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INVESTIGATIONS INTO THE ROLES AND MOLECULAR MECHANISMS OF LANC-LIKE PROTEINS

BY

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DISSERTATION

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ABSTRACT

Lanthipeptides are a family of ribosomally synthesized and posttranslationally modified natural products defined by the characteristic thioether crosslinks lantionine and methyllanthionine. Among the various biological activities that lanthipeptides exhibit, the anti-microbial activities shown by many members of the family (hence named lantibiotics) have drawn the most attention and make them promising antibacterial drug candidates. One of the posttranslational modifications necessary for lanthipeptide synthesis and activity is a Michael-type addition of a cysteinyl thiol to dehydroalanine and dehydrobutyrine, catalyzed by the prokaryotic lantibiotic cyclase (LanC) or the cyclase domain in a multi-functional lantibiotic synthetase. Intriguingly, despite the lack of evidence of the presence of lanthipeptides in eukaryotes, sequence homologs of lantibiotic cyclases (LanC-like proteins or LanCL) have been found in a variety of eukaryotic species. The exploration of the biochemical and biological functions of LanC-like proteins may extend our understanding of the catalytic mechanisms of LanC or reveal novel modes of regulation of general interest.

In this work, our understanding of the functions of mammalian LanC-like proteins has been broadened in different ways and novel mechanisms of action have been revealed. The characterization of LanCL2 function in mammalian cells is described in Chapter 2 and a novel function of LanCL2 as a regulator of Akt activity was identified. LanCL2 promotes Akt phosphorylation and cell survival in liver cells and this regulation is through directly facilitating Akt phosphorylation by mTORC2. Chapter 3 describes the establishment of LanCL knockout mouse lines and the phenotypic characterization of Lancl2−/− mice. Shortened life span was observed in Lancl2−/− mice, accompanied with the symptom of cardiomyopathy. These mice also showed increased susceptibility to microbial infections, which is suggestive of a compromised immune system. In Chapter 4, the role of LanCL proteins in lantionine ketimine biosynthesis was investigated. The detection of lantionine ketimine in LanCL triple knockout mice argues against an essential role of LanCL proteins in this pathway and suggests the need for a systematic screening for potential LanCL substrates. Lastly, Appendices A and B describes a functional study of LanCL2 in HEK293 and MCF-7 cells and the identification of novel LanCL2 interacting proteins, respectively.
To my parents for their love and support
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CHAPTER 1. INTRODUCTION

1.1 LANTHIPEPTIDES AND THEIR BIOSYNTHESIS

Lanthipeptides, or lanthionine-containing peptides, are the largest and best studied group of ribosomally synthesized and posttranslationally modified peptides (RiPPs). A range of different activities have been reported for lanthipeptides, including antimicrobial (Schnell et al, 1988), antiviral (Ferir et al, 2013), antiallodynic (Meindl et al, 2010), and morphogenetic (Willey & Gaskell, 2011). Lanthipeptides with antimicrobial activity are called lantibiotics. Lantibiotics have potent antimicrobial activity against a wide range of Gram-positive bacteria as well as some Gram-negative bacteria (Piper et al, 2009). Nisin, the most intensively studied lantibiotic, has been used as food preservative for almost 50 years without development of substantial bacterial resistance (Delves-Broughton et al, 1996; Hansen, 1994). Other than nisin’s use in the food industry, lantibiotics have much potential to treat human and animal infections, with several of them currently in clinical development (Ghobrial et al, 2010; Grasemann et al, 2007; Sedgwick, 2009).

The biosynthesis of lanthipeptides starts with a ribosomally synthesized precursor peptide, which consists of an N-terminal leader and a C-terminal core peptide. The core peptide undergoes a series of post-translational modifications including the dehydration of serines and threonines to the α,β-unsaturated residues 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), respectively. The dehydrated amino acids are subject to a Michael-type addition of a cysteinyl thiol to form thioether crosslinks (Fig 1.1). The removal of the leader peptide yields the mature product, which is exported from the producing cells.

To date four classes of lanthipeptides have been classified based on the types of enzymes in their biosynthesis pathway (Fig 1.2). For class I lanthipeptides, the dehydration and cyclization reactions are catalyzed by two distinct enzymes: LanB and LanC. LanB-catalyzed dehydration involves the glutamylation of the side chains of serines and threonines followed by elimination (Garg et al, 2013; Ortega et al, 2015). LanC is a zinc-binding protein and catalyzes the cyclization through zinc-mediated activation of the Cys thiolate for nucleophilic attack (Li et al, 2006). The cleavage of the leader peptide and the export of the mature peptide are carried out by the enzymes LanP
and LanT, respectively. For class II lanthipeptides, a single bifunctional enzyme LanM carries out both the dehydration and cyclization reaction. LanM is comprised of an N-terminal dehydratase domain bearing no sequence homology to LanB and a C-terminal cyclase domain having ~ 25% sequence homology to LanC. The dehydration reaction carried out by LanM also has a different mechanism, involving an initial phosphorylation of the serines and threonines and subsequent elimination of the phosphate group (Chatterjee et al, 2005). Similarly, the function of proteolysis and transport is also combined in a single enzyme LanT. Class II LanT has an additional protease domain at the N-terminus in comparison to the class I LanT.

Class III and IV lanthipeptides are synthesized by multifunctional synthetases, named LanKC and LanL, respectively. They both possess an N-terminal OspF-like phosphothreonine lyase domain and a central kinase domain, which catalyze the dehydration in a phosphorylation/β-elimination pathway that is similar to LanM (Goto et al, 2010; Goto et al, 2011; Muller et al, 2010). Differences between LanKC and LanL reside in the C-terminal cyclase domain. Although the cyclase domain in both class III and IV lanthipeptide synthetases is homologous to LanC and LanM, only class IV synthetases (LanL) have the conserved Zn$^{2+}$ ligands (Goto et al, 2010). LanKC catalyzes the cyclization reaction in a Zn$^{2+}$-independent manner and the reaction also generates an additional structure called labionin (Kodani et al, 2004; Meindl et al, 2010; Wang & van der Donk, 2012). LanT-like transporters have been reported in class III and IV lanthipeptides (Knerr & van der Donk, 2012). The protease for class III lanthipeptides has been recently identified from *Kribbella flavida* (Voller et al, 2013), but gene clusters encoding a protease or protease domain for class IV lanthipeptides have not been found to date.

Although at least three different catalytic mechanisms have been discovered for the dehydration of serines and threonines, all cyclases in lanthipeptide synthesis are similar in sequence and catalytic mechanism. Interestingly, neither LanB nor LanM has any clear sequence homologs in the protein database other than the lanthionine dehydratase (or domain) in their own class. In contrast, the homologs of the lanthionine cyclases exist in a wide variety of eukaryotic organisms, including animals, plants and microorganisms. A blast search of eukaryotic model organisms revealed sequence
homologs of LanC in almost all of them except in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Caenorhabditis elegans*. Based on phylogenetic analysis LanC-like (LanCL) proteins actually contain more sequence homology to the cyclization domain of class II LanM proteins than the class I LanC enzymes, despite their namesake (Yu et al, 2013).

1.2 LANC-LIKE PROTEINS: DISCOVERIES AND FUNCTIONS

1.2.1 Expression, structure and proposed function for LanC-like proteins.

LanCL proteins have conserved hydrophobic domains, zinc-binding residues, and other putative active site residues as in prokaryotic LanCs. Three LanCL proteins are encoded in the human genome, with their genes located on chromosome 2 (Mayer et al, 2001), 7 (Wang et al, 1998) and X, respectively. Human LanCL1 and LanCL2 have the highest expression in various parts of the brain and testis and more ubiquitous expression in other tissues based on northern blot analysis (Mayer et al, 2001; Mayer et al, 1998). A major transcript of 4.8 kb was identified for LanCL1 in all tissues (Mayer et al, 1998). The expression pattern of LanCL1 was also confirmed in both human and mouse tissues by Western blotting (Bauer et al, 2000; Huang et al, 2014). For LanCL2, two major transcripts (4.7 and 4.1 kb) were present in the brain but only one much shorter transcript (1.8 kb) was present in the testis (Mayer et al, 2001). The distribution of mouse LanCL2 in various tissues was examined by Western blotting (Ike Joewono unpublished data) and was in agreement with previous northern blot data. Interestingly, the *LANCL2* gene was found to be co-amplified with the epidermal growth factor receptor (EGFR) in glioblastoma at significant frequencies (50%) (Eley et al, 2002). Elevated expression of LanCL2 was observed in these tissues as well, representing a unique expression pattern in glioblastoma (Eley et al, 2002). The tissue expression pattern of human or mouse LanCL3 has not been examined so far due to the lack of specific antibodies.

Since the discovery of the human LanCL1 and LanCL2 genes in 1998 (Mayer et al, 1998) and 2001 (Mayer et al, 2001) an understanding of their functions and molecular mechanisms has become an important focus of research. Resolved by the Zhang group in 2009, the crystal structure of human LanCL1 has a two-layer barrel like structure, with the Zn$^{2+}$ ion located at the top of the barrel, resembling the structure of NisC, the LanC of
nisin (Li et al, 2006; Zhang et al, 2009). A similar structure was observed for the human LanCL2 (Dr. Neha Garg unpublished data). The structural similarity combined with the conservation of the zinc ligands and other essential amino acids for the catalytic activity of LanC suggests that LanCL may possess similar enzymatic activity (Fig 1.3). However, the absence of the homologs of lanthipeptide dehydratases in the eukaryotic genomes and the likely absence of dehydroamino acids in most tissues also raise the possibility that eukaryotic LanCL proteins may utilize different substrate(s) from LanC.

To date, many interesting features and functions of LanCL1 and LanCL2 have been revealed after years of investigation. An enzymatic activity has also been described for LanCL1 recently (Huang et al, 2014). These novel identified functions suggest an interesting and important role of LanCL proteins in evolution rather than a mere replication of the LanC genes in eukaryotes.

1.2.2 LanCL2 discovery and its role in regulating adriamycin sensitivity.

LanCL2 was discovered by the Tsuruo group in a screen looking for adriamycin sensitivity genes and was therefore named “testis-specific adriamycin sensitivity protein” (Sugimoto, 1999). The molecular cloning and detailed characterization of the gene were carried out by Prohaska and colleagues (Mayer et al, 2001).

Following the discovery of LanCL2 as a “testis-specific adriamycin sensitivity protein”, two more papers supported this function of LanCL2 and proposed different mechanisms of action (Landlinger et al, 2006; Park & James, 2003). Park et al. examined the relationship of LanCL2 with the multi-drug resistance gene MDRI (Park & James, 2003). MDRI encodes the protein P-glycoprotein (P-gp), which is known to increase the cellular resistance to a range of antibiotics including adriamycin. MES-SA/Dx5 is a multidrug resistant derivative of the sarcoma MES-SA cell line, where the P-gp protein level is elevated. Exogenous expression of LanCL2 in MES-SA/Dx5 suppressed P-gp expression. LanCL2 expression level was also reverse-correlated with P-gp expression in three LanCL2 stable cell lines. In accordance with the lowered P-gp expression all three LanCL2 stable cell lines were rendered more sensitive to adriamycin. Moreover, the authors showed that it was the MDRI mRNA, instead of the protein, that was decreased by exogenous LanCL2 expression. LanCL2 was shown to reside in all three
compartments within the cells (plasma membrane, cytosol and nucleus) by fractionation. Therefore, the nuclear LanCL2 may be involved in the transcriptional regulation of MDR1 expression, although it is equally possible that LanCL2 is not a direct regulator, and that the activity of LanCL2 may arise in other compartments in the cell by initiating a signaling cascade. Further investigation is needed to establish a causal relationship between the decreased P-gp expression and the increased cell sensitivity to adriamycin in LanCL2 overexpressing cells.

A different mechanism of LanCL2-induced adriamycin sensitivity was proposed by Prohaska and colleagues, where the plasma membrane localization of LanCL2 plays an essential role (Landlinger et al., 2006). In UAC (human epithelial cells of amniotic origin) cells, LanCL2 seemed to strongly localize to the plasma membrane and some juxta-nuclear vesicles while LanCL1 was mostly in the cytosol and nucleus. Comparison of the sequences between the two proteins revealed an extra N-terminal extension of eighteen amino acids in LanCL2, which contains a potential myristoylation site. A LanCL2 mutant without the first seventeen amino acids or a mutant with the potential “myristoylation” glycine mutated to alanine was shown to be absent from the plasma membrane. The same result was obtained when the cells were treated with an inhibitor of N-myristoyltransferases, suggesting the myristoylation is required for the membrane localization of LanCL2. More direct evidence came from the use of 3H-myristic acid supplemented cell culture media and the subsequent detection of the tritiated LanCL2. A stretch of basic residues in the N-terminus of LanCL2 likely contribute to LanCL2’s membrane association as well by binding to negatively charged phospholipids, particularly to phosphotidylinositol monophosphates. Similar to what was observed in the sarcoma cell line, LanCL2 overexpression rendered UAC cells more susceptible to adriamycin-induced cell death. The phenotype was abolished if the myristoylation-defective (G→A) mutant of LanCL2 was used, suggesting the membrane localization of LanCL2 is essential for its function in adriamycin sensitization.

The two distinct mechanisms described above may be attributed to the different subcellular localization of LanCL2 in MES-SA/Dx5 and UAC cell lines. In fact, the membrane localization of LanCL2 was only reported once so far. LanCL2 was shown to reside mainly in the cytoplasm and nucleus in three different cell lines in the Human
Protein Atlas (http://www.proteinatlas.org/ENSG00000132434-LANCL2/subcellular). It is also noted that LanCL2 expression was not found to be associated with adriamycin sensitivity in some high-throughput screening studies (Ahn et al, 2004; Cleator et al, 2006; Gyorffy et al, 2005), suggesting that the function of LanCL2 in regulating adriamycin sensitivity may be cell-type dependent.

1.2.3 The role of LanCL2 in mediating ABA signaling in mammalian cells.

The plant hormone abscisic acid (ABA) regulates many growth and developmental processes, including seed and bud dormancy, senescence, and abscission. ABA is also important in response to a range of stresses, such as drought, salt, low temperature, and pathogens (Cutler et al, 2010; Manoj K.Rai, 2011). The signaling pathway of ABA in plants has been extensively studied since its discovery in the 1960’s, yet the direct receptor of ABA still remains unclear despite many proposed candidates. In 2007, Liu et al. reported that the ABA receptor may belong to the G protein-coupled receptor (GPCR) protein family (Liu et al, 2007). GCR2 was identified as a GPCR in Arabidopsis that mediates all the functions of ABA (Liu et al, 2007). However, this notion was soon disputed by three follow-up papers demonstrating (1) that GCR2 does not belong to the GPCR family, but instead belongs to the LanC-like protein family, and (2) that the role of GCR2 in ABA sensing could not be reproduced by the same genetic manipulation (Gao et al, 2007; Guo et al, 2008; Johnston et al, 2007). The interaction between GCR2 and ABA was also challenged (Risk et al, 2009).

ABA’s function as a plant hormone has been the most intensively studied, but ABA is also found in animals (Le Page-Degivry et al, 1986; Zocchi et al, 2001). An activation pathway involving a pertussis (PTX)-sensitive G protein complex has been proposed (Bruzzon et al, 2007). ABA promotes the production of cAMP and the protein kinase A (PKA)-dependent phosphorylation of ADP-ribosyl cyclase. The subsequent increase of cyclic ADP-ribose leads to increased intracellular Ca^{2+} concentration (Bruzzon et al, 2007). It has been reported that ABA modulates the immune responses in leukocytes (Bruzzon et al, 2007), and stimulates the insulin release in pancreatic cells (Bruzzon et al, 2008) and the expansion of mesenchymal stem cells (Scarfi et al, 2008). In addition, ABA has also been shown to be associated with anti-proliferation and pro-
apoptotic activities in cancer cells (Livingston, 1976; Ma et al, 2006). A more comprehensive review of the function of ABA in animal cells can be found elsewhere (Li et al, 2011).

Interestingly, despite the controversy with regard to GCR2 as the receptor of plant ABA signaling, the role of mammalian GCR2 homologs (i.e. LanCL proteins) in mediating ABA sensing in animal cells was still tested. Surprisingly, LanCL2 was shown to have a critical role in mediating ABA signaling in different biological contexts (Sturla et al, 2009). The authors reported that LanCL2 knockdown lowered ABA-activated increase of cAMP and Ca\(^{2+}\) concentration in human granulocytes. Conversely, LanCL2 overexpression further increased the Ca\(^{2+}\) concentration. Moreover, the various biological responses of granulocytes stimulated by ABA, including chemotaxis, chemokinesis, ROS production, and particle phagocytosis, were also suppressed by LanCL2 knockdown. The role of LanCL2 in mediating the ABA’s insulin release function in rat insulinoma cells was also reported (Sturla et al, 2009). The whole ABA pathway can be reproduced in an ABA-unresponsive cell line (HeLa) by overexpressing LanCL2 and the ADPRC protein (CD38), further demonstrating an indispensable role of LanCL2 in mediating ABA signaling (Sturla et al, 2009). Follow up studies reported LanCL2’s function in mediating ABA signaling in other cell types as well, such as monocytes, macrophages, and keratinocytes (Bassaganya-Riera et al, 2011; Bruzzone et al, 2012; Magnone et al, 2012).

Despite the myriad of biological activities that ABA regulates, the role of LanCL2 in the ABA signaling pathway has been mostly studied in only one biological context: inflammatory responses. Interestingly, ABA was reported to have conflicting roles in modulating the innate immune and inflammatory responses. ABA has been initially recognized as a pro-inflammatory cytokine (Bruzzone et al, 2012; Bruzzone et al, 2007; Magnone et al, 2009; Magnone et al, 2012; Sturla et al, 2009). By activating Nuclear Factor-κB (NF-κB) nuclear translocation and activity, ABA up-regulates the expression of many NF-κB-targeted pro-inflammatory cytokines, such as Tumor Necrosis Factor-α (TNF-α), prostaglandin E2, and cyclooxygenase-2 (Magnone et al, 2009). ABA also stimulates Reactive Oxygen Species (ROS) and Nitric Oxide (NO) production, immune cell migration, and phagocytosis (Bruzzone et al, 2012). However, Bassaganya-Riera and colleagues showed that ABA exerted anti-inflammatory activity through PPARγ and that
it was the endogenous PPARγ level that determined whether ABA had pro- or anti-inflammatory function in a particular cell type (Bassaganya-Riera et al, 2011). In spite of the conflict, LanCL2 is necessary for ABA function in both cases and therefore, possesses dual and conflicting functions in immune regulation.

