The N-terminus of a1 Subunit and Na/K-ATPase-Mediated Signal Transduction

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In partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science

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Date of Defense: August 27, 2009
The N-terminus of α1 Subunit And Na/K-ATPase-Mediated Signal Transduction

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I dedicate this work to my dear parents
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INTRODUCTION

The Na/K-ATPase is an ion transporter that mediates active transport of Na\(^+\) and K\(^+\) across the plasma membrane by hydrolyzing ATP (Lingrel and Kuntzweiler, 1994; Skou, 1957). The functional Na/K-ATPase mainly consists of \(\alpha\) and \(\beta\) subunits. The \(\alpha\) subunit plays the catalytic role for the whole enzyme as it contains both the nucleotide and the cation binding sites (James et al., 1999). So far, four isoforms of \(\alpha\) subunit (\(\alpha_1\)~\(\alpha_4\)) have been discovered and each shows a distinct tissue distribution pattern (Lingrel et al., 1990; Sweadner, 1989). Interestingly, studies from the past several years have revealed many non-pumping functions of Na/K-ATPase (Haas et al., 2002; Kometiani et al., 1998; Li and Wattenberg, 1998; Xie, 2001; Xie et al., 1999). Moreover, a key concept emerged from the study of the non-pumping pump is that it mainly resides in the special plasma membrane lipid domain called caveolae and forms a big signaling complex by interacting with many other proteins (Liang et al., 2007; Tian et al., 2006; Wang et al., 2004; Xie and Cai, 2003).

Based on its 3D structure, the Na/K-ATPase \(\alpha\) subunit contains three major intracellular cytosolic domains, the N-terminal cytosolic tail (NT), the second cytosolic domain (CD2) and the third cytosolic domain (CD3). All three domains are able to interact with other proteins (Done et al., 2002; Efendiev et al., 2005; Kimura et al., 2007; Tian et al., 2006; Yuan et al., 2005; Yudowski et al., 2000; Zhang et al., 2006). Among the three intracellular domains, NT appears to be most interesting as it contains binding motifs to various signaling proteins and the protein-protein interactions play important roles in many signaling events. For example, in response
to G protein-coupled receptor (GPCR) signals, association of Phosphoinositide 3-kinase (PI3K) with the proline-rich domain within NT led to recruitment of clathrin and adaptor protein 2 (AP2) that mediated endocytosis of the Na/K-ATPase (Yudowski et al., 2000). In another example, it was demonstrated that Inositol triphosphate (IP3) receptor interacted with NT dynamically that regulated intracellular Ca\(^{2+}\) signaling events (Yuan et al., 2005; Zhang et al., 2006). Furthermore, thanks to the pioneering work of my labmates, it has been revealed that NT is capable of interacting with caveolin-1, the molecular marker of caveolae and the interaction between the two facilitates the Na/K-ATPase-mediated signal transduction (Wang et al., 2004).

Caveolae are flask-shaped vesicular invaginations in the plasma membrane and are enriched with cholesterol, glycosphinglipids and sphingomyelin (Fielding and Fielding, 2000). Since the discovery of its marker protein, caveolin, in the early 1990s (Rothberg et al., 1992), the studies on the cellular functions of caveolae at the molecular level progress dramatically. It is now widely recognized that numerous signaling proteins are localized in caveolae and it serves as an important signal transduction site (Thomas and Smart, 2008). More interestingly, the caveolin proteins are not only capable of interacting with various signaling molecules to regulate signal transduction events (Williams and Lisanti, 2004) but also involved in intracellular cholesterol trafficking and homeostasis (Razani et al., 2002b; Uittenbogaard et al., 1998).

Cholesterol is an essential molecule for all mammalian cells. It is
indispensable for the normal functions of the cell membranes and one of the structure components of caveolae. Moreover, it plays an important role in signal transduction and generation of cell surface polarity (Simons and Toomre, 2000). High blood cholesterol level is one of the major risk factors for hypertension and many cardiovascular diseases.

Our recent studies have gradually revealed that the Na/K-ATPase and caveolin-1 protein interact with and affect the functions of each other. (Cai et al., 2008; Liu et al., 2005; Liu et al., 2003; Wang et al., 2004). Since cholesterol is known to interact with caveolin-1 (Murata et al., 1995) and affect the signaling function of the Na/K-ATPase (Wang et al., 2004), this project is to further explore whether and how the Na/K-ATPase and caveolin-1 complex is involved in cholesterol metabolism. Moreover, we have generated the Na/K-ATPase α1 NT-expressing mice and studied the physiological role of NT.
**LITERATURE**

**Na/K-ATPase**

The Na/K-ATPase or sodium pump is an integral membrane protein and belongs to the P-type ATPase superfamily (Kaplan, 2002). As its name implies, it mediates active import of $K^+$ and export of $Na^+$ across the plasma membrane by hydrolyzing ATP.

**Subunits and isoforms of the Na/K-ATPase**

The functional Na/K-ATPase is mainly composed of two subunits: $\alpha$ subunit and $\beta$ subunit in equimolar ratios. The $\alpha$ subunit shows the molecular weight around 110 kDa. Based on the primary sequence and membrane topology analysis, $\alpha$ subunit is considered to contain ten transmembrane segments with both N-terminal and C-terminal tails facing the cytosolic side (FIGURE 1). Besides the N-terminal cytosolic tail, the amino acids between the second and the third transmembrane segments constitute the second cytosolic domain (CD2) and those between the fourth and the fifth transmembrane segments constitute the third cytosolic domain (CD3). Four isoforms of the $\alpha$ subunit ($\alpha_1$-$\alpha_4$) have been discovered so far. Interestingly, the four isoforms display distinct tissue distribution patterns (Lingrel et al., 1990; Sweadner, 1989). While the $\alpha_1$ is ubiquitously expressed in all mammalian cells, the $\alpha_2$ expression appears to be restricted to neuronal or muscle cells and the $\alpha_3$ is mainly found in the brain. Finally, the $\alpha_4$ expression is only detected in sperm cells. The
FIGURE 1. Schematic drawing of the Na/K-ATPase α and β subunits. Different colors of the amino acids denotes different homology levels among isoforms. Green: identical among all isoforms; Blue: identical except one isoform; Yellow: identical except two isoforms; Red: different among all isoforms.

