitor cells.

5.9 Discussion

Identification of LBH as an intrinsically disordered protein (IDP) was an invaluable finding, as it implicates a high degree of functional diversity. IDP’s are unique to eukaryotes, and the number of proteins with large disordered regions increases with organism complexity (Fuxreiter et al., 2008). Recently, IDP’s have been associated with and enriched for in many diseases (Uversky et al., 2009). Thus, aside from its roles in breast cancer, heart development, and congenital heart disease (CHD) as described in this thesis and previously published work (Briegel et al., 2005), LBH may be a key player in numerous diseases and developmental processes. Additionally, hub proteins at the center of
complicated protein interaction networks tend to be highly disordered (Haynes et al., 2006). As such, the LBH interactome is almost certainly large and highly complex, likely giving LBH multiple activities depending upon its conformation. It therefore becomes increasingly important to identify such networks as these interactions not only affect the function of LBH, but will likely dictate the conformational structure of LBH.

Post-translational modification of IDPs largely impacts conformation and protein interactions (Uversky et al., 2009). Aside from the proposed effect of phosphorylation on cellular localization, phosphorylation of LBH may also be influencing protein interactions, structural conformation, or activity. Our preliminary mutational analysis of LBH indicated that disruption of predicted phosphorylation sites S34, T69, and T98 did not largely alter the cellular localization of LBH. However, mutation at S34 and at least one other predicted phosphorylation site resulted in a small proportion of cells displaying evenly spaced LBH nuclear foci. Phosphorylation at these sites may actually exclude LBH from the nucleus (Brunet et al., 2001; Macian et al., 2001), and it is therefore necessary to perform reciprocal experiments in which these residues are mutated to phosphomimetic Aspartic acid or Glutamic acid.

The functional significance of LBH nuclear foci formation is unknown. One potential cause of focus formation may be that LBH is accumulating in ND10 or PML bodies. PML bodies have numerous functions including apoptosis and transcriptional regulation (Bernardi and Pandolfi, 2007; Takahashi et al., 2004) and have demonstrated tumor suppressor function (Salomoni and Pandolfi,
For a general review of PML bodies, please refer to (Bernardi and Pandolfi, 2007). PML body numbers vary during the cell cycle (Bernardi and Pandolfi, 2007), which could explain why only a small percentage of cells transfected with mutated constructs displayed the LBH foci phenotype. Cell synchronization should be performed to identify if phosphomutant LBH foci formation is related to the cell cycle. Even if cell cycle analysis does not reveal changes in LBH localization to PML bodies, it may still reveal changes in LBH nuclear localization as has been demonstrated with other transcription factors such as TBX2 (Bilican and Goding, 2006). Phosphomutant LBH foci may also be due to recruitment to DNA repair foci. However, as there was no damaging agent applied to the cells, this type of foci may be unlikely. A third possibility, is that the phosphomutant form of LBH interacts with large transcriptional complexes, as has been shown for steroid hormone receptors (Baumann et al., 2001; Black et al., 2004; Graham et al., 2009), which could also localize with sites of active transcription with PCNA. Co-localization studies will need to be performed with components of different nuclear foci.

Biochemical characterization of proteins is important for understanding their cellular function. In this chapter we have demonstrated that LBH is highly conserved among vertebrate species (58-99%), and, in all likelihood, has numerous functional roles due to its inherent lack of structure. We have also shown that endogenous LBH is primarily localized to the nucleus, and is phosphorylated. Finally, both potential protein interaction motifs and miRNA
binding sites have been identified within the LBH protein sequence and 3’ UTR, respectively, providing further insight into the function and regulation of LBH.
Chapter 6: Summary and Future Directions
6.1 Summary

Lbh is an embryonic transcription co-factor with important roles in embryonic development, congenital heart disease, and now breast cancer. The findings in this thesis support all previously published results suggesting that Lbh positively affects proliferation and maintains progenitor cells in an undifferentiated state. In addition, we identified a novel pro-survival function of LBH in basal breast tumor cells. Further work is needed to solidify the role of Lbh in both normal and neoplastic mammary development particularly because LBH has the potential to serve as an important prognostic and therapeutic target in breast, and probably numerous other cancers.