Although the binding of ABA to GCR2 was questioned, in silico docking of ABA and LanCL2 using the LanCL1 crystal structure (Zhang et al, 2009) as the template suggested an ABA binding site in LanCL2 (Bassaganya-Riera et al, 2011). Moreover, overexpressing LanCL2 significantly stimulates ABA binding to the plasma membrane of HeLa cells, suggesting that LanCL2 may be the endogenous ABA receptor (Sturla et al, 2009). A variety of binding assays were also carried out, which provided evidence to support the direct interaction between ABA and LanCL2 (Sturla et al, 2011). However, it is still unclear how LanCL2 transduces the signal. First, there is no evidence, thus far, showing that LanCL2 is a transmembrane protein. Therefore, unless ABA can penetrate into cells it remains elusive how LanCL2 interacts with ABA in vivo. Furthermore, a PTX-sensitive G protein complex is involved in ABA signaling, but LanCL2 is not a GPCR. So either LanCL2 can signal to the G protein complex or an unidentified GPCR is involved. Regardless, much information is still missing and LanCL2’s function warrants further investigation.

ABA has shown medicinal potential in treating inflammation-related diseases, diabetes, and cancer (Guri et al, 2010; Guri et al, 2007; Guri et al, 2010; Livingston, 1976; Ma et al, 2006). As a direct interacting protein and mediator of ABA function in animal cells, LanCL2 has become a new focus of the therapeutic targeting of the ABA signaling pathway. A synthetic analog of ABA that competes for LanCL2 binding showed an inhibitory effect of ABA-induced inflammatory responses in granulocytes (Grozio et al, 2011). In silico docking analysis also revealed a new ligand of LanCL2 that activates PPARγ and ameliorates experimental colitis (Lu et al, 2012). Targeting LanCL2 grants more drug specificity than targeting any other components in the pathway because LanCL2 functions upstream in the pathway.
1.2.4 LanCL1 discovery and its potential function in erythrocytes.

LanCL1 was isolated and characterized in an effort to identify interacting proteins of stomatin in erythrocytes (Mayer et al, 1998). Stomatin is a membrane protein that may have a function in regulating an ion channel in erythrocytes. To identify the associated ion channel, different fragments of stomatin were used in an affinity chromatography purification of proteins from erythrocyte ghosts. LanCL1 was the only protein identified and was named p40 because of its size. Similar to GCR2 in plants, LanCL1 was proposed to be a GPCR due to its seven hydrophobic domains, despite the lack of sequence homology to any other proteins in the GPCR super family. This incorrect assignment was corrected by the same group two years later when studies showed that p40 was completely solubilized in alkaline buffer, indicating that it is a peripheral membrane protein instead of an integral membrane protein (Bauer et al, 2000). This finding is in striking contrast to any known GPCR. Refined sequence analysis revealed that p40 was homologous to prokaryotic lantibiotic cyclases (LanC and LanM), hence, it was renamed to LanC-like protein 1 (LanCL1). Nevertheless, LanCL1 and stomatin may still be bona fide interacting partners, but the functional significance of this interaction was, unfortunately, not further investigated.

Interestingly, the function of LanCL1 in erythrocyte biology was re-examined in the context of erythrocyte pathogenesis independently by another research group (Blisnick et al, 2005). PfSBP1 is a protein from the parasite Plasmodium falciparum, which is an integral part of the Maurer’s cleft, a membrane structure important in parasite protein export. LanCL1 was identified to be an interacting protein of the carboxyl-terminal of PfSBP1, which is located in the erythrocyte cytosol. As reported previously (Bauer et al, 2000), LanCL1 has a tight membrane association in hypotonic buffer, but is mostly solubilized in isotonic buffers. However, a significant amount of LanCL1 is recruited to the plasma membrane of P. falciparum infected erythrocytes, even in the isotonic buffer. These results suggested an interaction-dependent subcellular localization change of LanCL1. Furthermore, immunofluorescence experiments showed a Maurer’s cleft-like localization pattern of LanCL1 within infected erythrocytes and co-localization of LanCL1 and PfSBP1. The question remains whether LanCL1 participates in the parasite protein transport and pathogenesis, warranting further investigation.
1.2.5 The anti-oxidant role of LanCL1 in the central nervous system.

In an attempt to identify novel reduced glutathione (GSH)-binding proteins in the bovine brain affinity chromatography was performed and LanCL1 was the only new interacting protein identified (Chung et al, 2007). Other proteins identified include glutathione-S-transferase-µ (GST-µ) and GST-π, which are known GSH conjugating enzymes. LanCL1 was shown to have similar affinity to free GSH and oxidized glutathione (GSSG). The dissociation constant ($K_d$) for LanCL1 and GSH or GSSG were both below 1 µM, which means LanCL1 has a greater affinity for GSH than GST-A or GST-B (Jakobson et al, 1979). LanCL1 was identified in another independent study looking for GSH-binding proteins in breast cancer cells (Mladkova et al, 2010), suggesting that the LanCL1-GSH interaction is not tissue specific. Additional evidence of the LanCL1-GSH interaction was revealed when the co-crystal structure of LanCL1 and GSH was solved (Zhang et al, 2009). LanCL1 exhibits a double seven-helix barrel fold structure complexed with a Zn$^{2+}$ ion, similar to its prokaryotic homolog NisC, the LanC for nisin (Li et al, 2006). Intensive interactions mediated by hydrogen bonds were identified between GSH with LanCL1. The nitrogen atoms in LanCL1 residues R4, K317 and R364 directly interact with the oxygen atoms in glycine and glutamic acid residues in GSH based on the co-crystal structure and mutagenesis analysis (Fig 1.4). The sulfhydryl group in GSH cysteine also binds to the Zn$^{2+}$ ion as the fourth ligand with the other three ligands supplied by LanCL1. Disruption of the Zn$^{2+}$ binding site in LanCL1 (C322A) abolished GSH binding, suggesting a Zn$^{2+}$-dependent binding of GSH. The authors reported a $K_d$ of 0.7 mM between LanCL1 and GSH based on surface plasma resonance (SPR) experiment, which showed a large disparity from the previous report (Chung et al, 2007). The $K_d$ discrepancy may be attributed by the different assays performed or indicates different binding affinities of bovine and human LanCL1 to GSH. LanCL2 also bound specifically to the GSH-Sepharose resin under the same conditions, but with much lower affinity (Zhang et al, 2009). One explanation for LanCL2’s lower affinity to GSH has been suggested to be a consequence of an N-terminal histidine (H12) that is only present in LanCL2, which may serve as a fourth Zn$^{2+}$ ligand and block the GSH binding (Zhang et al, 2009).
The high expression level of LanCL1 in the brain and spinal cord as well as the binding between LanCL1 and GSH, the major antioxidant in cells, indicate possible functions of LanCL1 in the central nervous system, particularly in the antioxidant defense. Indeed, the expression level of LanCL1 was significantly regulated during the onset of amyotrophic lateral sclerosis (ALS) in an experimental mouse model (SOD1<sup>G93A</sup>), which suggested the possible involvement of LanCL1 in this disease (Chung et al, 2007). In a different study LanCL1 was found to interact with a small thioether compound lanthionine ketimine (LK) in the mammalian brain (Hensley et al, 2010). Interestingly, LK exhibited neuroprotective and neurogenic activities both in cell culture and ALS mouse models (Hensley et al, 2010; Hensley et al, 2010), suggesting a role for LanCL1 in central nervous system diseases through binding to sulfur-containing small molecules. LanCL1 was also indicated to have a function in neurite outgrowth in PC12 cells by interacting with the SH3 domain of the signaling protein Eps8 (Zhang et al, 2009).

Mostly recently, Huang et al. reported an anti-oxidant function of LanCL1 in the central nervous system based on studies in LanCL1 knockout mice (Huang et al, 2014). LanCL1 expression is induced by normal neuronal development, increased neuronal activity and oxidative stress (Huang et al, 2014). Reactive oxygen species (ROS) were accumulated in Lancl1 -/- mice, causing enhanced damage to lipid, protein and DNA, as well as mitochondria dysfunction. These mice also exhibited increased cell apoptosis in the brain starting from 8- to 12- weeks old, accompanied with neuroinflammatory responses. More importantly, cortical neurons derived from lancl1 -/- mice which had a conditional expression of LanCL1 in the brain displayed resistance to H<sub>2</sub>O<sub>2</sub>-induced lipid peroxidation and cell death, demonstrating a role of LanCL1 in protecting neuronal cells from oxidative stress (Huang et al, 2014). Interestingly, the authors also found that LanCL1 had GST-like catalytic activity, conjugating GSH to common reporter substrates such as 1-chloro-2,4-dinitrobenzene (CDNB) and p-nitrophenyl acetate (Huang et al, 2014). It is noteworthy that the same attempt was made when LanCL1 was identified as an interacting protein of GSH, but no GST-like or other GSH-related activities was reported (Chung et al, 2007). Regardless, it was the first time that LanC-like proteins
were directly involved in sulfur chemistry in eukaryotes, as an important extension of functions of their prokaryotic homologs.

1.2.6 The role of LanCL1 in binding and inhibiting cystathionine-β-synthase (CβS).

Cystathionine-β-synthase (CβS) is a trans-sulfuration enzyme that condenses serine and homocysteine to form cystathionine, which in turn is transformed into cysteine by cystathionine γ-lyase. CβS was also reported to catalyze the reaction between serine and cysteine to form lanthionine \textit{in vitro}, which is converted to lanthionine ketimine (LK). Since LanCL1 binds LK (Hensley et al, 2010), its relationship with CβS is intriguing and was therefore investigated by Luo and colleagues (Zhong et al, 2012). LanCL1 was found to interact with CβS in HEK293 cells and in mouse cortex. The N-terminal regulatory and the C-terminal auto-inhibitory domains in CβS are required for the interaction. A more detailed truncation strategy was used for LanCL1 mapping because of the lack of distinct domain structures in LanCL1 and the minimum required fragment identified was amino acids 158-169. As expected, a peptide including LanCL1_{153-173} but not the scrambled control dramatically disrupted the interaction of LanCL1 and CβS. It is interesting that amino acids 158-169 actually reside in the inner helix barrel based on LanCL1 structure, raising the question how this “hidden” fragment participates in the protein-protein interaction.

The interaction between LanCL1 and CβS was shown to have an inhibitory effect on CβS as either LanCL1 knockdown or the use of the decoy peptide LanCL1_{153-173} increased CβS activity (Zhong et al, 2012). H_{2}O_{2}-induced decrease of the intracellular GSH/GSSG ratio was shown to disrupt LanCL1-CβS interaction. This regulation might be mediated by the ability of LanCL1 to bind GSH because the interaction between a GSH-binding defective LanCL1 mutant and CβS was resistant to the regulation (Zhong et al, 2012). The authors proposed that oxidative stress induced a change in the GSH/GSSG ratio, which disrupted LanCL1-CβS interaction and increased the activity of CβS. The subsequently elevated synthesis of cystathionine boosted the level of cysteine and GSH, protecting cells from the oxidative stress (Zhong et al, 2012). This mode of regulation, however, was questioned regarding to whether the increased cystathionine can be efficiently transformed to GSH in brain and protect cells from the oxidative stress.
Since an increase in total glutathione in cells was not reported (Zhong et al., 2012), it remains elusive how the disruption of LanCL1-CβS interaction exhibited protective effects on cells from oxidative damage. On the other hand, it is intriguing that LanCL1 displayed a negative effect on neuroprotection, which is in contrast with another report (Huang et al., 2014). Further investigations are warranted to understand the function of LanCL1 in neuroprotection.

1.3 SUMMARY OF INVESTIGATIONS PRESENTED IN THIS THESIS

Since the discovery of human LanCL1 and LanCL2 at the beginning of the century, many studies have been carried out towards the understanding of their functions and interesting findings have been made, especially in the most recent 5 years. However, much is still unknown about these proteins. In this work, efforts towards a better understanding of LanC-like proteins were made and novel functions and mechanisms of action were revealed. The characterization of LanCL2 function in mammalian signaling pathways is described in Chapter 2 and a novel function of LanCL2 as a regulator of Akt activity was identified. LanCL2 promotes Akt phosphorylation and cell survival in liver cells and this regulation is through directly facilitating Akt phosphorylation by mTORC2. Chapter 3 describes the establishment of LanCL knockout mouse lines and the phenotypic characterization of Lancl2−/− mice. Shortened life span was observed in Lancl2−/− mice, accompanied with a symptom of cardiomyopathy. These mice also showed increased susceptibility to microbial infections, suggesting a compromised immune system. In Chapter 4, the role of LanCL proteins in lanthionine ketimine biosynthesis was investigated. The detection of lanthionine ketimine in LanCL triple knockout mice argues against an essential role of LanCL proteins in this pathway and suggests the need of a systematic screening for potential LanCL substrates. Lastly, Appendices A and B describe a functional study of LanCL2 in HEK293 and MCF-7 cells and the identification of novel LanCL2 interacting proteins, respectively.
Fig 1.1 Illustration of the dehydration and cyclization steps in lanthipeptide synthesis.
Fig 1.2 Schematic representation of the four classes of lanthipeptide synthetases.
Fig 1.3 Sequence alignment of NisC and human LanCL proteins. Protein sequences of NisC from *Lactococcus lactis* and LanCL1, LanCL2 and LanCL3 from *Homo sapiens* were aligned by Clustal Omega. The conserved zinc ligands among the proteins are indicated by purple triangles and the conserved active site residues are indicated by red triangles.
Fig 1.4 Crystal structure showing the interaction between LanCL1 and GSH. GSH is shown by a green stick model. Selected residues in LanCL1 are depicted by yellow stick models. Zn$^{2+}$ is shown as a magenta sphere. Oxygen, nitrogen, and sulfur atoms are colored red, blue, and yellow, respectively. Hydrogen bonds are shown as dashed black lines. Amino acid residues in the binding site and those of GSH are labeled as single letters.
1.5 REFERENCES


CHAPTER 2. LANTIBIOTIC CYCLASE-LIKE PROTEIN 2 (LANCL2) IS A NOVEL REGULATOR OF AKT

2.1 INTRODUCTION

The Ser/Thr protein kinase Akt belongs to the AGC kinase family and plays a central role in a variety of cellular functions, including cell proliferation, cell survival and glucose metabolism (Lawlor & Alessi, 2001). Deregulation of Akt activity is closely associated with several human diseases, such as cancer, diabetes, and cardiovascular and neurological diseases. Hyperactivation of Akt is one of the most common hallmarks in human malignancy, making Akt and its signaling pathways important therapeutic targets in cancer treatment (Bellacosa et al, 2005). On the other hand, Akt has an established function to suppress neuronal death (Datta et al, 1997; Dudek et al, 1997). Restoration of decreased activity of Akt represents an important approach in treating neurodegenerative diseases, including Parkinson’s disease (Namikawa et al, 2000; Ries et al, 2006).

Akt can be activated by a wide range of extracellular signals, including many growth factors and cytokines. In the case of insulin signaling, insulin receptor (IR) is activated and recruits insulin receptor substrate (IRS) to the plasma membrane, where IRS is phosphorylated and in turn provides a docking site for the p85 regulatory subunit of phosphatidylinositide 3-kinase (PI3K). The interaction with IRS induces a conformational change in p85, which activates the catalytic subunit p110 of PI3K (Myers et al, 1992; Ruderman et al, 1990). Phosphatidylinositol (3,4,5)-trisphosphate (PIP3), the product of PI3K, recruits phosphoinositide-dependent kinase 1 (PDK1) and Akt to the plasma membrane where Akt is phosphorylated by PDK1 at Thr308 in the T loop (Alessi et al, 1997). Akt is also phosphorylated in the C-terminal hydrophobic motif at Ser473 by mammalian target of rapamycin complex 2 (mTORC2) (Sarbassov et al, 2005). Dual phosphorylation of Akt results in its full activation. In addition, Akt activity is negatively

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regulated by protein phosphatases. The phosphatases responsible for dephosphorylating Thr308 and Ser473 sites belong to the protein phosphatase 2A (PP2A) and PP2C family, respectively (Gao et al, 2005).

The activity of endogenous Akt is also subject to context-dependent regulation/fine-tuning by many interacting proteins. In the past two decades, more than 20 of these proteins have been discovered, including both activators and inhibitors of Akt function (Franke, 2008). Many of these regulators do not have discernable enzymatic activity, and they regulate Akt function either by directly stimulating/inhibiting its kinase activity, or affecting its subcellular localization or access to other regulators.

As described in Chapter 1, the eukaryotic LanC-like proteins are homologs of prokaryotic LanC, which is a cyclase involved in the biosynthesis of lantibiotics (Knerr & van der Donk, 2012). The human genome encodes three LanC-like proteins, LanCL1, 2 and 3, the functions of which are largely unknown. Human LanCL2 has been suggested to have a role in adriamycin sensitizing and the abscisic acid (ABA) signaling pathway (Landlinger et al, 2006; Magnone et al, 2012; Park & James, 2003; Sturla et al, 2009). But the biochemical nature and mechanism of action of LanCL2 remain elusive. Here, I investigated the potential function of LanCL2 in mammalian cells by examining its role in major signaling pathways. The findings reveal LanCL2 to be a novel regulator of Akt activity and cell survival.

2.2 MATERIALS AND METHODS

Antibodies and reagents – The antibodies used were obtained from the following sources: Anti-LanCL2 antibody was generated by Proteintech Group Inc using full-length recombinant LanCL2 protein as the antigen. Anti-LanCL2 was also purchased from Abcam. Anti-LanCL1 antibody was from Abnova. Anti-FLAG M2 was from Sigma-Aldrich; anti-HA (16B12) from Covance, anti-HA (rabbit) from Promega, anti-tubulin from Abcam, anti-pPKC from Millipore, anti-His and anti-GST from Santa Cruz, anti-rictor and anti-raptor (for IP) from Bethyl Lab, and all other antibodies from Cell Signaling Technology. Cobalt beads and anti-HA agarose beads were purchased from Thermo Fisher. TNFα was from Cell Signaling Technology. Adriamycin and okadaic acid were from LC laboratories. Fibronectin was from Calbiochem. GST-Akt was from
SignalChem. His-Akt was from Millipore. All other reagents were obtained from Sigma-Aldrich.

**Plasmids** – Human LanCL2 cDNA was subcloned in the p3×FLAG-CMV-14 vector (Sigma-Aldrich), with the 3xFLAG tag fused at the C-terminus of LanCL2.

Human Akt1 cDNA was subcloned in the pCDNA3 vector (Invitrogen) with an HA tag at the N-terminus. pCMV6myristoylated-HA-Akt (HA-myr-Akt) was previously described (Erbay et al, 2003). pCDNA3HA-Akt1-K179M was subcloned from pLNCX-HA-Akt1-K179M (Addgene plasmid #9007) (Ramaswamy et al, 1999).