Extracellular

Cytoplasm

tissue-specific expression patterns of different isoforms may indicate different physiological roles. The four isoforms display high sequence homology (87% identity among α1, α2 and α3, 78% identity between α1 and α4). However, the amino acid sequence of the N-terminal cytosolic tails (NT) is less conserved than the other parts (50%–70% identity among α1, α2, α3 and α4), which suggests that the NT may confer the distinct physiological roles of the four isoforms. For example, there is a cholesterol recognition/interaction amino acid consensus sequence within NT (\(^{51}\text{LHRKYGTDSLRS}^{61}\)) of human α1 subunit (the consensus sequence will be discussed in more details later). Human α2 and α4 also contain the consensus
sequence but two of the amino acids within this sequence are different in α2 and four in α4. Moreover, human α3 does not have the sequence within NT.

The β subunit is smaller than the α subunit and highly glycosylated in vivo. The overall molecular size is around 55 kDa with the protein portion accounting for 35 kDa (Lingrel and Kuntzweiler, 1994). There are three isoforms of β subunit (β1~β3). Like the isoforms of α subunit, different isoforms of β subunit also display distinct tissue distribution patterns. For example, the β1 is ubiquitously expressed in mammalian cells while the β2 is mainly expressed in neural cells. Moreover, the β3 mainly localizes to skeletal, lung, muscle and brain tissues (Wang et al., 1996). Via its primary sequence and membrane topology study, the β subunit is supposed to contain a single transmembrane domain with its N-terminal tail facing cytoplasm (FIGURE 1). Functionally, the β subunit is essential for the maturation of the whole enzyme and responsible for proper plasma membrane localization (Geering, 1990; 1991; Lutsenko and Kaplan, 1993; McDonough et al., 1990). Furthermore, it is involved in stabilization of a K+ -bound intermediate form of the protein (Eakle et al., 1994).

Besides the α and β subunits, the γ subunit has been identified in kidney tissues with molecular weight around 6.5 kDa (Mercer et al., 1993). It belongs to the family of small, single transmembrane proteins characterized by a FXYD motif (Therien et al., 2001). Recent studies have shown that the γ subunit modulates the function of the Na/K-ATPase in a tissue-specific manner (Garty and Karlish, 2006). However, compared to the α and β subunits, still little is known about the functions of the γ subunit.
Function of the $\alpha$ subunit and its interacting proteins

The $\alpha$ subunit is considered as the catalytic part of the whole enzyme because it contains the cation ($\text{Na}^+$ and $\text{K}^+$) and ATP binding sites (Lingrel et al., 1990). Additionally, the cardiac steroids such as ouabain, which is a well-known specific ligand for the Na/K-ATPase, also binds to the $\alpha$ subunit. As the major part of an ion transporter, it couples transport of two $\text{K}^+$ into and three $\text{Na}^+$ out of the cells by hydrolyzing ATP as its energy source. As a result of its pumping function, the mammalian cells are able to maintain steep ion gradients across the plasma membrane with low sodium, high potassium intracellularly and high sodium, low potassium extracellularly. Moreover, the sodium gradient across the plasma membrane created by the Na/K-ATPase provides the driving force for several facilitated membrane transport proteins that import glucose, amino acids and other nutrients into the cells.

Recent studies have revealed that the $\alpha_1$ subunit is capable of interacting with various proteins, which play important roles in protein trafficking and signal transduction processes (Devarajan et al., 1994; Done et al., 2002; Efendiev et al., 2008; Efendiev et al., 2005; Kimura et al., 2007; Lee et al., 2001; Tian et al., 2006; Wang et al., 2004; Yuan et al., 2005; Yudowski et al., 2000; Zatti et al., 2005; Zhang et al., 2006). Depending on the binding site on the $\alpha_1$ subunit, they can be categorized into two groups. The first group of proteins bind to NT of the $\alpha_1$ subunit such as 14-3-3 Zeta (Efendiev et al., 2005), PI3K (Yudowski et al., 2000), IP3 receptor (Zhang et al., 2006), and caveolin-1 (Wang et al., 2004). The second group constitutes
those bind to CD2 or CD3 of the α1 subunit such as arrestin, spinophilin, GPCR kinases, 14-3-3 epsilon (Kimura et al., 2007), adaptor protein-1 (Efendiev et al., 2008), adaptor protein-2 (Done et al., 2002), ankyrin (Devarajan et al., 1994) and Src (Tian et al., 2006). On the other hand, depending on the function of the α1-association, they can be divided into three groups. The first group includes those bind to the α1 subunit and modulate trafficking of the Na/K-ATPase. For example, in response to dopamine and its corresponding GPCR signals, several proteins were recruited to the α1 subunit including arrestin, spinophilin, GPCR kinase and 14-3-3 epsilon, which mediated endocytosis of the Na/K-ATPase (Kimura et al., 2007). In another example, 14-3-3 Zeta associated with the α1 subunit and facilitated binding of PI3K to the α1 subunit that subsequently led to endocytosis of the Na/K-ATPase (Efendiev et al., 2005; Yudowsky et al., 2000). Conversely, in response to Angiotensin II, adaptor protein-1 attached to the α1 subunit and facilitated the recruitment of the Na/K-ATPase to the plasma membrane (Efendiev et al., 2008). Furthermore, the interaction between α1 and caveolin-1 appears to be important for ouabain-induced endocytosis of the Na/K-ATPase (Liu et al., 2005). The second group consists of those dynamically interact with α1, which influences Ca^{2+} signaling events or kinases activity. For example, binding of the IP3 receptor and PLC gamma1 to α1 formed the signaling complex that regulated intracellular Ca^{2+} signal (Yuan et al., 2005; Zhang et al., 2006). Additionally, interaction of the α1 subunit with Src kinase inhibited its kinase activity. Binding of ouabain to the α1 subunit released Src and activated its kinase activity, which led to activation of Mitogen-activated Protein Kinase (MAPK) pathway (Tian
et al., 2006). The last group contains those directly enhance the Na/K-ATPase activity such as coflin and polycystin-1 (Lee et al., 2001; Zatti et al., 2005).

**Caveolae and caveolin proteins**

**Caveolae, the structure and function**

The caveolae were first identified in the 1950s by electron microscopy and defined as little pits in the plasma membrane with diameter around 60–80 nm (Yamada, 1955). They appear as flask-shaped vesicular invaginations and, unlike the clathrin-coated pits, show no obvious coat on the cytoplasmic surface (Stan, 2005). The caveolae structures are highly abundant in specific cells such as smooth-muscle cells, fibroblasts, endothelial cells and adipocytes (Parton and Simons, 2007). Although the morphology of caveolae was well characterized more than half a century ago, it was not until the discovery of its protein marker, caveolin, in the early 1990s (Rothberg et al., 1992) that the studies on the cellular functions of caveolae at the molecular level were made possible and have progressed dramatically since then.