In Chapter 3, I demonstrated that Lbh is a direct transcriptional target of the oncogenic WNT signaling pathway. The Lbh genomic locus contains four conserved canonical TBE sites, all of which were bound by TCF4 in vitro and β-catenin in vivo, and had physiological activity in luciferase reporter assays. Only Wnt3a induced Lbh expression, whereas Wnt5a and Wnt7a, which signal primarily through non-canonical pathways, had slight inhibitory effects on basal Lbh levels, and Wnt7a effectively blocked Wnt3a mediated Lbh transcription. These findings, and the relationship between WNT and breast cancer, led us to investigate Lbh expression in MMTV-Wnt1 driven mouse mammary tumors, as well as human breast tumors and cell lines. Indeed we found Lbh overexpression in MMTV-Wnt1 transgenic mouse mammary tumors as well as in human basal-subtype, triple-negative breast tumors and cell lines. Interestingly, Wnt7a administration to HCC1395 breast cancer cells dramatically reduced both LBH and DKK1 expression levels, suggesting that Wnt7a could be used to inhibit LBH
expression in breast tumor cells. In addition, Wnt7a may act as a tumor suppressor in breast cancer, similar to what has been described in lung cancer.

We further investigated the functional role of LBH in basal subtype breast cancers in Chapter 4 by utilizing three cell lines which highly express LBH: HCC1395, HCC1187, and MDA-MB-231. In doing so, we identified a novel correlation between high LBH expression and a \( CD44^{\text{high}}/CD24^{\text{low}} \) tumor cell phenotype. Knockdown of LBH in HCC1395, in particular, resulted in a significant decrease in the \( CD44^{\text{high}}/CD24^{\text{low}} \) population, in part because of decreased survival of this population, and also the result of increased transcript and protein levels of luminal differentiation marker CD24. I also identified a reduction in colony formation upon knockdown of LBH in HCC1395 cells, and reciprocal enhancement upon overexpression of LBH in BT549 cells, further supporting that LBH effects the growth and survival of basal breast cancer cells.

Finally, Chapter 5 focused on the biochemical properties and regulatory mechanisms of LBH. For the first time, I identified endogenous cellular localization of LBH in both tissue culture and embryonic development, and found that LBH is phosphorylated in tissue culture cells. In attempt to identify interacting partners and alternative regulatory mechanisms, I discovered numerous predicted protein interaction motifs, and predicted microRNA binding sites in the 3’UTR, several of which have functional relevance in undifferentiated stem-like cells.

From this body of work and previously published data, we have derived the following working model (Figure 6.1). Transcription of \( LBH \) is activated in
response canonical Wnt signaling, but is inhibited by Wnt7a potentially through a non-canonical pathway involving LMX1B. Nuclear localization of LBH, probably regulated by phosphorylation/de-phosphorylation events, leads to the formation of transcriptional complexes to activate transcription of genes involved in proliferation and survival and block transcription of genes regulating differentiation.

**Figure 6.1:** Model for Lbh regulation and function. Canonical Wnt signaling is activated by the binding of canonical Wnt ligands, such as Wnt3A or Wnt1, to co-receptors low density lipoprotein receptor-related protein (LRP5/6) and Frizzled (FZD). This activates dishevelled, which in turn inactivates a destruction complex containing glycogen synthase kinase 3 beta (GSK3β), adenomatous polyposis coli (APC), and Axin. β-catenin is then released from this complex where it accumulates in the cytoplasm and translocates to the nucleus. Nuclear β-catenin forms a transcriptionally active complex with T-cell factor (TCF)/lymphoid enhancing factor (Lef) transcription factors to directly activate transcription of Lbh. Non-canonical Wnt7a/Lmx1b signaling blocks Lbh activation mediated by canonical Wnt signaling. We propose that the function of Lbh is to complex with other transcription factors and chromatin remodeling enzymes to activate transcription of proliferative and pro-survival genes and repress genes involved in differentiation.
6.2 Future Directions

On account of the novelty of LBH, each finding results in several additional questions that need to be addressed. In the remainder of this chapter I will outline experiments that have not previously been addressed in discussion sections of other chapters that should be performed in the future to further characterize and understand the molecular role of LBH in development and disease.