**Cell culture and transfection** – All cell lines used in this study were obtained from ATCC. The following media were used to maintain various types of cells: Dulbecco's Modified Eagle Medium/Ham's F-12 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) for HepG2 cells, DMEM containing 10% FBS and 1% pen/strep for HEK293 cells, MEM containing 10% FBS and 1% pen/strep for Hep3B cells, MFE support medium (Xenotech) for Ea1C-35 cells, and BEGM (LONZA) supplemented with 70 ng/ml phosphoethanolamine and 5 ng/ml EGF for THLE-2 cells. All cells were maintained at 37 °C with 5% CO2. Plasmid DNA transfection was performed using TransIT-LT1 (Mirus Bio LLC) in HepG2 cells or Polyfect (QIAGEN) in HEK293 cells, according to the manufacturers’ instructions.

**Lentivirus-mediated RNAi** – LanCL1 and LanCL2 lentiviral shRNA constructs in the pLKO vector were obtained from Sigma-Aldrich (TRC library). Their clone IDs are: LanCL2 #1 (L1), TRCN0000045403; LanCL2 #2 (L2), TRCN0000045406; LanCL1, TRCN0000011687. mTOR and rictor lentiviral shRNA constructs were from Addgene, as plasmid 1855 and plasmid 1853, respectively (Sarbassov et al, 2005). Lentivirus packaging was performed by co-transfecting pLKO-shRNA, pCMV-dR8.91, and pCMV-VSV-G into HEK293T cells using TransIT-LT1 at 0.5, 0.45, and 0.05 µg, respectively (for 1 well in a 6-well plate). Media containing viruses were collected 48 h after transfection. HepG2 and other cells were infected with the viruses in the presence of 8 µg/ml polybrene for 24 h and were then subjected to selection with various concentrations of puromycin.

**Western blotting** – Cells were lysed in MIPT lysis buffer (50 mM Tris-Cl, pH 7.2, 137 mM NaCl, 25 mM NaF, 25 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 10
mM sodium pyrophosphate, 0.3% Triton X-100, and 1x protease inhibitor cocktail (Sigma-Aldrich)) and microcentrifuged at 16,100 x g for 10 min at 4 °C. The supernatant was mixed 1:1 with 2 x SDS sample buffer and heated at 95 °C for 5 min. Proteins were resolved on SDS-PAGE, transferred onto PVDF membranes (Millipore), and incubated with various antibodies following the manufacturers’ recommendations. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Inc.), and images were developed on x-ray films.

**Immunoprecipitation** – Cells were lysed in MIPT lysis buffer or NP40-based lysis buffer (20 mM Tris-Cl, pH 7.5, 0.2% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1.5 mM MgCl$_2$, 137 mM NaCl, 50 mM NaF, 1 mM NaVO$_3$, 12 mM β-glycerophosphate, 1x protease inhibitor cocktail (Sigma-Aldrich)) and microcentrifuged at 16,100 g for 10 min at 4 °C. The supernatant was incubated with anti-FLAG beads or anti-HA beads (Sigma-Aldrich) for 2 h. The beads were then washed 3 times with lysis buffer and boiled in 2x SDS sample buffer for 5 min followed by Western blotting. For immunoprecipitation of endogenous IRS1, incubation with anti-IRS1 antibody was followed by incubation with protein A beads.

**His-LanCL2 pull down** – For His-LanCL2 pull down of endogenous Akt, cells were lysed in His pull down buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 25 mM NaF, 25 mM β-glycerophosphate, 0.1 mM NaVO$_3$, 20 mM imidazole, 0.3% Triton X-100, and 1x protease inhibitor cocktail (Sigma-Aldrich)) and incubated with 10 µg His-LanCL2 protein for 2 h at 4 °C, followed by incubation with Cobalt beads for another 1 h. The beads were then washed 3 times with the lysis buffer and boiled in 2x SDS sample buffer for 5 min. For LanCL2-Akt in vitro binding, His-LanCL2 and GST-Akt were directly mixed in His pull down buffer for 2 h followed by incubation with Cobalt beads. The beads were then washed and boiled as described above.

**mTORC1 and mTORC2 kinase assay** – mTORC1 and mTORC2 were immunoprecipitated from cell lysates with anti-raptor or anti-rictor antibody, respectively. The kinase assays were performed as described (Ikenoue et al, 2009). mTORC2 kinase assay was carried out at 37 °C for 30 min in mTORC2 kinase buffer (25 mM HEPES, pH 7.4, 100 mM potassium acetate, 1 mM MgCl$_2$, and 500 µM ATP) with
62 ng His-Akt as the substrate. mTORC1 kinase assay was carried out at 30 °C for 30 min in mTORC1 kinase buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 10 mM MgCl$_2$ and 250 µM ATP) with 16 ng GST-S6K1 (aa332–421) as the substrate. Reactions were stopped by adding 2xSDS buffer and boiling.

**TUNEL assay and immunostaining** – TUNEL assays were performed following manufacturer’s manual (Roche). For immunostaining, cells were fixed with 3.7% formaldehyde followed by permeabilization with 0.1% Triton X-100 and blocking with 3% BSA in PBS. Cells were then incubated with antibodies against cleaved PARP and HA for 2 h at RT followed by incubation with Alexa Fluor-labeled secondary antibody and DAPI for 30 min. The stained cells were examined with a Leica DMI 4000B fluorescence microscope, and the fluorescent images were captured using a RETIGA EXi camera, and analyzed with Q-capture Pro51 software (QImaging™).

### 2.3 RESULTS

#### 2.3.1 LanCL2 positively regulates Akt phosphorylation.

To probe the function of LanCL2 in mammalian cells, I used lentivirus-delivered shRNA to knock down LanCL2 in multiple cell lines and examined the response of several major signaling pathways. LanCL2 knockdown suppressed serum-stimulated Akt phosphorylation on both Ser473 and Thr308 in human hepatocarcinoma HepG2 cells (Fig 2.1A), suggesting impaired Akt activation. This effect on Akt was further validated by decreased phosphorylation of GSK3β, a known substrate of Akt, in LanCL2 knockdown cells (Fig 2.1B). Insulin stimulation of Akt phosphorylation was similarly affected by LanCL2 knockdown, and two independent shRNAs elicited similar effects (Fig 2.1C), confirming the on-target specificity of the RNAi. The phosphorylation levels of two other kinases in the AGC family, S6K1 and cPKC, were not affected by LanCL2 knockdown, and neither was phosphorylation of Erk (Fig 2.1A). Hence, the effect of LanCL2 knockdown appears to be specific to Akt. Conversely, overexpression of FLAG-tagged LanCL2 modestly, but reproducibly, increased Akt phosphorylation (Fig 2.1D).

Interestingly, the effect of LanCL2 knockdown was only observed in HepG2 cells and not in several other human cell lines examined under the same conditions, including HEK293, HeLa, and MCF-7. Hence, we wondered if this might be a liver cell-specific
function of LanCL2 and set out to test it. I knocked down LanCL2 in three other liver cell lines – hepatocarcinoma Hep3B cells, and SV40 large T antigen-immortalized Ea1C-35 and THLE-2 liver cells, and found that serum-stimulated Akt phosphorylation was impaired in all three cell lines (Fig 2.1E). Collectively, these data suggest a positive role of LanCL2 in the regulation of Akt activation in liver cells. Of note, LanCL2 was found to be expressed in all cell lines I examined, with some variation in expression levels that did not correlate with its knockdown effect on pAkt (Fig 2.2). Knockdown of LanCL1, which is also expressed in HepG2 cells, did not affect Akt phosphorylation (Fig 2.1F).

2.3.2 LanCL2 does not regulate Akt phosphorylation through the canonical insulin signaling pathway or its phosphatase PP2A.

In search of the mechanism by which LanCL2 regulates Akt phosphorylation, I first asked whether LanCL2 functions through the insulin receptor-IRS-PI3K pathway. As shown in Fig 2.3A, LanCL2 knockdown had no effect on the level of insulin receptor or its phosphorylation at the activation sites Tyr1150/1151 in response to insulin. Moreover, the insulin-stimulated interaction between endogenous IRS1 and the p85 subunit of PI3K was also unaffected by LanCL2 knockdown (Fig 2.3B). Thus, LanCL2 does not appear to regulate Akt phosphorylation through these canonical upstream components. I then investigated whether LanCL2 functions by regulating the phosphatase PP2A. As shown in Fig 2.3C, okadaic acid, a specific inhibitor of PP2A, did not rescue Akt phosphorylation at T308 in LanCL2 knockdown cells, suggesting that LanCL2 does not function through inhibiting PP2A.

2.3.3 LanCL2 interacts with Akt.

With the known upstream regulators of Akt and its phosphatase PP2A ruled out as targets of LanCL2 regulation, we considered the possibility of a direct interaction between LanCL2 and Akt. This possibility was first tested in HEK293 cells as an exogenous system because of the ease to achieve higher transfection efficiency. Co-immunoprecipitation (co-IP) of transiently expressed FLAG-LanCL2 and endogenous Akt was observed (Fig 2.4A), and the reciprocal co-IP performed with HA-Akt and FLAG-LanCL2 further confirmed this interaction (Fig 2.4B). Next, I examined and
confirmed this interaction in HepG2 cells by either pulling down endogenous Akt with His-LanCL2 purified from bacteria, or performing co-IP of transiently expressed FLAG-LanCL2 and HA-Akt (Fig 2.4C and D). The interaction was also detected between purified His-LanCL2 and GST-Akt (Fig 2.4E), suggesting that the interaction is direct and not mediated by other protein(s).

To map the LanCL2 interaction site on Akt, various deletion mutants of Akt were generated and examined for their binding to LanCL2. As shown in Fig 2.4F, the Akt fragment aa120-433, containing mostly the kinase domain, bound to LanCL2 to a similar extent as intact Akt. It is not feasible to map the Akt-interacting domain on LanCL2, because our preliminary data suggests a double seven-helix barrel fold structure of LanCL2 similar to that of NisC and LanCL1 (Li et al, 2006; Zhang et al, 2009), making it unlikely for any fragment of LanCL2 to maintain its native fold.

Next, we asked whether the interaction between LanCL2 and Akt was affected by the activation status of Akt. Interestingly, serum-stimulation diminished Akt interaction with LanCL2 as shown in Fig 2.5A. Furthermore, LanCL2 displayed higher affinity for a kinase-inactive mutant of Akt (K179M) (Franke et al, 1995) than for a constitutively active Akt (myr-Akt) (Andjelkovic et al, 1997) (Fig 2.5B). These observations suggested that LanCL2 might have a higher affinity for inactive Akt. Alternatively, the interaction might simply be influenced by the subcellular localization of Akt, as both serum stimulation and tagging by a myristoylation signal (myr-Akt) translocate Akt to the plasma membrane. To distinguish between the two possibilities, Akt-K179M was compared to Akt-WT under serum stimulation, as Akt translocation is dependent on its PH domain but independent of its kinase activity. As shown in Fig 2.5C, LanCL2 displayed higher affinity for Akt-K179M than for Akt-WT. Taken together, these findings suggest that LanCL2 favors binding to inactive Akt.

2.3.4 LanCL2 enhances Akt phosphorylation by mTORC2.

In light of the physical interaction between Akt and LanCL2, and the lack of any known catalytic activity for LanCL2, I set out to test a simple model in which LanCL2 facilitates Akt recruitment to its kinases. PDK1 and mTORC2 are known to phosphorylate T308 and S473, respectively (Alessi et al, 1997; Sarbassov et al, 2005),
although other kinases may also be involved in a context-dependent manner. Indeed, we found that LanCL2 co-immunoprecipitated mTOR and rictor (core components of mTORC2) (Fig 2.6A), but not PDK1 (data not shown). While knockdown of rictor did not affect LanCL2-mTOR pulldown, knockdown of mTOR clearly weakened LanCL2-rictor interaction (Fig 2.6B and C), suggesting that mTOR, rather than rictor, may directly interact with LanCL2. Indeed, I also consistently found a small amount of raptor in the LanCL2 immunoprecipitates, as would be expected if LanCL2 interacted with mTOR.

A straightforward scaffold model would predict the dependence of Akt-rictor interaction on LanCL2. However, that is not the case, as knockdown of LanCL2 had no detectable effect on the Akt-rictor interaction (Fig 2.6D). Nevertheless, this observation does not rule out the possibility that LanCL2 binding may be important for optimal catalytic activity of mTORC2 toward Akt. To test this model, in vitro kinase assays were performed with immunoprecipitated endogenous mTORC2 and recombinant Akt as the substrate, with and without the addition of His-LanCL2 purified from bacteria. As shown in Fig 2.6E, recombinant LanCL2 clearly enhanced Akt Ser473 phosphorylation in vitro. Importantly, mTORC1 kinase activity toward S6K1 was not affected by recombinant LanCL2 (Fig 2.6E), which is consistent with the observation that LanCL2 knockdown did not affect S6K1 T389 phosphorylation in cells (Fig 2.1A). These observations strongly suggest that LanCL2 specifically regulates Akt phosphorylation by mTORC2, even though LanCL2 may interact with both mTORC1 and mTORC2. The physical interaction between LanCL2 and Akt may have contributed to this specificity, as I did not detect any LanCL2-S6K1 interaction (Fig 2.6F).

The effect of LanCL2 on mTORC2 kinase activity toward Akt prompted us to examine another substrate of mTORC2, SGK1 (Garcia-Martinez & Alessi, 2008). Phosphorylation by mTORC2 activates SGK1, which in turn phosphorylates NDRG1 at Thr346. The latter is widely used as the readout of SGK1 activity in cells (Murray et al, 2004). Interestingly, NDRG1 phosphorylation was suppressed by LanCL2 knockdown (Fig 2.7), suggesting that LanCL2 may facilitate SGK1 phosphorylation by mTORC2 as well. However, I was not able to reliably assess whether LanCL2 interacted with SGK1 because of difficulty detecting endogenous SGK1 with available antibodies.
2.3.5 LanCL2 promotes cell survival through Akt.

Among a wide range of cellular functions controlled by Akt is cell survival. Activated Akt protects a variety of cell types from apoptosis (Duronio, 2008). We therefore wondered whether LanCL2 was involved in this cellular process because of its role in regulating Akt phosphorylation. Indeed, I observed cleavage of poly (ADP-ribose) polymerase (PARP), a marker of apoptosis, induced by LanCL2 knockdown in HepG2 cells (Fig 2.8A). Apoptosis induced by TNFα/cycloheximide (CHX) or adriamycin was also enhanced by LanCL2 knockdown (Fig 2.8A). DNA fragmentation, another important marker of programmed cell death, was also significantly increased in LanCL2 knockdown cells as measured by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays (Fig 2.8B). Importantly, expression of a constitutively active form of Akt (myr-Akt) effectively suppressed TNFα/CHX-induced apoptosis in LanCL2 knockdown cells (Fig 2.8C), suggesting that LanCL2 promotes cell survival through activation of Akt.

2.4 DISCUSSION AND OUTLOOK

In the current study, I have established LanCL2 as a novel regulator of Akt phosphorylation. LanCL2 interacts with Akt and promotes the maximal phosphorylation of Akt in response to mitogenic signals. I also show that LanCL2 binds mTORC2 and directly promotes the mTORC2 kinase activity towards Akt. Furthermore, we demonstrate the biological significance of this novel regulation by showing that LanCL2 promotes cell survival through Akt. These findings add another player to the growing list of modulators for Akt, a central regulator of myriad cellular and developmental processes. The observation that LanCL2 favors binding to inactive Akt is reminiscent of several other positive regulators of Akt, such as APPL (Adaptor protein containing PH domain, PTB domain and Leucine zipper motif) and APE (Akt-phosphorylation enhancer) (Anai et al, 2005; Mitsuuchi et al, 1999). Upon activation, Akt undergoes a dramatic conformational change in the catalytic domain (Yang et al, 2002), where LanCL2 binds, which may lead to the dissociation of LanCL2. This binding preference for inactive Akt also implies that LanCL2 is involved in the activation of Akt rather than maintaining its phosphorylation or activity. Supporting this notion, LanCL2 does not
function through the phosphatase of Akt – PP2A, but acts instead directly to enhance Akt phosphorylation by mTORC2.

Enhancing Akt phosphorylation through its kinases is one of the reported mechanisms for Akt interacting proteins. Freud-1/Aki1 and BSTA are two examples in this category, functioning as scaffold proteins to facilitate Akt-PDK1 and Akt-ri tor binding, respectively (Nakamura et al, 2008; Yao et al, 2013). LanCL2, however, does not seem to be necessary for the tethering of Akt and mTORC2 despite its interaction with both proteins. Nevertheless, LanCL2 may contribute to optimal catalysis by orienting the substrate (Akt) for the kinase (mTORC2), or rendering Akt more accessible to mTORC2 for subsequent phosphorylation. In other words, LanCL2 may help to achieve a catalytically more effective interaction between Akt and mTORC2. This catalytic function of scaffold proteins has been proposed before. For instance, the yeast scaffold protein Ste5 is found to primarily function through tethering components of the MAPK pathway but it most likely also contributes to orient kinase/substrate for optimal catalysis (Park et al, 2003). Interestingly, NDRG1 phosphorylation is also suppressed in LanCL2 knockdown cells, indicating a dampened SGK1 activity. LanCL2 may facilitate SGK1 phosphorylation by mTORC2 in the same way as Akt. On the other hand, the absence of a LanCL2-S6K1 interaction coincides with the absence of a LanCL2 effect on mTORC1 kinase activity towards S6K1, corroborating our hypothesis that the physical interaction is necessary for LanCL2-mediated enhancement of substrate phosphorylation by mTOR complexes.

It is noteworthy that the phosphorylation of Akt on T308 is also affected by LanCL2 knockdown, yet LanCL2 does not interact with PDK1. This is in agreement with previous reports that Akt phosphorylation on T308 by PDK1 is dependent on the prior phosphorylation on S473 (Scheid et al, 2002; Yang et al, 2006). Therefore, when S473 phosphorylation is attenuated by LanCL2 knockdown, so is the phosphorylation on T308.