Now caveolae have been implicated in endocytosis, trancytosis, calcium signaling and numerous other signal transduction processes (Kurzchalia and Parton, 1999; Lisanti et al., 1995; Williams and Lisanti, 2005). Many signaling proteins have been discovered to be localized and even concentrated within caveolae (Fujimoto et al., 1992; Lisanti et al., 1994; Lockwich et al., 2000; Wang et al., 2004), which suggests that caveolae may serve as a good place for compartmentalization of signal transduction and
facilitate the regulation of various signaling events in a time and space-specific manner. Additionally, caveolae have been exploited by pathogens as entry sites for invading the cells (Pelkmans et al., 2001).

**Caveolins, from gene to protein structure and function**

The caveolin proteins are structural proteins that are necessary for the formation of caveolae (Williams and Lisanti, 2004). There are three genes (CAV-1, CAV-2 and CAV-3) encoding for four caveolin proteins (caveolin-1α, caveolin-1β, caveolin-2 and caveolin-3) with the molecular weight around 22 kDa. Caveolin-1 (Cav-1) and caveolin-2 (Cav-2) are co-expressed in most cell types while caveolin-3 (Cav-3) is expressed mainly in the muscle cells. As membrane proteins, all the caveolins show unusual membrane topology with both N and C-termini lying within the cytoplasm and a long hairpin-like intramembrane domain. Gene ablation studies have revealed that Cav-1 and Cav-3 are indispensable for biogenesis of caveolae (Drab et al., 2001; Galbiati et al., 2001) while loss of Cav-2 has no apparent effect on caveolae formation *in vivo* (Razani et al., 2002a). An interesting feature of caveolins is that they form oligomers via cytoplasmic N-terminal domain (Sargiacomo et al., 1995), which is believed to play a role in the formation of caveolae.

Cav-1 is found to interact with many signaling proteins via its N-terminal scaffold domain (residues 82~101) and modulate various signal transduction pathways. For example, it interacts with transient receptor potential canonical channel 1 (TRPC1) and IP3 receptor to regulate Ca\(^{2+}\) store release-induced Ca\(^{2+}\) entry.
(Sundivakkam et al., 2009). It also dynamically associates with endothelial Nitric Oxid synthases (eNOS) that controls NO production and signaling (Kone, 2000). Other Cav-1-interacting proteins include epidermal growth factor receptor (EGFR), G-protein α subunits, GPCR kinase, insulin receptor, MAP kinases, PI3K, protein kinase A (PKA), Src kinase, etc (Krajewska and Maslowska, 2004).

The signaling Na/K-ATPase and caveolae

In the past several years, accumulating evidence has demonstrated that besides ion transport function the Na/K-ATPase can also be a signal transducer (Xie, 2003; Xie and Askari, 2002; Xie and Cai, 2003). Interestingly, previous work from my labmates has shown that the Na/K-ATPase α1 subunit interacts with Cav-1 via NT and is highly concentrated in caveolae (Liu et al., 2003; Wang et al., 2004) (FIGURE 2). The signaling Na/K-ATPase mainly resides in caveolae (Liang et al., 2007). Disruption of the caveolae structure leads to disassembly of the signaling complex and abolishes the activation of Na/K-ATPase-mediated signal transduction by ouabain (Wang et al., 2004). Moreover, loss of Cav-1 interrupts endocytosis of the Na/K-ATPase induced by ouabain (Liu et al., 2005). Expression of NT in LLC-PK1 cells disrupts the interaction between the Na/K-ATPase and IP3 receptor, which inhibits ATP-induced Ca\(^{2+}\) release (Chen et al., 2008). Taken together, NT of the Na/K-ATPase α1 is an important domain for the interaction between the Na/K-ATPase and its signaling partners like Cav-1 and IP3 receptor, which is essential for the localization, trafficking and function of the signaling pump.
FIGURE 2. Schematic drawing of the Na/K-ATPase-mediated signaling complex localizing in the special lipid domain, caveolae.

On the other hand, it has been demonstrated recently that the interaction between NT and Cav-1 is also important for the membrane trafficking of Cav-1 (Cai et al., 2008). Like other proteins, Cav-1 is synthesized in the endoplasmic reticulum (ER), where it goes through a first stage of oligomerization and then travels to the Golgi complex. It is further processed within the Golgi complex and at some point in the biosynthetic pathway it associates with lipid rafts, becomes detergent-resistant and is organized into higher-order oligomers, which resemble the characters of the cell surface Cav-1 proteins (Parton and Simons, 2007). Nevertheless, trafficking of Cav-1
into the plasma membrane appears to be regulated by the plasma membrane pool of the Na/K-ATPase. Graded knockdown of Na/K-ATPase correspondingly decreases the plasma membrane pool of Cav-1 and accumulation of Cav-1 in the perinuclear regions. Furthermore, this effect is independent of the pumping function of the Na/K-ATPase. Instead, it is mediated by interaction between NT and Cav-1. Further database search has revealed that there is a caveolin-binding motif (CBM) localized within NT. Subsequent site-directed mutagenesis analysis has confirmed that CBM is responsible for binding of NT to Cav-1 (Cai et al., 2008; Wang et al., 2004). Thus, interaction of NT with Cav-1 stabilizes the plasma membrane pool of Cav-1.

**Caveolin and cholesterol homeostasis**

Besides the role of caveolin proteins in the biosynthesis of caveolae and signal transduction, many evidences have implicated them in cholesterol homeostasis (Razani et al., 2002b). For instance, it has been reported that Cav-1 directly binds to free cholesterol in a 1:1 ratio (Murata et al., 1995). It not only participates in the trafficking of cholesterol from ER to the plasma membrane (Uittenbogaard et al., 1998) but also is involved in the trafficking of cholesterol from the plasma membrane to the internal membranes (Uittenbogaard et al., 2002). Additionally, the expression of Cav-1 appears to be under control of sterol regulatory element binding proteins (SREBP), the master regulator of cellular cholesterol level. High cellular free cholesterol levels decrease the transcription of Cav-1, which is accompanied by reduction of caveolae in the plasma membrane (Fra et al., 2000; Hailstones et al.,
Furthermore, knockout of Cav-1 \textit{in vivo} significantly alters cellular cholesterol homeostasis (Frank et al., 2006).