6.2.1 Mechanism of Wnt7a inhibition of canonical Wnt signaling

Based upon developmental genetics results in Chapter 3, we proposed that repression of Wnt3a mediated LBH expression by Wnt7a may be mediated by poorly understood LMX1B signaling. This hypothesis was further supported by the identification of a conserved LMX1B binding site located within the first intron of the LBH gene. To test this hypothesis we first knocked down LMX1B in 293T cells by RNAi, and monitored LBH expression in response to both Wnt7a alone as well as Wnt7a and Wnt3a treatment in combination. However, knockdown of LMX1B had no effect on the ability of Wnt7a to repress Wnt3a mediated LBH transcription. One reason for this result could be that conditions in this preliminary experiment were not optimal, as knockdown efficiency was low. Therefore, the experiment should be repeated before absolute conclusions are drawn. Nonetheless, we also monitored LMX1B expression in HCC1395 cells treated with Wnt7a and surprisingly found a 10 to 80 percent reduction in LMX1B expression levels depending up the timepoint observed. Taken together, these findings suggest that Wnt7a inhibition of Wnt3a target genes in these systems
does not occur via LMX1B signaling, and therefore requires further investigation. In all likelihood, the inhibitory effects of Wnt7a on canonical Wnt signaling occur through one of the non-canonical Wnt pathways such as the PCP or Wnt/Ca2+ pathways (Jessen, 2009; Wang, 2009). To approach this possibility, expression levels of the different FZD receptors should be determined in HCC1395 and 293T cells by qPCR analysis, as these receptors often determine whether Wnt ligands signal though canonical or non-canonical pathways (Carmon and Loose, 2008). Immunofluorescent co-localization studies should be performed to determine pathway specific factors, such as Vangl for the PCP pathway or PKC for the Wnt/Ca2+ pathway (Jessen, 2009; Wang, 2009), that are recruited to the cell membrane upon Wnt7a treatment.

6.2.2 Targets of LBH regulation

LBH has been termed a transcription co-factor, and one of the most frequent questions that arise is: what are the target genes of LBH? Thus far, we have potentially identified CD24 and ESR1 as targets of LBH, but direct transcriptional regulation has not been assessed.

CD24

CD24 expression is upregulated in response to knockdown of LBH in basal breast cancer cell lines. Identification of direct CD24 regulation by LBH will be performed in a similar manner to ChIP analysis performed in Chapter 3. In this case, however, we will immunoprecipitate endogenous LBH from HCC1395 or MDA-MB-231 cells with affinity purified Lbh antibody and screen CD24 regulatory regions to screen for LBH binding. Should segments of CD24
genomic locus be identified, we will for the first time be able to conclude that LBH acts as a direct transcriptional repressor.

*Estrogen receptor alpha*

We predict that LBH regulates the stability of ERα at the post-transcriptional or post-translational level because ERα protein but not mRNA levels are reduced in HC11 cells ectopically expressing Lbh. First, ERα protein levels will be monitored in response to proteasomal inhibition with MG-132. If Lbh levels increase after blocking the proteasome, we will need to identify factors that mediate this response through protein interaction studies. Alternatively, if no change in ERα protein levels is observed upon proteasomal inhibition, we can infer that other regulatory mechanisms, such as microRNAs, may be directly acting upon *ESR1* transcripts. To assess this possibility, miRs known to be associated with ERα stability including miR 18a (Wanâ€“Hsin et al., 2009), miR-206 (Adams et al., 2007), miR 221/222 (Zhao et al., 2008) to name a few, will be assessed by either Northern blot or qPCR analysis on extracts from HC11 +/- Lbh stable cell lines. We will also assess changes in ERα protein and mRNA levels upon overexpression of LBH in ER positive breast cancer cell lines including MCF7.

*LBH mediated gene expression changes*

Ideally we would like to identify genes regulated by LBH that are downstream of Wnt activation. However, we must first identify genes regulated
by LBH in general, to ensure that changes in gene expression are mediated by LBH alone and not by other canonical Wnt targets. Because every cell line behaves differently, we will have to perform these studies in several cell lines to derive a consensus set of genes regulated by LBH.