LanCL2 was identified in a screen for genes whose downregulation results in anticancer drug resistance and therefore was designated “testis-specific adriamycin sensitivity protein (TASP)” (GenBank accession no. AB035966). Two other reports also showed that LanCL2 overexpression in human epithelial cells of amniotic origin (UAC) and a human uterine sarcoma cell line (MES-SA) increased adriamycin-induced cell
death rate (Erbay et al, 2003; Landlinger et al, 2006). The observation that LanCL2 knockdown cells are more sensitive to adriamycin appears to contradict those earlier reports. However, it is important to note that the function of LanCL2 may be highly dependent on cell type and cellular context. Indeed, in another report using human gastric, pancreatic, colon and breast cancer cell lines, the expression level of LanCL2 was not correlated with adriamycin sensitivity (Gyorffy et al, 2005). Of the various cell lines examined, inhibition of Akt activation by LanCL2 knockdown is only consistently observed in liver cells. While it remains to be determined what other types of cells may harbor the same mechanism, this novel function of LanCL2 is observed in several liver cell lines including both hepatocarcinoma and non-cancerous cells. It is not clear why LanCL2 regulates Akt only in liver cells while its expression appears to be ubiquitous, but the stoichiometry of various components in the Akt pathway likely varies from tissue to tissue, which could determine the relative contribution of these regulators. As Akt is a well-established regulator of glucose metabolism and organ growth in the liver, future probing of the physiological functions of LanCL2 in those contexts is warranted.
2.5 FIGURES

Fig 2.1 LanCL2 is necessary for maximal Akt phosphorylation. All experiments were performed in HepG2 cells unless otherwise indicated, with cell lysates subjected to Western analysis. (A) Cells were infected with lentiviruses expressing LanCL2 shRNA (“L”) or a hairpin of scrambled sequence as control (“C”) for 1 day, followed by puromycin selection for 2 days. The cells were then either maintained in growth medium (“growth”), or serum-starved overnight (“starved”) and re-stimulated with 10% FBS for 20 min (“stimulated”). (B) Cells were infected as in A and maintained in growth medium before lysis. (C) Cells were infected with lentiviruses expressing LanCL2 shRNAs (“L-1” and “L-2”), serum-starved overnight, and then stimulated with 30 nM insulin for 20 min. (D) Cells were transfected with FLAG-tagged LanCL2 together with a plasmid carrying a puromycin-resistant gene, and selected in puromycin for 3 days. (E) Three cell lines as indicated were treated as in A, serum-starved overnight, and then stimulated with 10% FBS for 20 min. (F) Cells were infected with lentivirus expressing LanCL1 shRNA for 1 day, followed by puromycin selection for 2 days. The cells were then serum-starved overnight and re-stimulated with 10% FBS for 20 min.
Fig 2.2 LanCL2 expression in various cell lines. Lysates were prepared from cells maintained in growth medium, and subjected to Western analysis.
Fig 2.3 LanCL2 does not function through the insulin-IRS-PI3K pathway or PP2A.

(A) HepG2 cells were infected with lentiviruses expressing LanCL2 shRNA ("L-1, L-2") or control shRNA ("C") for 1 day, followed by puromycin selection for 2 days. The cells were then serum-starved overnight and stimulated with 30 nM insulin for 20 min, followed by cell lysis and Western analysis.

(B) Cells treated as in A were serum-starved overnight and stimulated with 100 nM insulin for 10 min prior to lysis and immunoprecipitation (IP) of endogenous IRS1.

(C) Cells were treated as in A, serum-starved, and then stimulated with 10% FBS for 20 min with or without pre-treatment with 1 µM okadaic acid ("OA") for 15 min, followed by Western analysis.
Fig 2.4 LanCL2 interacts with Akt. (A) FLAG-tagged LanCL2 was transfected in HEK293 cells and FLAG IP was carried out 24 h after transfection. Endogenous Akt was detected by Western blotting. (B) FLAG-tagged LanCL2 and HA-tagged Akt were co-expressed in HEK293 cells, and HA IP was carried out after 24 h, followed by Western analysis. (C & E) His pulldown assay was performed with purified His-LanCL2 protein and HepG2 cell lysates (C) or purified GST-Akt protein (E) and analyzed by Western blotting. (D) HepG2 cells were co-transfected with FLAG-LanCL2 and HA-Akt followed by IP with FLAG antibody. (F) FLAG-tagged LanCL2 was co-expressed with different HA-tagged Akt deletion mutants and FLAG IP was carried out 48 h after transfection.
Fig 2.5 LanCL2 favors binding to inactive Akt. (A) HepG2 cells were co-transfected with FLAG-LanCL2 and HA-Akt for 24 h, serum-starved overnight, and then stimulated with 10% FBS for 20 min prior to cell lysis and IP with anti-FLAG antibody. (B) HepG2 cells were co-transfected with FLAG-LanCL2 and myr-Akt or K179M-Akt for 24 hr, followed by IP with anti-FLAG antibody. (C) HepG2 cells were co-transfected with FLAG-LanCL2 and HA-Akt-WT or HA-Akt-K179M, and treated as in (A), followed by IP with anti-FLAG antibody.
Fig 2.6 LanCL2 binds mTOR and facilitates Akt phosphorylation by mTORC2. (A) HepG2 cells were transfected with FLAG-tagged LanCL2, and FLAG IP was carried out 48 h after transfection. (B, C) HepG2 cells were infected with lentiviruses expressing a control shRNA (“C”), rictor shRNA (“ric”), or mTOR shRNA (“M”) for 2 days, followed by puromycin selection for 2 days. Cells were then transfected with FLAG-tagged LanCL2 and FLAG IP was carried out 48 h after transfection. (D) HepG2 cells were infected with lentiviruses expressing control shRNA or LanCL2 shRNA (“L”), and selected by puromycin. Cells were then transfected with HA-Akt, and HA IP was carried out 48 h after transfection. (E) Endogenous raptor and rictor were immunoprecipitated from HepG2 cells, and subjected to mTORC1 and mTORC2 kinase assays, using GST-S6K1 and His-Akt as substrates, respectively. Phosphorylation at T389-S6K1 or S473-Akt was detected as a readout of the kinase activity. Purified His-LanCL2 (5 µg) was added before the kinase assay in the indicated samples. (F) HEK293 cells were transfected with FLAG-LanCL2 or an empty vector, followed by FLAG IP and Western analysis.
**Fig 2.7 LanCL2 knockdown impairs NDRG1 phosphorylation.** HepG2 cells were infected with control (“C”) or LanCL2 shRNA (“L-1, L-2”) lentiviruses, and selected by puromycin. Cells were starved overnight and stimulated with 10% FBS for 10 min, followed by cell lysis and Western analysis.
Fig 2.8 LanCL2 depletion sensitizes cells to apoptosis through downregulating Akt phosphorylation. (A) HepG2 cells were infected with lentiviruses expressing LanCL2 shRNA ("L1, L2") or control ("C"), selected with puromycin, and then treated with 10 ng/ml TNFα and 10 µg/ml cycloheximide (CHX) for 3.5 h or 5 µM adriamycin for 17 h, followed by Western analysis. (B) Cells treated with TNFα and CHX as in A were subjected to TUNEL assays. Data shown are average of three independent experiments, and ~30,000 cells were examined for TUNEL signals for each data point. (C) Lentivirus-infected and puromycin-selected cells were transfected with HA-myr-Akt or a control plasmid, followed by TNFα/CHX treatment as in A. The cells were fixed and immunostained for cleaved PARP and HA. The percentage of transfected cells positive for cleaved PARP was calculated. Data shown are average of three independent experiments. Paired t-test was performed to compare each data to control. *P <0.05.
2.6 REFERENCES


CHAPTER 3. GENERATION OF LANCL KNOCKOUT MICE AND CHARACTERIZATION OF LANCL2-/- MICE

3.1 INTRODUCTION

Gene knockout mouse models are valuable research tools for studying protein functions. This method permits probing of protein function in a physiological context and can thus help to establish gene-disease correlations. Furthermore, gene knockout also guarantees complete elimination of endogenous protein compared with RNAi-mediated gene knockdown. However, the lengthy procedure and low success rate of the traditional gene KO method has hampered this approach. In the past twenty years, new DNA cleavage-based genome engineering technologies have been discovered and rapidly developed, including Zinc Finger Nuclease (ZFN) (Kim et al, 1996; Porteus & Carroll, 2005), Transcription Activator-like Effector Nuclease (TALEN) (Boch et al, 2009; Miller et al, 2011; Moscou & Bogdanove, 2009) and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and Cas9 system (Cho et al, 2013; Jinek et al, 2012; Wiedenheft et al, 2012). Although the enzymes involved in each system are different, these technologies feature similar improvements over the traditional method: the homologous recombination rate is significantly increased because of the new strategy of generating double strand DNA break (Rouet et al, 1994). Therefore the isolation of embryonic stem cells is no longer required and a much higher success rate has been achieved. Consequently, the procedure is much more efficient.

Zinc Finger Nuclease (ZFNs) are a class of engineered DNA-binding proteins for targeted genome editing. The two functional domains in ZFN are a DNA-binding domain composed of a chain of zinc finger proteins and a DNA-cleaving domain, which is the nuclease domain of the endonuclease FokI (Porteus & Carroll, 2005) (Fig. 3.1). ZFN binds to a specified locus in the genome, cuts the DNA and generates a double-strand DNA break (DSB), which is subsequently healed by non-homologous end joining (NHEJ), the endogenous DNA repair process, if no template is provided. The imprecise mechanism intrinsic to NHEJ leads to a DNA frameshift in the repaired area and the consequent appearance of pre-mature codons, which serve as a signal for the decay of mRNA by the cellular RNA surveillance system (Chang et al, 2007).
Our groups and others have been investigating the function of LanCL2 in various cell types, yet no in vivo study of LanCL2 has been carried out before this study. In this chapter, I will discuss my efforts towards generating a LanCL2 knockout mouse line using ZFN technology and characterization of the mouse phenotypes, focusing on gaining insight into the function of LanCL2 in regulating inflammatory responses. I will also briefly discuss our work in generating LanCL1 and LanCL3 knockout mouse lines, as well as LanCL double and triple KO lines.

3.2 MATERIALS AND METHODS

**Mouse husbandry and microinjection** – FVB mice were maintained on a 12 h/12 h light/dark cycle with access to water and food. Microinjections of ZFN mRNAs were performed in the Transgenic Mouse Facility in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. LanCL2 ZFN mRNA (Sigma-Aldrich) was diluted to 4 ng/µl and 2.5 ng/µl with 10 mM Tris buffer, 0.1 mM EDTA (pH 7.5) for the first injection and 2.5 ng/µl for the second and third injections. LanCL1 and LanCL3 ZFN mRNAs were diluted to 2.5 ng/µl in the same buffer and two injections were performed for each of them.

**Genomic DNA isolation from mouse tail tips** – For PCR amplification followed by Surveyor assay: The tail tip (2-5 mm) was dissolved by incubating with 600 µl of extraction buffer (20 mM Tris pH7.5-8.0, 50 mM EDTA, 100 mM NaCl, 0.5% SDS and 500 µg/ml proteinase K) for 2-3 h at 55 °C. Then 240 µl of high salt solution (4.21 M NaCl, 0.63 M KCl, 10 mM Tris pH 8.0) was added to the dissolved tail and the sample was incubated for 30 min at 4 °C to precipitate proteins. The precipitated proteins were removed by centrifugation at 16,100 x g for 10 min at 4 °C and the supernatant was transferred to a new tube. Genomic DNA was precipitated by adding 2 x volume of ethanol followed by centrifugation at 16,100 x g for 10 min at 4 °C. The precipitated DNA pellet was washed once with 80% ethanol and dissolved in 200-400 µl of TE buffer (1 mM Tris pH 8.0, 0.1 mM EDTA) after a brief air dry. For PCR amplification followed by agarose gel examination: Genomic DNA was extracted from mouse tail tips using KAPA Express Extract Kits following the manufacturer’s instructions.
PCR for Surveyor assays – LanCL1: LanCL1ZFNF: 5’ TCC ATA TGT GGT TTC TGA AAA GC 3’; LanCL1ZFNR: 5’ AGC GCC AGG CAT GAA TAC 3’. LanCL2: LanCL2ZFNF: 5’ CAA AGC TGG AGA AGA TTC AAT TTA GG 3’; LanCL2ZFNR: 5’ AAG CAG AGG CTG GGT GAT AA 3’. LanCL3: LanCL3ZFNF: 5’ GTC TTG TCA CCT CCC GTC TC 3’; LanCL3ZFNR: 5’ GCT CTG GGA GAC GTG GTA GA 3’. The annealing Tm is 55 °C, 58 °C and 56 °C for LanCL1, LanCL2 and LanCL3 PCR, respectively. NEB Taq DNA polymerase and standard Taq buffer were used. The following PCR program was used for PCR for Surveyor assay and genotyping PCR with different annealing temperatures: 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 58 °C /55 °C/56 °C for 1 min, 72 °C for 30 s, and 72 °C for 10 min.

Genotyping of Lancl1-/-, Lancl2-/-and Lancl3-/- mice – LanCL1 and LanCL3: the same PCR as for Surveyor assay was performed. LanCL2: line #99 used the same PCR as for Surveyor assay; line #52: Two pairs of primers were designed to genotype the 2-nt deletion mutant: 2ntWT, CTT CCT GAT GAA CTG CTG TAT; 2ntMUT, TTC CTG ATG AAC TGC TGT GG; 200bpREV1, GAA TTA CTC CCA CCT AGA AGC; 400bpREV1, GCT CTA TTT GGT TAT GTG GGT. The annealing Tm is 55 °C. Primer 2ntWT and 200bpREV1 were paired to amplify a 200 bp DNA fragment around the ZFN cutting site only from WT mice; while primer 2ntMUT and 400bpREV1 were used to amplify a 400bp DNA fragment around the ZFN cutting site only from the 2 nt deletion mutant. PCR products were resolved by 1.5% agarose gel electrophoresis. NEB Taq DNA polymerase and standard Taq buffer were used.

RT-PCR and cloning – LanCL2 RT-PCR: The reverse transcription of mRNA to cDNA used 1 µl of Superscript II reverse transcriptase (Invitrogen) with oligo(dT) primer (Invitrogen). PCR primers are: mLanCL2RTforward: 5’ GGA AAG GTT ATG GAA TCT GC 3’; mLanCL2RTreverse: 5’ TCC ATG TGC TCC GTA ATC TAG 3’. These primers are located in mouse LanCL2 exons 5 and 6. The annealing Tm is 55 °C. PCR amplification cycle number was optimized to be semi-quantitative: a total of 24 cycles was used. NEB Taq DNA polymerase and standard Taq buffer were used. Primers designed to clone full length (FL) and truncated (Trun) mouse LanCL2: mFLLanCL2F: 5’ GAA AGA ATT CAA TGG GCG AGA CCA TGT CA 3’; mFLLanCL2R: 5’ GAA AGG ATC CAT CCT TCT GCA AAA AGC C 3’; mTrLanCL2F: 5’ GAA AGA ATT
CAA TGG GCG AGA CCA TGT CA 3'; mTrLanCL2R: 5' AAA AGG ATC CCA GTT CAT CAG GAA GCT C 3'. The annealing Tm: 55 °C. Platinum Pfx DNA polymerase (Invitrogen) was used.

**Founder identification using Surveyor mismatch endonuclease assay** – Genomic DNA was extracted from mouse tail tips cut from three week old pups and a 400 bp fragment surrounding the ZFN cutting site was amplified by primer sets ZFNforward and ZFNreverse. The PCR product (≥50 ng/µl) was denatured and hybridized using the following program: 95 °C for 10 min; 95 °C to 85 °C, –2 °C/s; 85 °C to 25 °C, –0.1 °C/ s; 4 °C, indefinitely. The rehybridized PCR product (about 15 µl) was incubated with 1 µl of enhancer and 1 µl of Nuclease S for 1 h at 42 °C. The cleaved products were resolved by 3% NuSieve DNA agarose gel electrophoresis.

**TA cloning and sequencing** – The PCR product amplified using primers ZFNforward and ZFNreverse was cloned using the TOPO TA cloning kit (Invitrogen) following the manufacturer’s instructions. At least 4 colonies were picked from each transformation and the plasmid DNA was sent for sequencing.

**RNA and protein extraction from mouse tissues** – Mouse brain, heart, liver and skeletal muscle were dissected and snap frozen in liquid nitrogen immediately. Frozen tissues were homogenized by grinding with a pestle and mortal. For protein extraction, the homogenized powder was lysed in tissue extraction buffer (200 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 2% triton X-100 and a protease inhibitor cocktail) and rotated for 30 min at 4 °C. After centrifugation at 16,100 x g for 10 min, the supernatant was transferred to a new tube and boiled with 1x volume of 2 x sample buffer for SDS page and Western blotting. RNA was extracted from the homogenized powder using the RNeasy Kit (QIAGEN) following the manufacturer’s instructions.

**Vaginal smear test** – The female mouse was held with its head slightly tilted down and vagina exposed. A plastic pipette tip filled with 10 µl of PBS was placed into the vagina and the PBS was used to flush the vagina gently 3 times. The final flush was placed on a glass slide and observed under a light microscope with a 10x objective.
**Microarray analysis** – Affymetrix GeneChip Mouse Gene 1.0 ST Array was used to compare the gene expression between WT and Lancl2-/- mouse brain. RNA was extracted from mouse brain using RNeasy Kit (QIAGEN) and the integrity of the RNA was confirmed by running the RNA on a bioanalyzer and a RNA agarose gel. Single sense-strand cDNA preparation was performed with 200 ng total RNA for Cycle 1 using Ambion P/N 4425209 Revison C and 15 µg cRNA for Cycle 2 using Ambion-manufactured Affymetrix Whole Transcript Expression Kit P/N 4411973. CDNA fragmentation, labeling and hybridization followed Affymetrix protocol P/N 702808 Rev 4 using Affymetrix WT Terminal Labeling Kit P/N 900670. The wash, stain and scan process followed Affymetrix P/N 702731 Rev 3 using Hybridization Wash and Stain Kit P/N 900720. Data were analyzed by R algorithm.

**Bone marrow-derived macrophage (BMDM) isolation** – The bone marrow was flushed from femurs of WT and Lancl2-/- mice with 2-3 ml of serum-free RPMI media. The cells were filtered through a 70 µm cell strainer and centrifuged at 300 x g at RT for 10 min. Cell pellets were suspended in ACK hypotonic buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4) for 1-2 min to remove red blood cells. PBS (2 x volume of ACK buffer) was added before centrifugation of the cells at 300 x g for 10 min. Cell pellets were suspended in RPMI complete medium supplemented with 40-50 ng/ml CSF at a concentration of 10⁵ cells /ml and plated on a 10-cm plate. Nine days later, cells were trypsinized, counted and plated on a 24-well plate at a concentration of 10⁶ cells/ml. Cells were subsequently starved in RPMI medium with 0.5% FBS overnight and stimulated with 1 µg/ml LPS in RPMI complete medium.