That caveolins play a role in lipid regulation is also supported by research on the caveolin mutants including the artificial truncated form and the point mutations observed in the patients with Limb Girdle Muscular Dystrophy. In one of the reports, a dominant negative Cav-3 mutant specifically associates with intracellular lipid droplets and leads to accumulation of free cholesterol in late endosomes, a decrease in surface cholesterol, cholesterol synthesis and efflux (Pol et al., 2001). It suggests that the caveolin is an important component for intracellular cholesterol balance and trafficking in the fibroblasts. Later studies have further uncovered the cholesterol regulating properties of caveolin. It has been shown that phosphorylation of a conserved serine residue (Ser80) in Cav-1 alters free cholesterol binding (Fielding et al., 2004). Consistent with this model, platelet-derived growth factor (PDGF) treatment leads to phosphorylation of Ser80 and increases cholesterol efflux.

One striking feature of caveolae is the high enrichment with cholesterol. It has been estimated that 144 molecules of caveolin are present in a caveolar structure while the amount of cholesterol might be 100 times higher than caveolins (Parton and Simons, 2007). Therefore, a simple 1:1 interaction between Cav-1 and cholesterol could not fully account for the dramatic enrichment of cholesterol within caveolae and other proteins and lipids are likely to contribute to the high packing of cholesterol within caveolae via interaction among proteins and lipids. One promising candidate is the Na/K-ATPase \( \alpha_1 \) subunit for the following reasons. Firstly, it shares many features
with cholesterol. It is ubiquitously expressed in the mammalian cells and is an abundant component of the plasma membrane. Like cholesterol, it is also highly concentrated within caveolae and interacts with caveolin proteins (Wang et al., 2004). Moreover, depletion of either $\alpha_1$ or cholesterol affects the number of surface caveolae (Cai et al., 2008; Dreja et al., 2002). Secondly, as mentioned before, the Na/K-ATPase $\alpha_1$ subunit regulates membrane trafficking of Cav-1 (Cai et al., 2008). Since Cav-1 trafficking may be coupled with intracellular cholesterol trafficking, Na/K-ATPase $\alpha_1$ subunit may also play a role in regulation of cholesterol transport.

**Cholesterol metabolism**

**General function**

Cholesterol is an integral component of all mammalian cell membranes. It is required for the cell membrane to establish proper membrane permeability and fluidity. Structurally, it contains a hydrophilic hydroxyl group that interacts with the polar head groups of the membrane phospholipids and sphingolipids and a bulky steroid ring with the hydrocarbon chain that is embedded in the membrane alongside the non-polar fatty acid chain of other membrane lipids. Therefore, presence of cholesterol tends to make the membrane lipid layer tightly packed and reduces membrane fluidity and permeability to hydrophilic molecules like protons and sodium ions. Moreover, cholesterol is essential for the structure and function of the special membrane lipid domain like caveolae and clathrin-coated pits. Depletion of
cholesterol disrupts caveolae structure (Dreja et al., 2002), which suggests that formation of caveolae requires cholesterol. Due to the importance of caveolae for signal transduction processes, transient changes in membrane cholesterol level may play a role in regulating signal transduction (Fielding and Fielding, 2004).

Cholesterol is also the precursor molecule for many essential biochemical molecules. For example, it is converted to bile acids in the liver, which solubilize fats in the digestive tract and facilitate intestinal absorption of fat molecules as well as fat soluble vitamins. It serves as the precursor for vitamin D and all the steroid hormones including cortisol, aldosterone, sex hormones (progesterone, estrogens and testosterone) and their derivatives. Additionally, it is suggested that cholesterol may serve as an antioxidant (Smith, 1991).

**Cholesterol de novo synthesis and regulation**

Cholesterol can be acquired from the food we eat but a large portion of cholesterol is from de novo synthesis. Liver and intestines are the major organs for de novo synthesis. On the cellular level, cholesterol synthesis takes place mainly in the ER from the two-carbon acetate group of acetyl-CoA, which is transported from the mitochondrion. The process has five major steps: 1. Acetyl-CoA is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). 2. HMG-CoA is converted to mevalonate. 3. Mevalonate is catalyzed to isopentenyl pyrophosphate (IPP) with the concomitant loss of CO₂. 4. IPP is converted to squalene. 5. Squalene is converted to cholesterol. Among the major synthesis steps, conversion of HMG-CoA to
mevalonate is the rate-limiting step, which is catalyzed by HMG-CoA reductase. Although cholesterol is essential for the cellular function, excess cholesterol is toxic to the cells (Attie, 2007). Therefore, cellular cholesterol levels are closely monitored and tightly regulated by a family of membrane-bound transcription factors called sterol regulatory element-binding proteins (SREBPs) (Horton et al., 2002). When the cellular cholesterol level is high, the SREBPs tightly associate with another membrane protein called SREBP cleavage-activating protein (SCAP), which is bound to the ER membrane resident protein, insulin regulated protein (Insig) and the whole protein complex is restricted within the ER membrane. Thus, the transcription factors, SREBPs, are kept in an inactive state (Goldstein et al., 2006). However, when the cellular cholesterol level drops below certain point, the SCAP protein is able to sense this change via its sterol-sensing domain and alters its conformation. This renders release of SCAP from Insig protein and trafficking of the SREBP-SCAP complex to Golgi network, where SREBPs are cleaved at two separate sites by two proteases called site-1 protease (S1P) and site-2 protease (S2P) respectively (Brown et al., 2000). Cleavage of the SREBPs results in release of the N-terminal domain of the SREBPs, which is its active form. Then the active SREBP protein is no longer membrane bound and able to migrate into the nucleus and activate the expression of many downstream genes that are involved in de novo cholesterol synthesis or uptake of the cholesterol from extracellular spaces. On the other hand, low levels of cholesterol tend to stabilize the key cholesterol synthesis enzyme, HMG-CoA reductase as it also contains a sterol-sensing domain while high levels of cholesterol
destabilize this enzyme (Goldstein et al., 2006). Thus, via SREBPs pathway and direct
effect of cholesterol on HMG-CoA reductase, a typical negative-feedback system is
established to make sure that cellular cholesterol level is kept within a normal range.