Because transient knockdown protocols have been optimized for breast cancer cell lines HCC1395 and MDA-MB-231, we would first assess gene expression changes in these two systems. To reciprocate findings, we would perform similar analysis on LBH negative cell lines stably overexpressing LBH, such as BT549. After 6 days of transient siRNA knockdown, or using stable knockdown or overexpression lines, mRNA extracts would be collected and subjected to microarray mRNA analysis to identify transcripts that are up and down-regulated in response to changes in LBH expression. Identified targets would be validated by qPCR.

Identification of LBH binding sites within the human genome

An alternative method to identify genes regulated by LBH, would be to perform ChIP-seq analysis (Valouev et al., 2008; Visel et al., 2009). In this case, LBH would be immunoprecipitated with affinity purified antibody from knockdown and overexpression cell lines. Immunoprecipitated DNA would then be isolated and sequenced by next generation sequencing. Results from this type of experiment would reveal the location of LBH binding within the entire human genome where. This method could also be used to tease out genes regulated by LBH directly downstream of Wnt, as we would only be assessing binding of LBH to DNA. The use of this requires additional in silico analysis to determine gene or
miR targets, but would provide a more accurate depiction of direct LBH regulation of genes. Any identified targets would be validated by qPCR analysis. Ideally, these experiments should also be performed in non-tumorigenic normal mammary epithelial cell lines such as HMEC or MCF10A to try and deduce the functional role of LBH in normal mammary epithelial cells.

6.2.3 *In vivo* functional role of LBH in basal breast cancer

Thus far, all LBH functional studies in breast cancer have been performed *in vitro* due to the lack of stable knockdown cell lines. Current attempts to generate a stable knockdown cell line have been unsuccessful, as shRNA mediated LBH knockdown in HCC1395 cells resulted in massive cell death, to the point where we were unable to derive a stable line. As such, we will attempt to establish stable lines expressing inducible shRNA vectors to reduce LBH levels in a controlled manner for a sustained period of time. Linsey Lindley will be working with lentiviral vector pTRIPZ, encoding turbo RFP, for easy visualization, and a Tet-on driven LBH shRNA. The shRNA targets the 3’UTR of LBH, so cDNA re-expression may be performed for experimental validation.

We are currently preparing to perform *in vivo* tumor formation assays using the BT549 +/- N-Lbh cell lines I generated. In collaboration with the Lippman laboratory, we will be injecting 2.5x10^6 cells mixed with 250 µl Matrigel/collagen I/PBS (40:40:20) into the 4th mammary gland on both the right and left side of *NOD SCID IL2γ−* mice. These mice lack mature lymphocytes and Natural Killer (NK) cells and are an ideal xenograft model for tumor
formation, as they are highly susceptible to tumor development, in some cases even from a single cell (Quintana et al., 2008). We will perform the injections on 6 mice for each of the following cell lines: BT549 parental, BT549 + pCDNA3, and BT549 + N-Lbh polyclonal cultures 2 and 3. Tumor formation will be monitored every few days, and mice will be sacrificed when tumors have reached 5% body weight or 90 days post-injection for tumor and metastasis formation analysis. Similar studies will be performed upon the creation of stable knockdown lines. Based upon the colony formation assays in Chapter 4, we would predict that overexpression of Lbh in BT549 cells will result in increased tumor formation, and knockdown of Lbh in basal breast cancer lines HCC1395 and MDA-MB-231 should result in decreased tumor formation.

We can also assess LBH effects on metastasis by performing tail-vein injection of the cell lines mentioned above modified from (Liang et al., 2005). We now also have stable luciferase MDA-MB-231 cells that can be used to visually analyze location of injected cells. Additionally, we would like to perform mammary gland reconstitution assays using both normal and transformed mammary epithelial cells expressing LBH (DeOme et al., 1959) to identify LBH effects on formation of normal mammary gland structures, as this is a method to evaluate stem-like properties of mammary epithelial cells.

6.2.4 Identification of LBH protein interactions

Protein interaction studies to date have failed to identify true interacting partners of LBH. In vitro studies are not suitable, as LBH is a highly flexible protein with many possible conformations (Al-Ali et al., 2009), and therefore
complexes with a multitude of unspecific proteins. All attempts to immunoprecipitate endogenous LBH from breast cancer cells have failed to identify interacting proteins due to technical difficulties. We will now immunoprecipitate LBH from BT549 breast carcinoma cell lines stably expressing Flag-tagged Lbh with M2 agarose instead of Lbh antibody. The resulting immunoprecipitation will yield a cleaner preparation and better resolution of interacting proteins since there will be no antibodies resulting in obstructive heavy and light chain bands.