**Mouse embryonic fibroblast (MEF) isolation** – Mouse embryos at age E12.5 to E14.5 were isolated, with the heads and visceral organs removed. The rest of the body was cut into small pieces using a razor blade and digested in trypsin for 5 min. Cell clumps were disrupted by passing through an 18 gauge needle several times and single cells were collected from the supernatant after centrifugation at 300 x g. Primary MEFs were only passaged up to 7 times for experiments before being discarded.

**Lipopolysaccharide (LPS)/GalN-induced septic shock** – Age (9 weeks old) and sex-matched WT, Lancl2+/- and Lancl2-/- mice were injected intraperitoneally with 1 mg/g body weight D-galactosamine combined with 0.1 µg LPS purified from *Escherichia*
coli 0111:B4 (Sigma). Mice were monitored for mortality every 20 min starting from 5 h to 10 h after injection.

**QRT-PCR** – RNA was extracted by TRIzol (Ambion). cDNA was synthesized using 1 µg of Superscript II reverse transcriptase (Invitrogen) using oligo(dT) primer (Invitrogen). Quantitative RT-PCR was performed on the StepOne Plus system using SYBR green in a MicroAmp 96-well reaction plate following the manufacturer’s protocols. β-Actin was used as a reference to obtain the relative change for target samples using the comparative $C^T$ method. The primers for TNFα were obtained from Qiagen.

**ELISA** – Mouse serum collected from the mandibular vein 1 h after LPS/GalN injection or cell medium collected 1 h and 3 h after LPS stimulation of BMDM were subjected to TNFα measurement using the ELISA kit from BD Sciences, following the manufacturer’s instructions.

### 3.3 RESULTS

#### 3.3.1 Generation of LanCL2 knockout mice.

The ZFN for knocking out LanCL2 in the mouse genome was designed by Sigma-Aldrich. It binds and cuts the fourth exon of LanCL2 (Fig. 3.1). Whole genome bioinformatics analysis by Sigma-Aldrich suggested that the chance of non-specific targeting by this ZFN pair was very low (Table 3.1). The *in vitro* activity of LanCL2 ZFN in mouse neuro2A cells was shown to be 10% or 12.6% by mRNA or DNA transfection, respectively (Sigma-Aldrich).

To generate LanCL2 KO founder mice, embryos at pronuclear stages were collected and microinjected with ZFN mRNA before they were transferred to pseudo pregnant females. A total of 102 pups were born and genotyped. Briefly, a 400 bp genomic DNA fragment around the ZFN cutting site was amplified by PCR and analyzed for potential mutation by a Surveyor mismatch endonuclease assay (Qiu et al, 2004). The genomic DNA from 7 founder mice showed consistent cleavage in the Surveyor assay (Fig 3.2A). The 400 bp PCR product from the seven mice was also cloned and sequenced to identify the exact genotype of each mouse. Of the seven founder mice, four (#3, #32, #41, #42) had a 1-nt deletion at the target site, two (#27, #52) had a 2-nt deletion and one (#99) had a 61-nt deletion (Fig 3.2B). The ratio of mutated colonies over all colonies sequenced...
suggests that these founder mice were all mosaic instead of being heterozygous (Table 3.2).

Six founder mice (all except #27) were bred with WT mice for germline transmission. The 61-nt deletion mutants were easily identified using the same PCR amplification as mentioned above. WT and mutant bands were separated by high concentration DNA gels. To genotype the 1-nt and 2-nt deletion mutants in a faster and more accurate way, primer pairs with only 1 or 2 nt difference at the 3’ end were designed to specifically amplify WT or mutant DNAs. While the strategy was successful for the 2-nt deletion mutants, it did not work for the 1-nt deletion mutants and, therefore, the Surveyor assay was still used for the latter. The genotyping results showed that all progenies from the 1-nt deletion founder mice were WT (data not shown). Only #52 (2 nt deletion) and #99 (61 nt deletion) founder mice had successful germline transmission (Fig 3.3), which means LanCL2 knockout heterozygotes were obtained.

Next, heterozygous breeding pairs were set up to breed for LanCL2 KO homozygotes. The genotyping result of the first litter (#52) is shown in Fig. 3.4A. WT, Lancl2+/− and −/− mice were sacrificed and RNA and protein were extracted from brain, heart, liver and hind limb skeletal muscle. Gene knockout was validated at the RNA level by semi-quantitative RT-PCR and at the protein level by Western blotting. RT-PCR showed a more than 90% decrease of the corresponding band in LanCL2 homozygous mice both in brain and muscle samples (Fig 3.4B), which is in agreement with the premature stop codon-induced RNA decay. The LanCL2 protein band was absent in all tissue samples (Fig 3.4C). For #99 founder mice, I obtained homozygotes much later because the heterozygous progenies from germline transmission were all females for the first several rounds of breeding. Therefore, I used #52 LanCL2 knockout mice for all subsequent experiments and cryopreserved line #99.

To test whether a truncated LanCL2 fragment that stopped right before the ZFN cutting site was still expressed but not detected by our antibody, both the FLAG-tagged full length and truncated mouse LanCL2 (nucleotides 1-588) were cloned and expressed in HEK293 cells. A similar expression level of the full length and truncated LanCL2 was achieved by adjusting the DNA amount for transfection (confirmed by FLAG antibody blotting). Subsequent Western blotting with a LanCL2 antibody gave an equally strong
detection of the two proteins (Fig 3.5), confirming that the antibody is capable of
detecting the truncated protein if present. Therefore, the LanCL2 knockout mice do not
express a truncated LanCL2, consistent with the nearly complete absence of the mutant
mRNA.

3.3.2 Generation and validation of LanCL1, LanCL3 and LanCL double and triple
KO mice.

LanCL1 and LanCL3 knockout mice were generated using ZFN technology by
Tong Hee Koh, a graduate student in the lab. The final LanCL1 KO mouse line bears a
19-nt deletion at the ZFN target site while the LanCL3 KO mouse line has a 37-nt
deletion (Fig 3.6A). Both LanCL1 and LanCL3 KO production was validated in this
work at the protein level by Western blotting as above for LanCL2 (Fig 3.6, B and C). To
check if any compensatory effect exists in the LanC-like protein family, the expression of
all three LanCL proteins was examined in side-by-side experiments for all three single
LanCL KO mouse lines (except for LanCL3 in Lancl1-/- mice). The results showed that
the KO of any single LanCL protein in mice did not affect the expression of the other two
proteins in the family (Fig 3.6 B, C and D).

LanCL proteins may have redundant functions that obscure the characterization of
each individual protein’s function. To gain a more thorough understanding of LanCL
functions, I crossed single knockouts to generate LanCL double knockout mice. After
three rounds of breeding, I obtained all three double KO mouse lines. Dr. Debapriya
Dutta, a postdoc in the lab, continued to cross these double KO mice to generate
LanCL1/2/3 triple KO mice (LanCL TKO). The complete KO of all three LanCL proteins
was validated in this work in mouse brain by Western blotting (Fig 3.6E).

3.3.3 Characterization of the growth and propagation of LanCL2 knockout mice.

Lancl2-/- mice were born alive from heterozygous breeding pairs at a ratio of 20.1%
(total mouse number: 235), close to what is expected based on Mendel’s law. The
body weight of WT and Lancl2-/- mice were monitored over the course of 18 weeks.
Lancl2-/- mice were slightly lighter in weight than WT mice both at 3-5 weeks old and
after 17 weeks (Fig 3.7A). Since litter size affects mouse weight more when they are
younger, a comparison was made between litter size-matched WT and Lancl2/- mice when they were 3-5 weeks old and the result confirmed the above observation (Fig 3.7B).

Both Lancl2/- males and females are fertile. However, Lancl2/- females seemed to have delayed pregnancy or lower pregnancy rate compared to WT mice. To dissect the problem, I monitored the mating behavior of 3 WT (M) x WT (F) and 4 WT (M) x Lancl2/- (F) breeding pairs. As shown in Table 3.3, Lancl2/- females have normal mating behavior based on the appearance of the mating plug, but do not have 100% pregnancy rate. However, a vaginal smear test suggested that Lancl2/- mice had normal estrous cycle. Therefore, it is still not clear why Lancl2/- female mice had a slight defect in reproduction.

3.3.4 Histology and microarray analyses of LanCL2 knockout mice.

Histology and microarray analyses were carried out to identify potential effect of LanCL2 KO in an unbiased manner. Age (8 weeks old) and gender (both male and female) matched WT and Lancl2/- mice were sacrificed and fixed in 10% neutral buffered formalin with the thoracic and abdominal cavity open. Major organs were dissected and examined by the veterinary diagnostic laboratory at the University of Illinois at Urbana-Champaign. No obvious defect was observed in Lancl2/- mice. Meanwhile, RNA was extracted from the brain of eight weeks old male WT and LanCL2 KO mice (n =3, each genotype) for microarray-based gene expression analysis in the Functional Genomics Unit in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. A significant decrease of mouse Lancl2 transcript (shown by all probe sets) was observed in Lancl2/- mouse brain (8.14 fold decrease with a p value of 2.06 x 10^-8). Western blot analysis of the same brain sample further confirmed the complete KO of the protein. However, no other transcripts were found differentially expressed based on the FDR corrected p-value. The possibility cannot be ruled out that a few transcripts at the top of the p-value list are still the real hits, but further confirmation with other methods such as qRT-PCR is needed. The data in general suggested a negligible transcriptome change in Lancl2/- mouse brain.
3.3.5 Characterization of the survival rate of LanCL2 knockout mice.

Although Lancl2/- mice appeared to be histologically normal when they were 8 weeks old, increased death rate was observed when they reached 4 months old, while Lancl1/- and Lancl3/- mice of the same age appeared to be fine. A total of 50 Lancl2/- mice and 24 WT mice were monitored for their 6 months survival rate (Fig 3.8A). Mice that were euthanized or used for other experiments before reaching 6 months old were recorded as censored data points. Two WT mice died during this period of time. One probably died from seizure (blood and saliva observed on the chin) and was included in the data. The other likely died from an accidental tail injury (blood on the tail) and was not included. Out of the 50 Lancl2/- mice, 14 died during the time of observation, with the majority dying between 4 and 6 months of age. The survived mice in this study were not continually monitored for a longer term. However, few pre-mature deaths of Lancl2/- mice were found at a later stage in general, indicating that aging did not increase the penetrance of the phenotype.

To investigate the possible mechanisms behind the increased death rate, Lancl2/- mice were submitted for histopathology analysis at RADIL IDEXX. Two male and two female Lancl2/- mice that were either moribund or found dead before the autolysis of the carcass were analyzed the first time. Lancl2/- mice were all diagnosed with cardiomyopathy, which is commonly observed in aged mice but usually not expected in mice 4-6 months of age. Multi-focal degeneration was found in all four mice and two of them also had cardiac myocyte hypertrophy and steatosis (Fig 3.8B and C). Mild liver steatosis was also detected in 3 out of the 4 mice. To confirm that this was not a postmortem secondary effect, four age-matched Lancl2/- mice that appeared to be normal were euthanized for histology analysis. Similarly, cardiac hypertrophy with multi-focal degeneration was found in all four mice. Mild hepatic steatosis was also identified in these mice. These results show that Lancl2/- mice are more likely to develop myocardial diseases at a young age, which may be the leading cause of their high death rate.
3.3.6 Investigation into the role of LanCL2 in inflammatory responses.

LanCL2 has been suggested to regulate the inflammatory responses *in vitro* by mediating the ABA signaling pathway (Bassaganya-Riera et al, 2011; Magnone et al, 2012). However, the exact function of LanCL2 in innate immune and inflammatory responses is unclear due to the conflicting role of ABA in these processes. Meanwhile, we observed a higher incidence of microbial infections in *Lancl2-/-* mice compared to their WT counterparts. *Lancl2-/-* mice were more prone to *Staphylococcus aureus*-induced eye infection. EDIM (Epizootic Diarrhea of Infant Mice) virus infection has also been detected in our mouse colony but was almost exclusively found in *Lancl2-/-* mice. These observations are suggestive of suppressed innate immune responses in *Lancl2-/-* mice.

To study the function of LanCL2 in innate immune and inflammatory responses *in vivo*, we used the lipopolysaccharide (LPS)-induced septic shock model. LPS is a prominent member of pathogen-associated molecular patterns (PAMPs), which is derived from gram-negative bacteria. LPS signals through Toll-like receptor 4 (TLR4) and induces the activation of NF-κB, MARK and JNK pathways in target cells such as macrophages and B cells. Molecules released from these cells, mainly cytokines, protect the cells and organism from harm caused by invading pathogens (Barton & Medzhitov, 2003; Newton & Dixit, 2012; Schletter et al, 1995). However, exposure to large amounts of LPS can be harmful to the host as a result of the massive stimulation of the innate immune system and the excessive production of cytokines and other mediator molecules. In severe situations, fetal symptoms such as septic shock can occur and result in the death of the host (Parrillo, 1993).

In these experiments, a slightly different model (LPS in combination with D-galactosamine (GalN)) was used to trigger septic shock in WT and *Lancl2-/-* mice (Galanos et al, 1979). GalN sensitizes the mice to LPS-induced septic shock several thousand fold, resulting in a fast development of the septic shock symptom that usually leads to the death of WT mice within a few hours (Galanos et al, 1979). As shown in Fig 3.9 A, all WT male mice died within 9 hours of the administration of LPS/D-GalN but two out of eight *Lancl2-/-* male mice survived the same dose of endotoxin. The remaining knockout mice also had a delayed onset of death compared to their WT counterparts.
Lancl2-/- females also had slight delay of death but their survival rate was not increased relative to WT mice (Fig 3.9B). This increased resistance of Lancl2-/- mice to septic shock suggests a weakened immune response, in agreement with their susceptibility to microbial infections.

Since TNFα is the major mediator of LPS/GalN-induced septic shock syndrome (Lehmann et al, 1987; Pasparakis et al, 1996; Pfeffer et al, 1993), we wondered if Lancl2-/- mice had decreased secretion of TNFα. Indeed, the serum TNFα level measured 1 h after LPS injection was lower in Lancl2-/- mice, although not statistically significant (Fig 3.9C). In addition, I harvested bone marrow-derived macrophages (BMDM) from WT and Lancl2-/- mice and stimulated them with LPS in vitro. The secreted TNFα in the medium was measured 1 h and 3 h after LPS stimulation, and, as expected, Lancl2-/- BMDM also had slightly lower TNFα secretion (Fig 3.9D).

Next we asked if the decreased TNFα secretion in Lancl2-/- mice was due to dampened NF-κB activation and consequently, lowered TNFα transcription. NF-κB activation was assessed by examining phosphorylation-mediated IκB degradation in mouse embryonic fibroblast (MEF) cells (Chen et al, 1995). In WT MEFs, IκB was phosphorylated and degraded as early as 15 min after TNFα stimulation but re-synthesized to the normal level 60 min after the stimulation, indicating NF-κB activation both in the cytoplasm and the nucleus. The same negative feedback loop was observed in Lancl2-/- MEFs, suggesting a normal NF-κB activation (Fig 3.10). The TNFα mRNA level was also examined by qRT-PCR in both MEFs and BMDMs, but the data were not conclusive. Nevertheless, the decreased TNFα secretion in Lancl2-/- mice is unlikely to be regulated through NF-κB.

It is noteworthy that I did not observe the same phenotype later when I repeated the experiment. Lancl2-/- mice seemed to have a similar survival rate as WT mice with the same LPS and D-GalN dose. We do not have a good explanation for this observation except for the change of mouse housing facilities between experiments. So far the identified microbes causing infections in Lancl2-/- mice were either Gram-positive Staphylococcus aureus or EDIM virus, while LPS is the endotoxin for Gram-negative bacteria. Therefore, using a different infection model may be considered for future experiments.
3.4 DISCUSSION AND OUTLOOK

LanCL2 has been reported to regulate cell sensitivity to adriamycin (Landlinger et al, 2006; Park & James, 2003) and mediate the function of ABA in animal cells (Bassaganya-Riera et al, 2011; Magnone et al, 2012; Sturla et al, 2009). In chapter 2, I also described the identification of LanCL2 as a novel regulator of Akt signaling in liver cells (Zeng et al, 2014). However, these studies have all been performed in vitro and the proposed functions of LanCL2 have been limited to certain cell types or biological contexts. The role of LanCL2 at the whole animal level remains elusive. To address this question, I generated LanCL2 knockout mouse line using ZFN technology and carried out a series of phenotype characterizations. LanCL2 knockout mice showed a 30% death rate at 4-6 months of age, accompanied with cardiomyopathy. They were also more susceptible to microbial infections and showed higher resistance to LPS-induced septic shock, which is suggestive of suppressed immune and inflammatory responses.

Cardiomyopathy is a type of heart abnormality that usually occurs in aged mice. It has been reported that certain gene disruptions can result in the occurrence of cardiomyopathy in younger mice and the subsequent premature death (Auger-Messier et al, 2013; Princen et al, 2009), similar to what we have observed. It is noted that the cardiac hypertrophy, degeneration and steatosis found in Lancl2-/- mice were not severe. Fibrosis was also not detected in Lancl2-/- mouse heart. Therefore, a functional characterization of the heart, under both normal and stressed conditions, is required to understand whether the observed cardiac abnormalities can indeed result in heart failures and a higher death rate in Lancl2-/- mice. It is interesting that the histological analysis of 8 weeks old Lancl2-/- mice did not reveal any abnormalities in the heart, which is probably because the development of the cardiomyopathy symptoms is a slow remodeling process.

The microarray analysis of WT and Lancl2-/- brain samples did not identify many differentially expressed genes, which is surprising given the high expression level of LanCL2 in the brain (Mayer et al, 2001). However, since no morphological changes have been found in Lancl2-/- mouse brain either, it may simply indicate that brain is not the right tissue for the analysis. Instead, a comparison of gene expression in WT and Lancl2
mouse heart may be more important based on my data. Moreover, a direct probing of the signaling pathways involved in cardiac hypertrophy and steatosis (Molkentin & Dorn, 2001) will also help understand the molecular mechanisms by which LanCL2 regulates these processes.