However, regarding the activation of SREBPs a fundamental question remains
to be resolved: “where is the cholesterol sensing pool?” Conceivably, the ER
membrane cholesterol pool is a good candidate as most members of the cholesterol
sensing machinery reside in the ER membrane. Nevertheless, ER membrane
cholesterol pool only constitutes a tiny portion of the cellular cholesterol. It has been
estimated that less than 1% of the cellular cholesterol appears in the ER membrane
(Lange et al., 1999). Moreover, the major function site for cholesterol is the plasma
membrane. Therefore, how could the ER cholesterol pool reflect whether cells have
enough cholesterol or not? One possibility is that the plasma membrane cholesterol
pool, which accounts for the majority of the cellular cholesterol, is able to monitor its
own cholesterol level and send a signal to ER. Actually, it has been reported that
plasma membrane cholesterol constantly travels to ER membrane (Lange et al., 1993)
and ER cholesterol level appears to be regulated by plasma cholesterol pool (Lange et
al., 1999). Thus, it has been hypothesized that a plasma membrane cholesterol sensor
exists, which controls the flux of plasma membrane cholesterol trafficking to the ER
membrane (Lange and Steck, 1996). When the plasma membrane cholesterol level is
too high, the sensor responses by directing more cholesterol towards ER. This tends to
upregulate ER cholesterol level. The increased ER cholesterol level is then detected
by ER membrane protein with the sterol-sensing domain such as SCAP and
HMG-CoA reductase so that the whole cholesterol regulation machinery will work to drive the cholesterol level back to normal. Therefore, discovery of this plasma membrane cholesterol sensor and the mechanism how it controls the cholesterol trafficking between the plasma membrane and ER will dramatically improve our knowledge on cellular cholesterol regulation.

**Intracellular cholesterol traffic**

As mentioned above, cellular cholesterol homeostasis is closely related to intracellular cholesterol transport. As a highly hydrophobic lipid molecule, cholesterol can hardly make a long-range movement within the cytoplasm by itself. Therefore, cholesterol is transported intracellularly either via small vesicles or by binding to the carrier proteins (Soccio and Breslow, 2004). Cholesterol is present in the membrane of little shuttle vesicles among different organelles. Generally, the vesicles attach to an intact cytoskeleton and move along it with ATP providing the energy. On the other hand, there are diffusible carrier proteins that contains hydrophobic cavity to hold cholesterol in it and carry it across the aqueous cytosol. For example, the steroidogenic acute regulatory protein (StAR) is found to transport cholesterol within the mitochondrion and stimulate conversion of cholesterol to steroids (Miller and Strauss, 1999). In another example, the sterol-carrier protein 2 (SCP2) is able to bind and transfer many lipids including cholesterol (Gallegos et al., 2001). Moreover, the Cav-1 protein is found to interact with cholesterol (Murata et al., 1995) and involved in cholesterol trafficking between the plasma membrane and internal membranes.
(Uittenbogaard et al., 2002; Uittenbogaard et al., 1998). Interestingly, most of the known cholesterol-binding proteins show a common cholesterol recognition/interaction amino acid consensus pattern (-L/V-(X)₁₋₅-Y-(X)₁₋₅-R/K-) (Li and Papadopoulos, 1998). This consensus pattern is characterized by a neutral and hydrophobic amino acid such as Leucine or Valine, a Tyrosine amino acid and a basic amino acid such as Arginine or Lysine (TABLE 1). One to five any amino acids can be placed between the three coding amino acids. According to the hypothesis, Leucine or Valine will interact with the hydrophilic side chain of cholesterol and Tyrosine will interact with hydrophilic hydroxyl group of cholesterol while the Arginine or Lysine helps to create a pocket to hold cholesterol. Further test by site-directed mutagenesis of Tyrosine to Serine in the cholesterol-interacting protein, peripheral-type benzodiazepine receptor, completely abolishes its cholesterol binding affinity (Jamin et al., 2005). Thus, the cholesterol recognition/interaction amino acid consensus pattern may be a common feature of the cholesterol-interacting proteins and plays a role in cholesterol trafficking.
The cholesterol is synthesized in the ER membrane. Nascent cholesterol must be transported to the plasma membrane to perform its major function. One trafficking route is via vesicular transport along the protein secretary pathway following the direction of ER, Golgi complex and plasma membrane. This pathway requires ATP as the energy source and intact cytoskeleton to travel along. However, it appears that the vesicular pathway is not the major way for the newly synthesized cholesterol to reach plasma membrane for the following reasons. Firstly, disruption of the cytoskeleton has no effect on the cholesterol transport from the ER to the plasma membrane (Kaplan and Simoni, 1985). Secondly, disassembly of the Golgi complex blocks more than 90% of protein secretion but only decreases 20% of the nascent cholesterol.
reaching the plasma membrane (Heino et al., 2000). Therefore, this pathway may contribute to a small part of the ER cholesterol trafficking to the plasma membrane but it is not likely the major one. Another possible mechanism is via cytosolic transport proteins like Cav-1 (Soccio and Breslow, 2004). It has been demonstrated that Cav-1 is one of the components that mediate cytosolic cholesterol trafficking (Uittenbogaard et al., 1998). Besides, newly synthesized cholesterol first appears in caveolae and then disperses to non-caveolae plasma membrane regions (Smart et al., 1996). Blockade of the cholesterol trafficking reduces cholesterol content of caveolae and expression of Cav-1 dramatically speeds up trafficking of newly synthesized cholesterol to caveolae. Taken together, Cav-1 may be an important cholesterol carrier protein that mediates transport of the ER cholesterol to the plasma membrane.

While the newly synthesized cholesterol is transported from ER to the plasma membrane, it also travels from the plasma membrane to the internal membranes including ER (Soccio and Breslow, 2004). However, many evidences suggest that the trafficking mechanisms for the two directions are different since many treatments affect one pathway and leave the other one intact (Field et al., 1998). One of the mostly studied pathways is the low density lipoprotein (LDL) receptor-mediated uptake of cholesterol from the extracellular spaces (Brown and Goldstein, 1986). Binding of LDL to the LDL receptor triggers the endocytosis of LDL receptor together with LDL. Then the whole complex goes to the endosome pathway and ends up in the acidic lysosomes, where LDL is hydrolyzed and releases free cholesterol. Subsequently, the unbound LDL receptor will return to the plasma membrane for the
next round of endocytosis and free cholesterol will be transported to the other internal membrane systems including ER or to the cell surfaces. Trafficking of the plasma membrane cholesterol back to ER is essential for the cellular cholesterol level regulation because it is considered to be part of the negative-feedback system to control cellular cholesterol content.

**Cholesterol trafficking defect and related diseases**

Intracellular cholesterol trafficking and regulation are vital for the proper functions of the cells. Interruption of this network may lead to serious problems like atherosclerosis and Niemann-Pick type C (NPC) disease.

Atherosclerosis is a condition that intracellular cholesterol build-up leads to thickness of the artery wall and accumulation of the macrophage white blood cells in the arterial blood vessels. Although typically asymptomatic for decades, atherosclerosis can progress continuously and eventually leads to atheromatous plaques that narrow the blood vessels causing insufficient blood supply to the tissues or even completely stop blood flow. In the advanced stage, it may lead to death of the tissues within minutes and such catastrophic events as heart attack and stroke. It is believed that a defect in terms of removal of the excess cellular cholesterol from the periphery tissues may underlie the molecular mechanisms of atherosclerosis (Rader et al., 2009).