Alternatively, we have now identified predicted protein interaction motifs within the LBH primary amino acid sequence. Protein microarrays are commercially available that comprise an array of spotted proteins containing interaction domains specific for a certain type of motif. We could use these arrays to perform in vitro binding assays using recombinant LBH. Several arrays are currently available, including WW domains, SH2, SH3 and PDZ. An important caveat of this technology, however, is that it is an in vitro assay, and may again result in a multitude of non-specific interactions.

In a direct targeted approach, we could perform co-immunoprecipitation studies followed by Western blot analysis to detect interactions using breast cancer cell lysates. MAPK is an ideal candidate, as LBH contains both a putative MAPK phosphorylation site, as well as a docking motif that aids recognition of the site. Antibodies to perform these experiments are commercially available.
6.2.5 Phosphorylation of LBH

In Chapter 5, we identified that LBH is a phosphoprotein, and that in a small percentage of cells, mutation of putative sites at S34 and T69 or T98 resulted in formation of LBH nuclear foci. Before further LBH phosphoprotein functional analysis is performed, we must identify phosphorylated residues in vivo. Mass spectrometry analysis will be performed on large amounts of endogenous LBH immunoprecipitated from HCC1187 cell extracts. HCC1187 cells are ideal for this study since we know LBH is phosphorylated in this cell line, and immunofluorescence studies have indicated both nuclear and cytoplasmic localization of LBH. Once phosphorylation sites have been identified, we can again perform mutational analysis, whereby we sites are mutated to both alanine to inhibit phosphorylation and aspartic or glutamic acid to mimic phosphorylation. Mutated constructs will be used to identify changes in localization and activity (once we have identified targets) of LBH. Additionally, we would validate the kinases predicted to phosphorylate LBH. Kinase activity would be modified by kinase specific activators and inhibitors specific for CK2, PKC, MAPK, and CDK1, and changes in LBH phosphorylation could be monitored by Western blot or mass spectrometry analysis.

6.2.6 Regulation of LBH by microRNAs

Several predicted miR binding sites in the LBH 3’UTR have been associated with breast cancer and linked to “stemness” properties. Therefore, these predicted miR binding sites should be validated to further strengthen the relationship between LBH expression and maintenance of an undifferentiated
phenotype. To validate these sites, we could create a luciferase reporter construct containing the 3’UTR of LBH fused downstream of the luciferase gene. As several of the predicted sites are spread out over the length of the 3’UTR, we would also generate a few deletion constructs to identify which sites are more functional in tissue culture cells. The luciferase reporter constructs would be transiently transfected into breast cancer cell lines that either highly or do not express LBH, such as HCC1395 or MCF7, respectively. We would expect luciferase activity to be reduced in cell lines that normally do not express LBH and vice versa, as expression of functional miRs within the breast cancer cell lines would interfere with translation of luciferase. Should we identify such changes we can validate responsible sites mutating the sequences in the 3’UTR luciferase constructs and monitoring activity. Please see (Martin et al., 2007) for a brief review on similar types of experiments. Alternatively, LBH expression will be monitored in response to transfection of LBH negative breast cancer cell lines with antagomiRs (Krutzfeldt et al., 2005), or in response to transfection of functional miRs (Yu et al., 2007) in LBH positive breast cancer cell lines. If a miR truly acts upon the LBH 3’UTR, we will observe changes in LBH protein expression in breast cancer cell lines.

6.3 Concluding remarks

LBH holds great promise as a future therapeutic treatment for basal breast cancer patients. Clearly, much more work is needed to dissect the functional role of LBH in both normal and cancerous cells in order for it to be considered useful as a therapeutic, but a strong collection of preliminary results suggests that LBH
functions to maintain cell viability and an undifferentiated phenotype in basal breast tumor cells. These findings suggest that direct downregulation LBH could be used to kill basal breast cancer cells dependent upon its expression and to push these cells towards differentiation, thus, making them more susceptible to conventional therapy. Identification of genes regulated by LBH and interacting protein partners will also be crucial for understanding the molecular role of LBH in development and disease.
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