If the cardiac diseases are not the only causes of the premature death of Lancl2-/- mice, the weakened immune system of these mice may be another important contributor. The susceptibility to microbial infections and higher resistance to LPS-induced septic shock suggested a compromised immune system in Lancl2-/- mice. LPS binds to Toll-like receptor 4, signals through the common adaptor protein MyD88 and activates NF-κB transcription factors in immune cells (Barton & Medzhitov, 2003). The activated NF-κB translocates into the nucleus and promotes the transcription of many pro-inflammatory genes, including TNFα. The results showed a lower serum TNFα level in Lancl2-/- mice post LPS injection, which is in agreement with their lower death rate. However, TNFα-stimulated NF-κB activation was not affected in Lancl2-/- MEFs. A straightforward explanation is that LanCL2 regulates TNFα production in a post-transcriptional manner. LPS-induced TNFα production in macrophages is regulated at multiple steps. For example, both the export of the TNFα mRNA from the nucleus to the cytoplasm and the processing of the pre-TNFα to the secreted form are subjected to the regulation by the MAP kinases ERK1 and ERK2 (Dumitru et al, 2000; Rousseau et al, 2008). LanCL2 may regulate TNFα production in a similar manner. On the other hand, it is also possible that NF-κB activation was not assessed in the best experimental system. MEF cells are good systems for signaling studies in general but are not physiologically relevant in LPS-induced inflammatory responses. Macrophages, instead, are the main source of LPS-induced TNFα production in vivo. In addition, although both LPS and TNFα activate NF-κB, different receptors and adaptor proteins are involved upstream of NF-κB activation (Barton & Medzhitov, 2003; Chen & Goeddel, 2002). Therefore, LanCL2 may be only important for NF-κB activation in macrophages by functioning at the signaling steps that are specific to LPS stimulation. Examining TNFα mRNA and protein level in LPS-activated macrophages from Lancl2-/- mice will certainly help to distinguish the two possibilities.
While it is worth dissecting the role of LanCL2 systematically in LPS-induced NF-κB activation in macrophages, a simple model is that LanCL2 functions by mediating ABA’s pro-inflammatory activity. ABA activates NF-κB and the subsequent transcription of its target genes by inducing a Ca\(^{2+}\) influx and PKC activation in various cell types (Bruzzone et al, 2012; Bruzzone et al, 2007). LanCL2 was shown to be an indispensable component in this process (Magnone et al, 2012). Therefore, Lancl2-/− mice, by disrupting the ABA signaling pathway, are expected to have a weakened inflammatory response. If the hypothesis is true, blocking ABA synthesis or using a synthetic ABA analog that competes for LanCL2 binding may lead to the same phenotype as a LanCL2 knockout.
3.5 FIGURES AND TABLES

**Fig 3.1** Schematic illustration of the binding and cutting sites of LanCL2 ZFN. ZFN functions as a dimer with a chain of zinc finger proteins (shown as yellow hexagons) that recognizes a specified DNA locus and a FokI domain that cuts the DNA and creates DSB. Red upper case: ZFN binding site in the *Lancl2* gene. Red lower case: ZFN cutting site in the *Lancl2* gene. Black bold and underlined: Primer binding sites for amplifying the 400 bp DNA fragment for Surveyor assay.

**5’ CAAAGCTGGAGATTCAATTTAGGATCTCCTATACTTTGAATACTGATCTATA**
**TACCCATCCCCATATATTGGATTCTTTAGTTTATTATTTTGAAGAACAGGAATCAAT**
**AGAGAGAGAGGTGGGATTGTGGTTATGAACCTGAGAGATGTTTTAGAAAATTTT**
**CTGAGATATGTCTTTGTTGGTCAGACTTCTACAGATGCATAGAACTATTGTCTG 3’**

**5’-TCAAGAGTCAGAGCTTCTGATGAAGACTGCGtgtatgGACGAGCGAGGATACCTGTAT-3’**
**3’-AGTTCTCAGTCTCGAAGGACTACTTGACGacatacCTGCTCGTCCTATGGAATA-5’**

**5’ GCCTTACTGTACCTGACACACAGTTGGCCTGGCACTGTTGGTGAAGACAGC**
**TATTAAAGGATTGGGCTACTGGAATTCTTGCCACTCGAAACTGTTACAT**
**GTAGTTATAATGACCCAGCGCTCTGGCTT 3’**
Fig 3.2 Surveyor assay and sequencing results for founder mice. (A) Genomic DNA was extracted from the seven founder mice, PCR amplified and subjected to Surveyor assay. The reaction was run on a 1.5% agarose gel. (B) Genomic sequences indicating the mutations in the 7 *Lanc12*/-/- founder mice. Red letters indicate ZFN cutting site. Deleted nucleotides in mutant mice are shown as dashes. Corresponding nucleotides are boxed in WT mice.
Fig 3.3 LanCL2 KO founder mice #52 and #99 had successful germline transmission. LanCL2 KO founder mice #52 and #99 were crossed with WT FVB mice and their progenies were genotyped by PCR of the tail DNA. (A) #52: primer pair 2ntMUT and 200bpREV1 was used. (B) #99: primer pair LanCL2ZFN F and LanCL2ZFN R was used. Positives (heterozygote mice) are indicated by arrows.
Fig 3.4 LanCL2 KO mice genotyping and KO confirmation. (A) Genotyping result of \textit{Lancl2} +/- breeding progenies. Upper gel: PCR of the tail DNA with primer pair 2ntMUT and 400bpREV1. Lower gel: PCR of the tail DNA with primer pair 2ntWT and 200bpREV1. Arrows indicate \textit{Lancl2}/-/- mice. (B) RT-PCR confirmation of LanCL2 KO. Total RNA was extracted from brain and skeletal muscle samples from \textit{Lancl2}+/+, +/- and -/- mice and subjected to RT-PCR (24 cycles) with LanCL2 specific primers (mLanCL2RTforward and mLanCL2RTreverse). (C) Brain, heart and liver samples from \textit{Lancl2} +/-, +/- and -/- mice were subjected to Western blotting analysis with the antibodies indicated.
**Fig 3.5 LanCL2 antibody recognizes truncated mLanCL2.** HEK293 cells were transfected with FLAG-tagged full-length (FL) and truncated (Trun) mouse LanCL2. Different DNA amounts were tested to achieve similar expression level. Antibodies used are indicated on the left side of the panel. The major band below FL-mLanCL2 in the LanCL2 blot is the endogenous human LanCL2 in HEK293 cells. Multiple truncated fragments of exogenously expressed FL-mLanCL2 were also present in cells, shown as the bands below the FL-mLanCL2 band in the FLAG blot.
Fig 3.6 LanCL protein expression in different LanCL KO mouse lines. (A) Genomic sequences indicating the mutations in Lancl1-/- (#36) and Lancl3-/- (#67) mice. Red letters indicate ZFN cutting site. Deleted nucleotides in mutant mice are shown as dashes. Corresponding nucleotides are boxed in WT mice. (B-E) Western blotting confirmed the complete knockout of LanCL proteins in the corresponding LanCL KO mouse lines. (B) Lancl1-/-, (C) Lancl3-/-, (D) Lancl2-/-, and (E) LanCL TKO mice were sacrificed with age- and gender-matched WT mice. Protein was extracted from different tissues and subjected to Western blotting for the detection of all three LanCL proteins with tubulin as the loading control.
Fig 3.7 Body weight comparison between WT and Lancl2-/- mice. (A) WT and Lancl2-/- female mice were weighed once per week over the course of 21 weeks. (B) Litter size-matched WT and Lancl2-/- mice, both males and females, were weighted once per week at 3-5 weeks of age. Unpaired t-test was performed to compare the weight of WT and Lancl2-/- mice. *P <0.05. **P <0.01.
Fig 3.8 Survival rate and cardiac morphology of Lancl2−/− mice. (A) Kaplan-Meier survival curve of WT and Lancl2−/− mice. Log-rank (Mantel-Cox) test of the two survival curves was performed: P<0.05. The mice survived only monitored for 7 months. However, few pre-mature deaths of Lancl2−/− mice were found at an older age than 6 months. (B) WT and Lancl2−/− hearts were cut along the longitudinal plane and stained with hematoxylin and eosin (H&E). Representative results of the left ventricular free wall are shown (40 x). The diameter of the myocytes are noted in the image. (C) H&E staining of the left ventricular free wall of one Lancl2−/− mouse with steatosis (10 x). Arrows indicate accumulated fat tissues in the heart.
**Fig 3.9 Lancl2−/− mice had higher survival rate and lower TNFα secretion in LPS-induced septic shock.** (A and B) Kaplan-Meier survival curve of WT and Lancl2−/− mice after LPS/GalD injection. Log-rank (Mantel-Cox) test of the survival curves was performed. (A) Male mice, *P*<0.05. (B) Female mice, *P*>0.05. (C) Serum TNFα level measured 1 h after LPS/GalD injection by ELISA. Five mice per genotype were examined. (D) BMDMs were starved in RPMI medium with 0.5% FBS overnight and then stimulated with 1 µg/ml LPS in RPMI complete medium. Secreted TNFα in the medium was measured 0, 1, 3 h after LPS stimulation. Data shown are average of three independent experiments except the 1 h data point, which is the average of two independent experiments.
Fig 3.10 TNFα-induced IκB degradation and re-synthesis are the same in WT and LanCL2 knockout MEFs. Early passage WT, Lancl2+/− and Lancl2−/− MEFs were treated with 10 ng/ml TNFα. Cells were harvested at 0, 15, 30 and 60 min after the treatment for Western blot analysis.
Table 3.1 Potential off-target binding sites of LanCL2 ZFN in the mouse genome.

Bioinformatics analysis revealed the top 6 potential off-target binding sites of LanCL2 ZFN in the mouse genome. All are located in the anti-sense strand of the DNA. Shown in the table are the chromosome locations, binding sequences (lower case letters indicate mismatches) and number of mismatches of the non-specific binding sites when bound by different ZFN heterodimer pairs.

<table>
<thead>
<tr>
<th>Chr No</th>
<th>Start</th>
<th>Binding sequence</th>
<th>No. of Mismatches</th>
<th>Name of Heterodimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chr 1</td>
<td>71338232</td>
<td>TAGCTTCCTtcTGAcCTGCTTACACaAtGAGCAGGAcActaCA</td>
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<tr>
<td>Chr 19</td>
<td>14364426</td>
<td>AGGcATCCTtcTtgGgCATAGGGGtAGgTCA TCAtGcAGCTG</td>
<td>8</td>
<td>NM133737-20919a1_N5_NM133737-r20912a1</td>
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<tr>
<td>Chr 7</td>
<td>133233694</td>
<td>AgeCTTCaTGcTcAACTtCCATGGGGAGGaGCAGGAcACCA</td>
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<td>NM133737-r20912a1_N5_NM133737-20919a1</td>
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<tr>
<td>Chr 3</td>
<td>88512822</td>
<td>GAGCTgCCTGATG AACgGgACTCTGG ACtgettGGATACCA</td>
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<td>NM133737-r20912a1_N6_NM133737-20919a1</td>
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<tr>
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<td>92971346</td>
<td>CccTAgCCTGCTgGT CAACCTtCAGTtgtTCAGGAAGCTA</td>
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<td>NM133737-20919a1_N5_NM133737-r20912a1</td>
</tr>
<tr>
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<td>GGGTAGCCacCTgGgCTAGGTTcACttTCtTCAGGAAGCTG</td>
<td>8</td>
<td>NM133737-20919a1_N6_NM133737-r20912a1</td>
</tr>
<tr>
<td>Founder mice</td>
<td>Number of sequenced colonies</td>
<td>Number of mutant colonies</td>
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<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----------------------------</td>
<td>--------------------------</td>
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<td></td>
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<td></td>
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<tr>
<td>#27 (2 nt deletion)</td>
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<tr>
<td>#52 (2 nt deletion)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>#99 (61 nt deletion)</td>
<td>10</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.2 Percentage of mutation in LanCL2 founder mice.** Tail DNA from LanCL2 founder mice was extracted. The 400 bp fragment including the ZFN cutting site was PCR amplified and TA-cloned into TOPO vectors. Different numbers of colonies from each founder mice were sent for sequencing and only a portion of the colonies were found to have mutations.
<table>
<thead>
<tr>
<th>Breeding pair</th>
<th>Plugs appearance (days)</th>
<th>Pups born (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT x WT</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>WT x WT</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>WT x WT</td>
<td>4</td>
<td>26</td>
</tr>
<tr>
<td>WT x Lancl2-/-</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>WT x Lancl2-/-</td>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>WT x Lancl2-/-</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>WT x Lancl2-/-</td>
<td>3</td>
<td>No pups</td>
</tr>
</tbody>
</table>

Table 3.3 Mating behavior of WT and Lancl2-/- mice. Three WT x WT and four WT x Lancl2-/- breeding pairs (males were around 3 months old and females were between 2 to 3 months old) were set up on the same day and monitored for three months. Shown in the table are days before mating plugs were first observed in the female mice and days before the first litter of pups were born.
3.6 REFERENCES


CHAPTER 4. INVESTIGATION OF THE ROLE OF LANCL IN LANTHIONINE KETIMINE BIOSYNTHESIS

4.1 INTRODUCTION

Lanthionine and methyl-lanthionine are the basic structural units in lanthipeptides (Chatterjee et al, 2005). Although lanthipeptides are not found in eukaryotes, the presence of free and protein-bound lanthionines has been reported in chick embryos and insect larvae (Rao et al, 1966; Sloane & Unich, 1966). In mammalian tissues, free lanthionine undergoes transaminase-catalyzed monodeamination, particularly by glutamine transaminase K (GTK), followed by a rapid cyclization to yield a thioether known as lanthionine ketimine (Fig 4.1) (Costa et al, 1987; Ricci et al, 1986). Cavallini and colleagues reported a method for detection and quantification of lanthionine ketimine (LK) that involves a reaction with phenylisothiocyanate (PITC) and subsequent analysis of the product absorbance at 380 nm (Cavallini et al, 1991; Pecci et al, 1988) (Fig 4.2). Using this method, LK has been detected in human and bovine brain at a concentration of 0.5-1 nmol/g tissue (Fontana et al, 1997; Ricci et al, 1989), as well as in human urine samples with a relative concentration of 3-140 µg/g creatinine (Pecci et al, 1988).

About 20 years ago, radioactive binding assays showed that LK bound to the bovine brain membrane in a specific, reversible manner with high affinity ($K_d = 58$ nM), suggesting a neurotransmitter-like function for LK (Dupre et al, 1993; Fontana et al, 1990). More systematic studies of the physiological role of LK have been limited until the most recent 5 years. A cell-permeable derivative of LK, LK ester (LKE), has been shown to have anti-inflammatory and anti-apoptotic function in NSC-34 motor neuron-like cells (Hensley et al, 2010). LKE-treated NSC-34 cells also showed a significantly increased neurite number and length (Hensley et al, 2010). The neuritigenesis effect of LKE was also assessed and confirmed in primary chick dorsal root ganglia (DRG) cultures (Hensley et al, 2010). Furthermore, the function of LKE in the central nervous system was investigated in animal models. LKE treatment delayed the onset of clinical paralysis and increased the survival of SOD1$^{G93A}$ mutant mice modeling amyotrophic lateral sclerosis (ALS) (Hensley et al, 2010). In the 3 x Tg-AD mouse model of Alzheimer’s disease, administration of LKE also substantially mitigated cognitive decline.
(Hensley et al, 2013). In addition, LKE was found to protect neuronal cells in a mouse model of cerebral ischemia (Nada et al, 2012). Taken together, the in vivo results confirmed the neuroprotection and neuritigenesis activity of LKE and supported its therapeutic potential in neuronal diseases.

Despite the therapeutic potential of LK, the biogenesis of lanthionine in mammals remains elusive. Lanthionine has long been thought to be a waste product from the transsulfuration pathway. The pyridoxal phosphate (PLP)-dependent enzyme cystathionine β-synthase (CBS) catalyzes the first step in the transsulfuration pathway, condensing homocysteine with serine or cysteine to form cystathionine (Fig 4.3A), another sulfur-containing bis-amino acid detected in the same organs as lanthionine (Fontana et al, 1997; Pecci et al, 1988). Cystathionine γ-lyase (CSE) also belongs to the PLP-dependent enzyme family and catalyzes the second step in the pathway, the α, γ-elimination of cystathionine to give cysteine, α-ketobutyrate and ammonia (Fig 4.3A), thus recovering cysteine from the harmful homocysteine waste. Alternatively, CBS and CSE-catalyzed condensation of cysteine to serine or another cysteine to form lanthionine was also observed in vitro but was quantitatively minor (Chiku et al, 2009; Singh et al, 2009) (Fig 4.3B). However, evidence of CBS or CSE being the biosynthetic enzymes for lanthionine in vivo is missing.

The relative abundance of LanC-like (LanCL) proteins and the coincidental detection of LK in the central nervous system suggest a potential correlation between the two, besides the similarity of nomenclature. The empirical connection, however, was established by a non-biased proteomic search in bovine brain indicating LanCL1 interacts with lanthionine ketimine (Hensley et al, 2010). Lanthionine could be an allosteric regulator of LanCL proteins or the product of LanCL-catalyzed reactions. However, although some biological functions of LanCL proteins have been revealed in the past few years, the biochemical function of these proteins remains unknown. The conservation of zinc ligands and other essential amino acids for catalysis, as well as structural similarities of LanCL1/2 and NisC, strongly suggests a similar sulfur catalytic chemistry for LanC-like proteins (Li et al, 2006; Zhang et al, 2009) (Dr Neha Garg unpublished data). Furthermore, evidence that LanCL1 and LanCL2 bind to the most abundant and important redox regulator glutathione suggests that glutathione could be one of its
substrates (Chung et al, 2007). Although the prokaryotic lanthipeptide dehydratase does not have mammalian sequence homologs, alternative routes of generating dehydrated amino acids do exist under certain conditions. Dehydroalanines (Dha) in proteins can be formed by base-catalyzed β elimination of one of the sulfur atoms in cystine or of phosphate in phosphoserine in human lenses (Linetsky et al, 2004). Under mild conditions, S-nitrosothiols can also be converted to Dha via phosphine-mediated cis-elimination from an azaylide intermediate (Wang et al, 2009). Therefore, it is plausible that LanCL catalyzes the reaction between glutathione and protein-bound Dha, followed by the cleavage of extra amino acids by peptidases to form lanthionine (Hensley et al, 2010) (Fig 4.4). A protein-free form of the proposed reaction intermediate, “gLan”, was synthesized in vitro and shown to have biological effect similar to that of LK in ALS mouse models, thus supporting the hypothesis (Hensley et al, 2010).

In this chapter, I will describe studies directed towards testing this hypothesis by detecting LK and related metabolites in LanCL knockout mice. If LanCL proteins are responsible for lanthionine biosynthesis, knockout of these proteins will result in the complete absence of lanthionine, and therefore LK, in the tissues. Two methods were utilized to detect LK in mouse tissue samples, mainly in the brain. LK was detected in both the WT and LanCL knockout mice using both methods, suggesting that LanCL proteins are not involved in lanthionine biogenesis in vivo.

4.2 MATERIALS AND METHODS

**Chemicals** – PITC was purchased from Perkin Elmer, d_6-DMSO from Cambridge Isotope Laboratories, triethylamine and pyridine from Fisher Scientific, acetonitrile from Macron Fine Chemicals. All other chemicals were from Sigma Aldrich.