The NPC disease is generally a complex lipid storage disorder (Liscum and Klansek, 1998). It may occur at any age but commonly inflicts preschool age children.
The patient may display clumsiness or hepatosplenomegaly (liver and spleen enlargement). Prominent manifestations include progressive dementia, cataplecty, dysarthria, dystonia, dysphagia and seizures, which are possibly due to progressive degeneration of central nervous system. Most of the patients die in their early years (less than 20 years old). At the molecular level, NPC disease is characterized by accumulation of cholesterol within late endosomes/lysosomes. As described in the previous paragraphs, in a normal cell LDL-derived cholesterol is released in the lysosomes and transported to the other internal membranes or the plasma membrane. However, in the NPC cells there appears to be a defect of the cholesterol trafficking between the plasma membrane and the late endosomes/lysosomes so that the cholesterol accumulates within the late endosomes/lysosomes (Lange et al., 2002). Certain amphiphiles such as U18666A, progesterone and imipramine are found to cause intracellular cholesterol accumulation similar to the NPC disease cells (Lange et al., 1998). Unfortunately, the molecular mechanism that underlies this phenomenon in the NPC disease remains to be uncovered.

**Hypertension**

Hypertension is a medical condition that the blood pressure is chronically elevated. In the developed countries, the chance to becoming hypertensive (blood pressure >140/90 mmHg) exceeds 90% during a lifetime. People with hypertension have higher chances of getting strokes, heart attacks, heart failure and arterial aneurism. Moreover, hypertension is the leading cause of chronic renal failure.
Depending on the cause of hypertension, it can be classified as primary hypertension and secondary hypertension. The primary hypertension has no specific medical cause of high blood pressure and therefore is also referred to as essential hypertension (Messerli et al., 2007). It accounts for ~95% of the hypertension cases. The secondary hypertension indicates that the high blood pressure is a result of another medical condition such as kidney disease, tumor, sleep apnea, disorders of the adrenal glands and pre-eclampsia. As the cause of high blood pressure is identified for the secondary hypertension, treatment and removal of the underlying medical condition or pathology normally solve the problem of hypertension.

**Mechanisms of blood pressure regulation**

The mammalian body has multiple mechanisms to control blood pressure and each one serves a specific and often different role in pressure regulation. In response to acute extraneous forces that act on the circulatory system, the nervous system can be modulated within seconds to prevent large rapid changes in blood pressure. After that, within minutes to hours, several blood pressure control mechanisms begin to react (Guyton, 1992). For example, the stress-induced relaxation of the arteries and arterioles leads to expansion of the blood vessels when the blood pressure is too high. By this way, the peripheral resistance gradually drops, which tends to drive the pressure back. However, the most powerful system within this time frame that affects blood pressure is the renin-angiotensin-aldosterone system (Hall, 1986). Renin, also known as angiotensinogenase is a circulating enzyme released by juxtaglomerular
apparatus when it detects a drop in arteriolar pressure. Then it breaks down angiotensinogen into the peptide angiotensin I, which is further converted to angiotensin II in the lungs by angiotensin converting enzyme (ACE). Angiotensin II affects blood pressure via several ways. First of all, it is a very powerful vessel constrictor by binding to the type 1 angiotensin II receptors (AT1) on the blood vessel cells. By this way, it increases systemic vascular resistance and arterial blood pressure. Secondly, it acts on the adrenal cortex to release aldosterone, which promotes sodium reabsorption in the kidney. The increased sodium reabsorption will lead to sodium and fluid retention that increase plasma volume and blood pressure. Thirdly, it stimulates the release of vasopressin from the posterior pituitary, which also increases fluid retention by the kidney. Moreover, it stimulates thirst centers within the brain and promotes water intake.

After hours to days, the kidney starts to take control and adjust the extracellular fluid and blood volumes to hold the blood pressure at a precise point. Therefore, in terms of long-term blood pressure regulation, the kidney-fluid volume system plays a dominant role compared to the other blood pressure control mechanisms (Guyton, 1992). According to the kidney-fluid volume system theory, the kidney is the key to balance fluid intake and output at various blood pressures. High blood pressures push kidney to excrete more sodium and fluid in the urine (pressure-natriuresis). Subsequently, fluid output exceeds fluid intake and tends to lower blood volume, which decreases blood pressure. On the other hand, low blood pressures will make kidney response in the reversed direction and ultimately increase
blood pressure. Thus, an equilibrium blood pressure will be reached eventually where the fluid intake equals output. Unlike the other control systems, the kidney-fluid volume system for blood pressure control is infinite. Therefore, no matter what the other control systems do, in the long run the kidney-fluid volume system always dominates and sets the blood pressure at certain point. Thus, it is important to study what molecular mechanism within kidney contributes to the regulation of sodium and fluid excretion in response to various sodium and fluid intake and what determines the setpoint of the equilibrium blood pressure.

The Na/K-ATPase and blood pressure regulation

It has long been established that sodium loading in either normotensive or hypertensive individuals is associated with an increase in blood pressure (Rodriguez-Iturbe and Vaziri, 2007; Weinberger, 1996). However, the exact mechanism between sodium loading and high blood pressure was not well understood until Guyton and his coworkers elegantly showed that kidney plays a key role in balancing salt intake and output by controlling a natriuresis response induced by changes in the blood pressure (Guyton et al., 1972). Therefore, regulation of the transport of salt ions especially sodium ion within the kidney may represent the molecular mechanism for long-term blood pressure regulation.

The renal Na/K-ATPase is an essential component for sodium reabsorption within the kidney. It resides on the basolateral plasma membrane and transports the sodium ion from cytosol to blood. On the apical plasma membrane resides the sodium
Figure 3. The renal Na/K-ATPase plays a role in sodium reabsorption, CTS induce endocytosis of both Na/K-ATPase and NHE3 that reduces renal sodium reabsorption, which affects plasma volume and blood pressure.