**Standard LK synthesis** – Standard LK synthesis was performed as described (Ricci et al, 1982). Briefly, 6 mmol of L-cysteine hydrochloride in 5 ml of water were added to 6 mmol of bromopyruvate acid in 2 ml of water. The mixture was swirled gently and a light yellow precipitate formed soon. The precipitate was washed with a small amount of cold water and dried under vacuum for an hour. About 1.0 g of crystalline material was obtained.
**PITC derivatization** – Mouse brain sample preparation and PITC derivatization were performed essentially as described with minor modifications (Fontana et al, 1997). Freshly harvested mouse brain (around 0.5 g) was homogenized in 5 ml of water or acetonitrile/H₂O mixture (v:v = 3:7) four times, 30 s each time with an OMNI TH homogenizer. The homogenate was deproteinated by adjusting the acetonitrile/H₂O ratio to 2:1 and centrifuged at 23,700 x g for 10 min. The supernatant was concentrated under N₂ flow to 1 ml before PITC derivatization. One ml of concentrated brain homogenate was mixed with 90 µl of PITC and 3 ml of coupling buffer (acetonitrile:pyridine: triethylamine:H₂O = 10:5:2:3) in a 5 ml reaction vial and stirred for 30 min at RT. The solution was then dried at 40 °C using a rotary evaporator and re-dissolved in 1 ml of 10 mM potassium acetate buffer (pH = 8.0). For standard LK, it was dissolved in acetonitrile/H₂O mixture (v:v = 3:7) and only a small aliquot was used for PITC derivatization, with 1 ml of coupling buffer and 30 µl of PITC.

**HPLC** – Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on an Agilent 1260 Infinity System with a Hypersil Gold C18 (250 mm x 4.6 mm, particle size 5 µ). The program ran from 98% buffer A (0.05 M ammonium acetate, pH 6.5) to 60% buffer B (acetonitrile:H₂O = 7:3) in 30 min and then to 100% buffer B in 5 min at a flow rate of 1 ml/min. All HPLC solvents were filtered with a Millipore filtration system equipped with a 0.22 µm PVDF membrane filter prior to use.

**LC/MS/MS** – Mouse tissue samples were homogenized and deproteinated in the same way as for PITC derivatization. The supernatant was then dried completely under N₂ flow and re-dissolved in acetonitrile/H₂O mixture (v:v = 3:7) for LC/MS/MS analysis (1 mouse brain dissolved in 400 µl solution). Samples were analyzed with the 5500 QTRAP LC/MS/MS system (AB Sciex, Foster City, CA) in the Metabolomics Lab of the Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) includes a degasser, an autosampler, and a binary pump. The LC separation was performed on an Agilent Zorbax SB-Aq column (4.6 x 50mm, 3.5µm.), with a gradient from 100% A (0.1% formic acid in water) to 99% B (0.1% formic acid in acetonitrile) in 6 min at a flow rate of 0.45 mL/min. The autosampler was set at 5 °C. The injection volume was 5 µl.
Positive and negative ion mass spectra were acquired under electrospray ionization (ESI) with the ion spray voltage at 5500 V and -4500 V, respectively. The source temperature was 500 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 33 psi, 65 psi, and 50 psi, respectively. Multiple reaction monitoring (MRM) was used to measure related metabolites.

4.3 RESULTS

4.3.1 Synthesis of lanthionine ketimine.

Lanthionine ketimine was synthesized by reacting equal moles of L-cysteine hydrochloride and bromopyruvic acid in H₂O (Fig 4.5). The product was dissolved in DMSO-d₆ and analyzed by ^1H NMR spectroscopy (Fig 4.6). Consistent with the literature (Ricci et al, 1982), both the ketimine and enamine tautomers were observed, with the enaminic form being the main structure under the condition of the assay (Fig 4.6).

4.3.2 LK detection in WT mouse brain by PITC derivatization.

The established method of LK detection by PITC derivatization and HPLC-UV detection was first validated using the synthetic LK. PITC derivatives of ketimines have an absorbance wavelength at 380 nm, distinguishable from PITC derivatives of other amino acids. The same reaction with lanthionine does not generate a peak at the corresponding retention time monitoring at 380 nm, confirming the specificity of the assay (Fig 4.7). A solvent screen to enhance the recovery rate of PTH-LK identified 10 mM potassium acetate (pH = 8) as optimal and hence, this solvent was used for all subsequent experiments.

With the optimized method in hand, I set out to detect LK in wild type mouse brain. The sample preparation was performed as described with minor modification (Fontana et al, 1997). Authentic LK was spiked into the mouse brain homogenates (spike in) before sample preparation for the identification of the endogenous LK. As shown in Fig 4.8, the peak with a retention time of 25.1 min has an increased intensity in the spiked sample as compared to the WT control, confirming that the peak corresponds to LK. Decreased relative intensity of other peaks in the spiked sample is because less brain homogenate was used. Brain samples prepared the same way but not exposed to PITC
were analyzed by HPLC as a negative control and, as expected, the peak corresponding to modified LK disappeared (Fig 4.9). To further confirm the identity of the peak, an LC/MS/MS method for the detection of PTH-LK was also developed using the PITC derivative of authentic LK as the standard. The MS/MS spectra are in agreement with a PTH-LK structure and the transition of m/z 307.1 to 126.1 in positive ionization mode was chosen for PTH-LK detection in the brain sample (Fig 4.10D). The HPLC fraction of brain sample eluted at around 25.1 min was subject to LC/MS/MS analysis and the peak corresponding to PTH-LK was detected (Fig 4.11D).

The brain LK level in 4 male and 9 female WT mice determined by the PITC method is summarized in Fig 4.12. The HPLC absorbance value at 380 nm is shown and suggests that the LK level in WT mouse brain is low. Female mice have a slightly higher LK level than male mice.

4.3.3 LK detection in LanCL knockout mouse brain by PITC derivatization and HPLC analysis.

If LanC-like proteins are responsible for lanthionine biosynthesis, complete elimination of these proteins from mice by gene knockout should lead to the absence of lanthionine, and hence, lanthionine ketimine. The mouse genome encodes three LanCL genes and all three of them are expressed in the brain (Fig 3.6). As discussed in Chapter 3, our lab generated all three LanCL knockout mouse lines and crossed them to obtain the double and triple KO lines. The brain LK level was first examined in all three single LanCL knockout mouse lines, as well as in LanCL1/LanCL2 double KO mouse line. To minimize the effect of day-to-day experimental variation on the data comparison, one WT mouse was always analyzed as a control together with LanCL knockout mice and the LK level detected in WT mouse was normalized to 1. The results are summarized in Fig 4.13. LK was detected in all of the LanCL knockout mouse lines, among which Lancl1-/-, Lancl3-/- and Lancl1-/-Lancl2-/- mice had similar levels of LK as WT but Lancl2-/- mice showed higher level of LK in 2 out of 3 assays. The three LanC-like proteins may have redundant or opposing functions that could complicate the result in single or double LanCL knockout mice. To obtain a more definitive result, the experiment was repeated in Lancl1-/-Lancl2-/-Lancl3-/- (LanCL TKO) mice. LK was clearly detected in the triple
KO mice (Fig 4.13), suggesting that LanC-like proteins are not essential for lanthionine synthesis in vivo.

4.3.4 Detection of LK, LK-Na and lanthionine in mice by LC/MS/MS.

The method of LK detection by PITC derivatization is specific because of the unique absorbance of PTH-ketimines at 380 nm. However, the purity of the peak declines and the relative error increases when the peak intensity drops. A full absorption spectrum taken at the top of the peak resembles that of the standard PTH-LK (λ_{max} at 380 nm) when the peak intensity is high, but not when the intensity is low. As shown in Fig 4.9, the detected PTH-LK in WT mouse brain is very low and approaches the detection limit of HPLC-UV in most experiments, therefore compromising the specificity and sensitivity of this assay in our experiments.

To detect LK with higher sensitivity and specificity, a triple quadrupole mass spectrometer (MS) coupled with electrospray ionization and HPLC was employed. Multiple reaction monitoring (MRM) was used for the measurement. Unlike the PITC method, which only detects LK, MS enables the detection of all related metabolites. To obtain a more comprehensive assessment of the in vivo lanthionine level, specific methods were developed to directly detect lanthionine and LK. The possible sodium adduct of LK (LK-Na) was also examined. The MS/MS spectra of the standards are shown in Fig 4.10. A specific transition was chosen to detect and quantify each metabolite in biological samples, as marked in the figure. Fig 4.11 shows the typical MRM chromatogram of these compounds in WT mouse brain. The relative signal intensity of LK in WT mouse brain was high, although the absolute concentration has not been determined in this study. Free lanthionines were also detected in mouse brain sample but with lower signal intensity. The LK-Na signal was very low, which may be caused by the inefficient fragmentation (Fig 4.10B).

With the methods established, we set out to detect LK and lanthionine in LanCL triple KO mice. Both LK and lanthionine were present in LanCL triple KO mouse brain (Fig 4.14A and B), confirming the previous result with PITC derivatization that LanCL proteins are not involved in lanthionine synthesis. We also tried to detect LK in tissues
other than brain. We found that LK was detected in mouse heart in both WT and LanCL triple KO mice (Fig 4.14C), but with lower signal intensity compared to that in brain.

4.4 DISCUSSION AND OUTLOOK

LK has been detected in human and bovine brain by PITC derivatization and HPLC-UV analysis (Fontana et al, 1997; Ricci et al, 1989). Here we report, for the first time, the successful detection of LK in mouse brain using the same method. A rough estimate of the LK level in WT mouse brain is around 0.1 nmole/g tissue, based on the comparison of the LK peak area in WT mouse samples with or without standard LK spiked in. It has been reported that although as low as 5 ng of LK/injection can be detected by HPLC-UV, the linearity between the HPLC peak heights and LK concentrations is observed in the range of 0.025 to 0.2 µg/injection of LK (Pecci et al, 1988). Based on our calculations one mouse brain (weighs about 0.5 g) only has about 10 ng of LK, which is out of the linear range and approaching the detection limit of HPLC-UV. To better quantify LK using this method, it is possible to pool multiple brains together for one HPLC analysis. The increased sample volume will also help minimize the percentage of sample loss during the preparation process. The only drawback will be the lack of information of LK level in each individual mouse and the variation among individuals.

Another problem of this method is the low specificity. As mentioned in the Results section, the purity of the PTH-LK peak decreases when the signal intensity drops, as shown by the full UV-Vis spectrum taken at the top of the peak. Therefore, we developed an LC/MS/MS method using a triple quadrupole mass spectrometer and used MRM to quantitate LK in mouse brain, which provided much higher sensitivity and specificity. The experimental procedure has also been largely shortened without the derivatization step. However, compared to PTH-LK, LK is very unstable in aqueous solution, either undergoing auto-oxidation at pH > 7 or yielding a red colored compound below neutrality (Cavallini et al, 1991). While shortening the extraction and analysis process helps minimize the variation caused by the instability of LK, the use of internal standard will eventually solve this problem. A standard LK that is added to and processed together with the brain sample likely undergoes the same deterioration as the endogenous
LK and therefore can be used for direct quantification of the latter. Stable isotope-labeled LK that has a few Dalton mass difference will be an even better control, as it can be easily separated from the endogenous LK by mass spectrometry.

Nevertheless, LK was detected in both WT and LanCL TKO mouse brains by both methods, suggesting that LanC-like proteins are not involved in LK biogenesis in vivo. However, my results did not rule out the possibility of other types of functional correlation between LanC-like proteins and LK. Interestingly, a clear increase of LK level was found in LanCL2 single knockout mice but not in LanCL1/2 double knockout or LanCL1/2/3 TKO mice. This suggests a potential functional interplay between LanCL1 and LanCL2. Given that LanCL1 single knockout and LanCL1/2 double knockout mice have the same phenotype in terms of LK level and that LanCL2 single knockout mice have increased LK level, LanCL2 could be an upstream inhibitor of LanCL1 function. A hypothesis is that LanCL1 is not required for LK biosynthesis at normal conditions but is able to promote excess LK generation if activated (inhibitor removed), which explains why LK is present in LanCL1 KO mice but increased in LanCL2 KO mice.

Now that we have shown that LK biogenesis is not mediated by LanC-like proteins, a systematic screening for potential LanCL substrates is warranted. The fact that both LanCL1 and LanCL2 bind to GSH (Chung et al, 2007; Zhang et al, 2009) still supports the hypothesis that GSH is one of the substrates. High throughput screening methods to identify GSH-conjugating metabolites in mammalian tissues (mainly liver) by mass spectrometry have been reported (Ma & Subramanian, 2006). The use of stable isotope-labeled GSH at an equal molar ratio of unlabeled GSH in these assays proved great sensitivity and reliability and can be applied in our experiments (Liao et al, 2012). By comparing the list of GSH conjugates in WT and LanCL KO animals, novel substrates of LanC-like proteins may be identified.
4.5 FIGURES

Fig 4.1 Lanthionine undergoes monodeamination and cyclization to form lanthionine ketimine. GTK catalyzes the transamination of lanthionine with α-keto acids. The intermediate of this reaction rapidly cyclizes to form lanthionine ketimine.
Fig 4.2 LK reacts with PITC to form PTH-LK.
Fig 4.3 Lanthionine formation through alternative reactions in the transsulfuration pathway. (A) The classic transsulfuration pathway catalyzed by CBS and CSE. (B) Alternative reactions catalyzed by CBS or CSE that lead to lanthionine formation. When the substrates are two molecules of cysteine, $\text{H}_2\text{S}$ is formed instead of $\text{H}_2\text{O}$. 
Fig 4.4 Hypothesized route of lanthionine synthesis by LanCL. Protein-bound Dha can be generated from protein-bound disulfide, phosphoserine or S-nitrosothiols. LanCL may catalyze the conjugation of protein-bound Dha to GSH, followed by the peptidase-mediated digestion of the reaction intermediate to form lanthionine.
Fig 4.5 LK synthesis from bromopyruvic acid and cysteine.
Fig 4.6 $^1$H NMR spectrum of LK in DMSO-$d_6$. 
Fig 4.7 PITC derivatization of LK, but not Lan, generates a specific peak in HPLC monitored at 380 nm. Equal amount of LK (A) or Lan (B) (3 µg) were mixed with 1 ml of coupling buffer and 30 µl of PITC. The reaction was stirred at RT for 30 min and dried using a rotary evaporator. The residue was dissolved in 1 ml of 10 mM potassium phosphate buffer (pH = 8) and an aliquot of 100 µl was injected for HPLC analysis.
Fig 4.8 HPLC chromatogram of PITC derivatized mouse brain extracts. One mouse brain was homogenized in 5 ml of 30% acetonitrile. Homogenate corresponding to 0.37 g brain was directly derivatized by PITC as WT control (A). The rest of the homogenate (corresponding to 0.13 g brain) was mixed with 30 ng of standard LK (spike in) for PITC derivatization (B).
Fig 4.9 WT brain derivatization with or without PITC. One mouse brain was homogenized and deproteinated. The supernatant was concentrated under N₂ flow to 1.2 ml and split into half for two PITC derivatization reactions. (A) The extract was added to 2 ml of coupling buffer and 60 µl of PITC. (B) The extract was added to 2 ml of coupling buffer only.
Fig 4.10 MS/MS spectra of authentic standards. (A) MS/MS spectra of LK. LK was synthesized as described in the methods and dissolved in 30% acetonitrile for MS/MS analysis. (B) MS/MS spectra of LK-Na. The standard solution was made by adding equal molar NaOH to the LK solution. (C) MS/MS spectra of Lan (negative mode). Lan was dissolved in 10 mM HCl in 30% acetonitrile as the standard solution. (D) Standard LK derivatization product was run on HPLC. The fraction collected at 25.1 min was used as the standard LK-PTH for MS/MS method development.
Fig 4.11 MRM chromatograms of LK and related metabolites in WT mouse brain. WT mouse brain was homogenized and deproteinated. The supernatant was completely dried under N₂ flow and dissolved in 400 µl of 30% acetonitrile. An aliquot of 5 µl was injected for LC/MS/MS analysis of LK, LK-Na and Lan. LK-PTH was from the HPLC fraction corresponding to endogenous LK in WT mouse brain. (A) MRM chromatogram of detected LK (transition of 190.1 → 73.1). (B) MRM chromatogram of detected LK-Na (transition of 212.0 → 168.1). (C) MRM chromatogram of detected Lan (transition of 202.0 → 120.0). (D) MRM chromatogram of detected LK-PTH (transition of 307.1 → 126.1).
Fig 4.12 Summary of LK level in WT mouse brain. The LK level was presented as the HPLC value monitored at 380 nm. Each dot represents one mouse. The mean of value of each gender group is shown.
Fig 4.13 Comparison of LK level in WT and LanCL KO mice. The LK level is presented as the HPLC value monitored at 380 nm. One WT mouse was euthanized and processed as a control with LanCL KO mice in each independent experiment and the results were normalized to WT control. Normalized results from 6 independent experiments are summarized here.
Fig. 4.14 LK and Lan detected in WT and LanCL TKO mice by LC/MS/MS. (A) LK detected in WT and LanCL TKO mouse brain. Results are from 3 independent experiments. (B) Lan detected in WT and LanCL TKO mouse brain from one experiment. (C) LK detected in WT and LanCL TKO mouse heart from one experiment. The results are all presented as the peak area in LC/MS.
4.6 REFERENCES


APPENDIX A. FUNCTIONAL CHARACTERIZATION OF LANCL2 IN HEK293 AND MCF-7 CELLS

A.1 MATERIALS AND METHODS

Antibodies and reagents – The antibodies used were obtained from the following sources: Anti-LanCL2 antibody was generated by Proteintech Group Inc using full-length recombinant LanCL2 protein as the antigen. Anti-pPKC was from Millipore, anti-tubulin from Abcam. All other antibodies were from Cell Signaling Technology. TNFα was from Cell Signaling Technology. LY294002 was from Cayman Chemicals. Akti-1/2, Wortmannin and U0126 were purchased from Calbiochem. Cycloheximide (CHX) was from Sigma Aldrich.

Cell culture – All cell lines used in this study were obtained from ATCC. Both HEK293 and MCF-7 cells were maintained with DMEM containing 10% FBS and 1% pen/strep at 37 ºC with 5% CO2.

TUNEL assay – TUNEL assays were performed following the manufacturer’s manual (Promega). The stained cells were examined with a Leica DMI 4000B fluorescence microscope, and the fluorescent images were captured using a RETIGA EXi camera, and analyzed with Q-capture Pro51 software (QImagingTM).