The diagram shows the renal cells with a hydrogen exchanger (NHE) that drives sodium ion from urine to cytosol. Renal sodium reabsorption mainly takes place in the proximal tubule section that involves apical sodium entry through NHE3 and basolateral sodium extrusion via the Na/K-ATPase. Recently, there are evidences that the signaling Na/K-ATPase controls expression and trafficking of NHE3 (Oweis et al., 2006). The Na/K-ATPase ligand such as ouabain not only decreases cell surface Na/K-ATPase activity but also downregulates activity and expression of NHE3. Thus, the functions of the Na/K-ATPase and NHE3 can be coupled via the signaling Na/K-ATPase and it may represent an important mechanism for the regulation of renal sodium reabsorption.
Cardiotonic steroids (CTS) including ouabain, digoxin, marinobufagenin (MBG) are well-known Na/K-ATPase ligands that are endogenously produced cardiotonic steroids (Hamlyn et al., 1991; Hilton et al., 1996; Schoner, 2002). At high concentration they can bind to the Na/K-ATPase and inhibit its pumping activity while at low concentration they trigger signal transduction via the Na/K-ATPase and regulate downstream events like trafficking of the Na/K-ATPase and Ca\(^{2+}\) signaling (Schoner and Scheiner-Bobis, 2007b). It has been demonstrated recently that the highly conserved cardiac glycoside binding site of the Na/K-ATPase is involved in blood pressure regulation (Dostanic-Larson et al., 2005) and the \(\alpha_1\) subunit ouabain-binding site of the Na/K-ATPase participates in the natriuresis response to salt loading (Loreaux et al., 2008). Enhanced production of the Na/K-ATPase ligand, MBG in the plasma is observed in the patients with essential hypertension (Gonick et al., 1998). Moreover, salt loading not only downregulates NHE3 but also induces Na/K-ATPase endocytosis in an MBG dependent manner (Amlal et al., 2001; Periyasamy et al., 2005). Taken together, the endogenous CTS may play a role in the natriuresis response to salt loading via binding to the Na/K-ATPase that leads to endocytosis of both Na/K-ATPase and NHE3. When the system goes wrong, it not only interrupts the normal natriuresis response but also contributes to hypertension.
MATERIALS AND METHODS

Materials

Chemicals of the highest purity and culture media were purchased from Sigma (St. Louis, MO). PP2, a Src kinase inhibitor, was from Calbiochem (San Diego, CA). Filipin, Mβ-CD and cycloheximide were obtained from Sigma. U18666A, the intracellular cholesterol trafficking inhibitor, was from Cayman Chemical (Ann Arbor, MI). NBD-cholesterol was from Molecular Probes (Eugene, OR). The antibodies and their sources are as follows: The monoclonal anti-α1 antibody (α6F) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. The polyclonal anti-Tyr(P)418-Src antibody was from Biosource (Camarillo, CA). The polyclonal anti-SREBP2 antibody was from Cayman Chemical. The polyclonal anti-HMG-CoA reductase antibody and the monoclonal anti-α1 antibody for immunostaining were from Upstate Biotechnology Inc. (Lake Placid, NY). The monoclonal anti-LDL receptor was from Calbiochem. The monoclonal anti-α-tubulin antibody was from Sigma. The monoclonal anti-GFP antibody and the monoclonal anti-caveolin-1 antibody for immunostaining were from BD Biosciences (San Jose, CA). All the other antibodies including the polyclonal anti-caveolin-1, the monoclonal anti-Src, the monoclonal anti-pERK1/2, the polyclonal anti-ERK1/2, the polyclonal anti-insulin receptor β subunit, the polyclonal anti-NHE3 were from Santa Cruz Biotechnology (Santa Cruz, CA). Optitran nitrocellulose membrane was from
Schleicher & Schuell Bioscience (Keene, NH). Enhanced chemiluminescence SuperSignal kit was purchased from Pierce (Rockford, IL). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). The Amplex Red Cholesterol Assay Kit was purchased from Molecular Probes. To purify GST-fused proteins, we obtained Glutathione beads from Amersham Bioscience (Uppsala, Sweden) and Probond Purification System from Invitrogen. Plasmids pEYFP-N1/C1 were from Clontech (Palo Alto, CA), pGEX-4T-1 was from Invitrogen, and RFP-Rab7 was requested from Addgene (Cambridge, MA).

**Plasmid Constructs**

Rat-α1 expression vector pRc/CMV-α1AAC was provided by Dr. Pressley. (Petrosian et al., 1998). Rat-α1 pump null mutant (D371N) and CBM mutant (F97A, F100A) were generated as previously described (Cai et al., 2008). Rat-α1 CRAC mutant (Y55S) was created by PCR-based site-directed mutagenesis with the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). YFP-α1 was created by inserting the full length rat-α1 gene into pEYFP-C1. GST-NT (amino acid 6–90) expression vectors were subcloned in frame into pGEX-4T-1 expression vector based on the sequence of pig kidney Na/K-ATPase α1 subunit. GST-NT-Y53S expression vectors were created by PCR-based site-directed mutagenesis. All constructs were verified by DNA sequencing.

**Cell culture, Transient transfection and Generation of a stable cell line**
The control LLC-PK1 and P-11 cell lines, caveolin-1 knockdown C2-9 cell line, the Na/K-ATPase α1 knockdown cell lines (A4-11, PY-17, and YCN23-19), the rat α1-rescued PY-17 cell line (AAC-19) or the caveolin-binding motif mutant rat α1-rescued PY-17 cell line (mCBM) and the α1 N terminus-YFP expressing LLC-PK1 cells (NT-YFP) were derived from the LLC-PK1 cells as previously described (Cai et al., 2008; Chen et al., 2008; Liang et al., 2006; Wang et al., 2004). The relative level of Na/K-ATPase α1 in control, α1 knockdown, and rescued cell lines is listed in Table 1. The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), penicillin (100 units/ml)/streptomycin (100 µg/ml), and 1 µg/ml puromycin (for LLC-PK1, no puromycin was added in the medium) in a 5% CO2-humidified incubator. After cells reached >90% confluence, they were serum-starved for 12 h and used for experiments unless indicated otherwise.

For transient transfection, when the cells reached ~70% confluence, they were transfected with the plasmid or empty vector by Lipofectamine 2000 as described previously (Cai et al., 2008). Experiments were performed 6 h after transfection for the pump-null mutant (D371N) transfection and 24 h after transfection for YFP-α1 and RFP-Rab7 transfection.

To generate the stable cell line (PY-17-Y55S), wild-type rat-α1 cDNA sequence (GenBank™ accession number NM_012504) was first mutated to CRAC mutant rat-α1 cDNA by mutating the $^{400}$taggaaca to $^{400}$tcggaaca using pRc/CMV-α1AACm1 as the template (Ohtsubo et al., 1990). The resulting plasmid
was called pRc/CMV-α1AACm1-Y55S. Then PY-17 cells were transfected with pRc/CMV-α1AACm1-Y55S. Selection was initiated with 3 μM ouabain because untransfected cells were very sensitive to ouabain. After about one week, ouabain-resistant colonies were isolated and expanded into stable cell lines in the absence of ouabain.