Cell lysis and Western blotting – Cells were lysed in MIPT lysis buffer (50 mM Tris-Cl, pH 7.2, 137 mM NaCl, 25 mM NaF, 25 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 10 mM sodium pyrophosphate, 0.3% Triton X-100, and 1x protease inhibitor cocktail (Sigma-Aldrich)) and microcentrifuged at 10,000 g for 10 min at 4 ºC. The supernatant was mixed 1:1 with 2 x SDS sample buffer and heated at 95 ºC for 5 min. Proteins were resolved on SDS-PAGE, transferred onto PVDF membranes (Millipore), and incubated with various antibodies following the manufacturers’ recommendations. Detection of horseradish peroxidase-conjugated secondary antibodies was performed with Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Inc.), and images were developed on x-ray films.

Lentivirus-mediated RNAi – LanCL2 lentiviral shRNA constructs in the pLKO vector were obtained from Sigma-Aldrich (TRC library). LanCL2 #1 (L1), TRCN0000045403; LanCL2 #2 (L2), TRCN0000045406. Lentivirus packaging was
performed as described in chapter 2. HEK293 and MCF-7 cells were infected with the viruses in the presence of 8 µg/ml polybrene for 24 h and were then subjected to selection with various concentrations of puromycin.

A.2 RESULTS AND DISCUSSION

A.2.1 LanCL2 knockdown increases Akt phosphorylation in HEK293 and MCF-7 cells.

To investigate the possible roles of LanCL2 in several most important signaling pathways in cells, we knocked down LanCL2 in HEK293 cells and performed a screen of seven key phosphorproteins both under starvation and in response to mitogenic signals (serum). They were p-Erk1/2 (Thr202/Tyr204), p-P38 (Thr180/Tyr182), p-JNK (Thr183/Tyr185), p-PKCa (Ser657), p-STAT3 (Tyr705 and Ser727), p-Akt (Ser473 and Thr308) and p-S6K1 (Thr389), as readouts of the MAPK, PKC, JAK/STAT, PI3K/Akt and mTOR pathways, respectively. Among these I found that p-Erk1/2 and p-Akt (S473 and T308) consistently increased in LanCL2 knockdown (KD) cells, and this change was not because of the change of total Erk or Akt amount (Fig A.1A). The phosphorylation of other proteins did not change (only p-S6K1 and p-PKC are shown in Fig A.1A). To determine if the inhibitory effect of Akt phosphorylation by LanCL2 is specific to HEK293 cells, we also knocked down LanCL2 in MCF-7 cells. Increased Akt phosphorylation was observed in MCF-7 cell as well (Fig A.1B), suggesting that the LanCL2 knockdown effect is not specific to HEK293 cells.

A.2.2 LanCL2 knockdown protects HEK293 cells from apoptosis.

Akt is the central mediator of cell survival (Duronio, 2008). ERK1 and ERK2 are also involved in cell survival regulation (Balmanno & Cook, 2009; Lu & Xu, 2006). Moreover, it has been reported that ERK1/2 activation antagonized TNFα-induced apoptosis (Pucci et al, 2009; Tran et al, 2001). Therefore we wondered whether LanCL2 is involved in this cellular process because of its effect on Akt and ERK phosphorylation. Control and LanCL2 knockdown HEK293 cells were induced to undergo apoptosis by serum starvation combined with TNFα/Cycloheximide (CHX). Indeed, decreased DNA fragmentation, measured by terminal deoxynucleotidyl transferase dUTP nick end
labeling (TUNEL) assays, was observed in LanCL2 knockdown cells (Fig A.2A). Cleaved caspase-3, another marker of apoptosis, was also decreased in LanCL2 knockdown cells (Fig A.2B), suggesting that LanCL2 has an inhibitory effect on cell survival in HEK293 cells.

Next we asked if LanCL2 regulates cell survival through Akt or ERK. Apoptosis was induced by starvation combined with TNFα/CHX in control and LanCL2 knockdown cells, with or without various PI3K/Akt inhibitors (LY294002, wortmannin and Akti-1/2) or ERK inhibitor (U0126). Surprisingly, I found that LanCL2 knockdown protected HEK293 cells from apoptosis even in the absence of Akt or ERK phosphorylation (Fig A.3). It is possible that the cell apoptosis machinery has already started after two days of starvation even before the TNFα/CHX treatment, therefore, the inhibition of Akt or ERK signaling may be too late to reverse the phenotype. On the other hand, prolonged inhibition of Akt or ERK, the key components of two major signaling pathways in cells, may induce secondary effects. Hence, for future experiments, shortened apoptosis induction time should be used.

If indeed LanCL2 regulates cell survival independently of its effect on Akt and ERK phosphorylation, further investigation into the mechanism of LanCL2-regulated cell survival is warranted. As shown in Fig A.2C, other apoptosis inducers such as etoposide and adriamycin were also used in HEK293 cells, but LanCL2 knockdown only protected cells from TNFα/CHX-induced apoptosis. By binding to TNF receptor-1 (TNF-R1), TNFα triggers three major signaling pathways, including the activation of the transcription factor NF-κB, the initiation of a protease cascade that leads to apoptosis, and the activation of c-Jun NH2-terminal kinase (JNK) with the subsequent activation of its downstream transcription factor c-Jun (Chen & Goeddel, 2002). Since the NF-κB and c-Jun effects are inhibited by the translation inhibitor cycloheximide, a reasonable speculation is that LanCL2 either regulates the caspase cascade or the functions of JNK that do not involve c-Jun (Yamamoto et al, 1999).
**Fig A.1 LanCL2 knockdown increases Akt and Erk phosphorylation in HEK293 and MCF-7 cells.** (A) HEK293 cells were infected with lentiviruses expressing control shRNA (C) or LanCL2 shRNA (“L-1” and “L-2”), and selected by puromycin. The cells were then either maintained in growth medium (“growth”), or serum-starved overnight (“starved”) and re-stimulated with 10% FBS for 20 min (“stimulated”), followed by Western blot analysis with antibodies indicated. (B) MCF-7 cells were treated as in (A).
Fig A.2 LanCL2 knockdown protects HEK293 cells from apoptosis. HEK293 cells were infected with lentiviruses expressing control shRNA (C) or LanCL2 shRNA (“L-1” and “L-2”), and selected by puromycin. Cells were then starved for 2 days and treated with 1 ng/ml TNFα and 1 µg/ml CHX for 24 h, followed by TUNEL assay (A) or Western blot analysis (B), or treated with 30 µg/ml etoposide or 5 µM adriamycin for 24 h (C).
Fig A.3 LanCL2 regulates cell survival independently of its effect on Akt and Erk phosphorylation. (A) HEK293 cells were infected with lentiviruses expressing control shRNA (C) or LanCL2 shRNA (“L-1” and “L-2”), and selected by puromycin. Cells were starved for 2 days and then treated with 1 ng/ml TNFα and 1 µg/ml CHX for 24 h, with or without pre-treatment with 10 µM LY294002 (“LY”), 1 µM Akti-1/2 (“Akti”) or 100 nM wortmannin (“wort”) for 1 h, followed by Western blot analysis. (B) HEK293 cells were infected with lentiviruses expressing control shRNA (C) or LanCL2 shRNA (“L”), and treated as in (A) except the pre-treatment with 5 µM or 10 µM U0126.
A.4 REFERENCES


APPENDIX B. PROTEOMIC SCREENING IDENTIFIES HSP90 AS A NOVEL LANCL2 INTERACTING PROTEIN

B.1 MATERIALS AND METHODS

Antibodies and reagents – The antibodies used were obtained from the following sources: Anti-LanCL2 antibody was generated by Proteintech Group Inc using full-length recombinant LanCL2 protein as the antigen. Anti-LanCL1 antibody was from Abnova. Anti-FLAG M2 was from Sigma-Aldrich; anti-HA (16B12) from Covance, anti-HA (rabbit) from Promega, anti-tubulin from Abcam, anti-HSP90 from Santa Cruz, and anti-pS473 Akt from Cell Signaling Technology. Geldanamycin was from LC laboratories. Anti-HA agarose beads were purchased from Thermo Fisher. All other reagents were from Sigma Aldrich.

Cell culture and transfection – All cell lines used in this study were obtained from ATCC. The following media were used to maintain various types of cells: Dulbecco's Modified Eagle Medium/Ham's F-12 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) for HepG2 cells, DMEM containing 10% FBS and 1% pen/strep for HEK293 cells. All cells were maintained at 37 °C with 5% CO2. Plasmid DNA transfection was performed using TransIT-LT1 (Mirus Bio LLC) in HepG2 cells or Polyfect (QIAGEN) in HEK293 cells, according to the manufacturers’ instructions.

Establishment of stable cell lines – Human LanCL1 and LanCL2 cDNA were subcloned in the PCDNA5F vector (derived from PCDNA3 from Life Technologies) between the restriction sites EcoRI and XhoI, with the 1xFLAG tag fused at the N-terminus of the genes. Human LanCL2 cDNA was also subcloned in the p3×FLAG-CMV-14 vector (Sigma-Aldrich) between the restriction sites HindIII and KpnI, with the 3xFLAG tag fused at the C-terminus of LanCL2. HEK 293 cells were transfected with N- or C-terminal FLAG tagged LanCL1 or LanCL2. One day after transfection, the cells were trypsinized and selected in G418-containing medium at a density of 20-30 cells per 10-cm plate. The cells were allowed to grow into visible colonies before being separately transferred to a 48-well plate using sterile filter paper soaked in trypsin. The cells were
then lysed and examined for exogenous LanCL1 and LanCL2 expression by Western blot after they reached confluency.

**Immunoprecipitation** – For LanCL2-Akt co-immunoprecipitation, cells were lysed in MIPT lysis buffer and microcentrifuged at 16,000 g for 10 min at 4 °C. The supernatant was incubated with anti-FLAG beads or anti-HA beads for 2 h. The beads were then washed 3 times with lysis buffer and boiled in 2x SDS sample buffer for 5 min followed by Western blotting. For mass spectrometry analysis, cells were also lysed in MIPT lysis buffer, ultra-centrifuged at 150,000 g to eliminate large ribosomal proteins and pre-cleared with non-specific IgG beads twice before incubation with anti-FLAG beads. The bound proteins were eluted with 100 µg/ml 3xFLAG peptide and resolved on 12% and 7.5% SDS-PAGE, followed by silver staining. For Akt-HSP90 and LanCL2-HSP90 co-immunoprecipitation, a previously reported lysis buffer (Sato et al, 2000) was used: 20 mM Tris-Cl, pH 7.5, 0.2% Nonidet P-40, 10% glycerol, 1 mM EDTA, 1.5 mM MgCl2, 137 mM NaCl, 50 mM NaF, 1 mM NaVO3, 12 mM β- glycerophosphate, 1x protease inhibitor cocktail.

**Lentivirus-mediated RNAi** – LanCL2 lentiviral shRNA constructs in the pLKO vector were obtained from Sigma-Aldrich (TRC library). LanCL2 #1 (L1), TRCN0000045403; LanCL2 #2 (L2), TRCN00000045406. Lentivirus packaging was performed as described in chapter 2. HepG2 cells were infected with the viruses in the presence of 8 µg/ml polybrene for 24 h and were then subjected to selection with various concentrations of puromycin.

**B.2 RESULTS AND DISCUSSION**

**B.2.1 Proteomic screening identifies HSP90 as a novel interacting protein of LanCL2.**

To identify potential interacting partners of LanCL2, we expressed N-terminal FLAG-LanCL2 in HEK293 cells and established three stable cell lines (#4, #9 and #20, Fig B.1A). Proteomic analysis of proteins coimmunoprecipitated with the recombinant LanCL2 was performed with stable cell line #20. A major band around 95 kDa was specifically associated with FLAG-LanCL2, as revealed by silver staining (Fig B.2A). Mass spectrometry analysis of this band was performed by the Protein Sciences Facility.
in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign and identified it as HSP90, including both HSP90α and HSP90β isoforms. The interaction between endogenous HSP90 and the stably expressed FLAG-LanCL2 in HEK293 cells was validated by co-IP (Fig B.2B).

LanCL2 was reported to be myristoylated at the N-terminal glycine residue, which is important for its recruitment to the cell membrane (Landlinger et al, 2006). The expression of an N-terminal FLAG tag, therefore, may impede the myristoylation and change the normal subcellular localization of LanCL2. To confirm the LanCL2-HSP90 interaction in a more physiological condition, I established new stable cell lines in HEK293 cells that express C-terminal FLAG-LanCL2 (#11, #14 and #16, Fig B.1B). Subsequent co-IP experiments performed in stable cell line #11 confirmed the interaction (Fig B.2C).

Furthermore, this interaction was reproduced in a different cell line, HepG2 cells, by pulling down transiently expressed C-terminal FLAG-LanCL2 (Fig B.2D). I had difficulty detecting endogenous LanCL2 upon immunoprecipitation of HSP90, possibly because HSP90 has many binding partners in the cell. Indeed, I was also not able to detect endogenous Akt, a well-established client of HSP90, from HSP90 immunoprecipitates.

**B.2.2 The interrelationship of the interactions between LanCL2, HSP90 and Akt.**

Given that LanCL2, Akt and HSP90 interact with each other (Fig 2.3, B.2) (Sato et al, 2000), we set out to examine the interrelationship of their interactions. LanCL2 knockdown did not affect the co-IP of Akt and HSP90 (Fig B.2A), suggesting that the Akt-HSP90 interaction is not mediated by LanCL2. However, LanCL2-HSP90 interaction appeared to be weakened by mitogenic stimulation (Fig B.2B), which correlated with increased Akt phosphorylation. We also wondered if these interactions were affected by geldanamycin (GA), an inhibitor of HSP90. Indeed, short-term (30 min) GA treatment dramatically decreased the interaction between HSP90 and Akt (Fig B.2C), as well as that between HSP90 and LanCL2 (Fig B.2D). At the same time, GA increased the interaction between LanCL2 and Akt (Fig B.2E). It is noteworthy that the phosphorylation status of the ectopically expressed HA-Akt was unchanged by GA
treatment, despite its physical release from HSP90, implying that the increased interaction between HA-Akt and LanCL2 in this case was not due to a change in Akt activity.

Contrary to previous studies in HEK293 and neuroblastoma SH-SY5Y cells (Meares et al, 2004; Sato et al, 2000), we found that short-term GA treatment dissociated Akt from HSP90 in liver cells. A similar mode of regulation has been reported for other kinase clients of HSP90, such as PKR and Src (Donze et al, 2001; Koga et al, 2006), in which cases the dissociation from HSP90 is necessary for the activation of the kinases. Our observation extends the current understanding of HSP90 regulation of Akt and suggests the existence of distinct regulatory mechanisms in different cell contexts.

Taken together, these observations suggest the possibility of a ternary complex and functional interaction between HSP90 and LanCL2. Although LanCL2 does not appear to mediate the HSP90-Akt interaction, HSP90 does seem to modulate the Akt-LanCL2 interaction. Inhibition of HSP90 leads to enhanced interaction between Akt and LanCL2, which may be a result of Akt dissociation from HSP90 and may contribute to Akt activation. Whether LanCL2 is involved in the regulation of any other clients of HSP90 would be an interesting question for future investigations.

**B.2.3 LanCL2 is required for GA-induced Akt activation.**

HSP90 is known to bind and stabilize Akt, and maintain its phosphorylation and activation state (Basso et al, 2002; Sato et al, 2000). On the other hand, HSP90 has also been reported to suppress Akt activation by mitogenic signals (Koga et al, 2006; Meares et al, 2004). Indeed, we observed that GA enhanced Akt phosphorylation in HepG2 cells (Fig B.3), confirming the suppressive role of HSP90 in this case. However, this effect of GA was abolished in LanCL2 knockdown cells (Fig B.2F). Similar results were obtained with a derivative of GA, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) (data not shown). Taken together, these data suggest possible involvement of HSP90 in LanCL2 regulation of Akt phosphorylation.
**B.3 FIGURES**

**A**

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<th>Cell line No.</th>
<th>C</th>
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<th>LanCL2</th>
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[Image: Short exposure of Western blot showing Tubulin, FLAG-LanCL2, and FLAG-LanCL1 bands.]

[Image: Long exposure of Western blot showing Tubulin, FLAG-LanCL2, Endogenous LanCL2, FLAG-LanCL1, and Endogenous LanCL1 bands.]

**Fig B.1 Establishment of LanCL2 and LanCL1 stable cell lines.** (A) HEK293 cells were transfected with control plasmid (“C”), N-terminal FLAG-tagged LanCL1 or LanCL2 and subjected to G418 selection. Three stably expressing cell clones were selected for both LanCL1 and LanCL2. The expression level of both exogenous and endogenous LanCL1 and LanCL2 were examined by Western blotting. The membrane was co-blotted with tubulin, LanCL1 and LanCL2. Short and long exposures of the film were shown with the identity of each band indicated on the right of the panel. (B) HEK293 cells were transfected with C-terminal FLAG-tagged LanCL2 and selected in G418-containing medium. The clones were tested for FLAG-LanCL2 expression by Western blot. Three clones that were selected for expansion and storage are indicated by arrows.
**Fig B.2 HSP90 interacts with LanCL2.**

(A) HEK293 cells stably expressing FLAG-LanCL2 were subjected to immunoprecipitation with anti-FLAG beads. Eluted proteins were analyzed by SDS-PAGE and silver-staining, followed by mass spectrometry analysis. (B and C) HEK293 cells with and without stable expression of N-terminal FLAG-LanCL2 (B) or C-terminal FLAG-LanCL2 (C) were subjected to immunoprecipitation with anti-FLAG antibody, followed by Western analysis. (D) HepG2 cells were transiently transfected with FLAG-tagged LanCL2, followed by immunoprecipitation with anti-FLAG antibody and Western analysis.
Fig B.3 LanCL2 is involved in HSP90 regulation of Akt. (A) HepG2 cells were infected with lentiviruses expressing LanCL2 shRNA (“L1, L2”) or control (“C”) for 1 day, selected with puromycin for 2 days, and then transfected with HA-Akt. IP was then performed with anti-HA antibody, followed by Western analysis. (B) HepG2 cells were transfected with FLAG-LanCL2, serum-starved overnight, and re-stimulated with 10% FBS for 20 min. IP was then performed with anti-FLAG antibody, followed by Western analysis. (C-E) HepG2 cells were transfected with the DNA indicated, followed by IP as indicated, with or without pretreatment with 10 µM geldanamycin (“GA”) for 30 min. (F) Cells were infected with lentiviruses expressing shRNA for LanCL2 (“L1”) or control (“C”), puromycin selected, and then serum-starved and restimulated with 10% FBS for 10 min, with or without pretreatment of 10 µM GA for 30 min. Western analysis was performed.
B.4 REFERENCES