**Experimental Animal**

Na/K-ATPase α1+/+ and α1+/− mice were generated as previously described (James et al., 1999). Cav-1 KO and control mice were purchased from Jackson Lab (Bar Harbor, ME). Genomic DNA was obtained from tail biopsies and used for PCR-based genotyping. Adult mice at 16-20 weeks were used for the study.

To generate the Na/K-ATPase α1 N terminus overexpressing mice (NT mice), Na/K-ATPase rat α1 N-terminus (1-160 amino acid) DNA sequence was obtained by PCR assay on rat α1 cDNA (GenBank, M28647) using sense primer: 5’-GGAATTCCTATGGGGAAGGGGGTTGGAC-3’ and antisense primer: 5’-GGGTACCAGGGATTCCATGATCTTGGAGC-3’. The PCR product was purified and sequence was verified by DNA sequencing. It was then inserted into the vector pEYFP-N1 (Clontech) between EcoRI and KpnI sites forming the construct pNT-EYFP. The construct was subsequently amplified by transforming into E. coli DH5α. After amplification, around 30ug of the construct were double digested by the endonucleases AseI and SspI at 37 °C for 3 hours to generate three DNA fragments: 2.5kb, 2.2Kb, and 550bp. As the 2.2Kb fragment (NT-EYFP) contains the CMV
promoter together with NT DNA sequence and the gene encoding EYFP protein, ~10ug of this fragment were purified and sent to University of Michigan Transgenic Animal Model Core Facility, where the mice with NT-EYFP DNA were generated by microinjection technique (Brinster et al., 1985). Genomic DNA was obtained from tail biopsies and used for PCR-based genotyping. A pair of PCR primers (sense primer: 5’-AACAACTCCGCCCCATTTGAC-3’ and anti-sense primer: 5’-CTCGGCTCAATCTGTTCCG-3’) targeting a 300 bp long DNA sequence within the NT-EYFP gene were used for PCR-based genotyping. The NT mice were backcrossed to the widely-used inbred mouse strain, C57BL/6 continuously. For hypertension study, 3-4 months old male NT mice with their male littermates without NT gene as control mice were used.

All mice were kept in a 12-h dark/light cycle and fed standard chow ad libitum unless indicated otherwise. All procedures were approved by the Institute Animal Care and Use Committee at the University of Toledo, Health Science Campus.

**Purification of Caveolin-rich Membrane Fractions**

Caveolin-rich membrane fraction were obtained via sucrose gradient fractionation as described previously (Wang et al., 2004). Briefly, cells were washed with ice-cold phosphate-buffered saline and scraped in 2 ml of 500 mM sodium carbonate, pH 11.0. The cell lysates were homogenized by a Polytron tissue grinder (three 6-s bursts) and subjected to sonication (three 40-s bursts). The homogenates were then adjusted to 45% sucrose by addition of 2 ml of 90% sucrose in MBS (25
mM Mes, 0.15 M NaCl, pH 6.5) and placed at the bottom of ultracentrifuge tubes. The ultracentrifuge tubes were then loaded with 4 ml of 35% sucrose and 4 ml of 5% sucrose (both in MBS containing 250 mM sodium carbonate) and centrifuged at 39,000 rpm for 16-20 h in an SW41 rotor (Beckman Instruments). Twelve gradient fractions of 1 ml were collected from the top to the bottom. Among the fractions, fraction 4 and 5 were combined and diluted with 4 ml of MBS, then centrifuged at 40,000 rpm in a Beckman type 65 rotor for 1 h. The pellet was resuspended in 250 µl of MBS and is considered as caveolin-enriched caveolar fraction.

**Separation of Cytosol and Cellular Membrane**

Cells were washed with ice-cold phosphate-buffered saline and scraped in 2 ml of sucrose buffer A (30 mM histidine, 250 mM sucrose, 1 mM EDTA-Na, pH 7.4). The cell lysates were homogenized by a Polytron tissue grinder (three 6-s bursts) and subjected to sonication (three 40-s bursts). Then cell lysates were transferred to a 15-ml Falcon centrifuge tube and subjected to centrifugation at 1,000 rpm for 5 min to remove cell debris. The supernatants were transferred to ultracentrifuge tubes and were centrifuged at 40,000 rpm in a Beckman type 65 rotor for 1 h. The supernatants (taken as cytosol fraction), were collected in the Eppendorf tubes. The pellets, taken as the membrane fraction, were resuspended in 250 µl of buffer A.

**Western blot analysis and Immunostaining**

Protein concentration of cell lysates was measured by Protein Assay Kit from
Bio-Rad (Hercules, CA). Cell lysates with equal amounts of proteins were loaded onto the gel and separated on 10% SDS-PAGE, transferred to an Optitran membrane, and probed with corresponding antibodies. Protein signals were detected with an ECL kit and quantified using a Bio-Rad GS-670 imaging densitometer.

Immunostaining of caveolin-1 or α1 was performed as previously described (Cai et al., 2008) with minor modifications. Cells were cultured for 24 h on glass coverslips. The cells were washed three times with PBS before being fixed with ice-cold methanol for 30 min. The cells were washed again with PBS and blocked with Signal Enhancer (Invitrogen). Primary antibodies were diluted in 3% BSA in PBS and coverslips were incubated with antibodies overnight at 4°C. After three washes with PBS, AlexaFluor 546/488–conjugated antibodies were added and incubated for 2-3 h at room temperature. Samples were washed and mounted onto slides. Cells were visualized using an inverted confocal laser scanning microscope (DM IRE2; Leica) equipped with a 63x/1.3 oil objective. An argon and a helium/neon laser were used for double fluorescence with excitation at 433 nm, 515 nm and emission at 505–510 nm, and 566–620 nm, respectively. To avoid the cross-talk between the two fluorescent dyes, we used the sequential method featured by the Leica confocal microscope to acquire the images for measuring colocalization of the two proteins.

**Cholesterol Assay**

Cholesterol was measured by Amplex Red Cholesterol Assay Kit according to
the manufacturer’s instructions, and the signals were detected by a microplate spectrofluorometer (excitation: 560 nm; cut off: 570 nm; emission: 590 nm).

Filipin Staining

Filipin staining was performed as described before (Carozzi et al., 2000; Chen et al., 2009). Briefly, cells were cultured on coverslips and grown to ~90% confluence. After three washes with PBS, cells were fixed with 4% paraformaldehyde at room temperature for 30 min. Cells were washed again with PBS for three times. Then cells were incubated with 50 mM NH₄Cl in PBS for 10 min to quench the background. Next, cells were permeabilized with 0.1% saponin in PBS for 10 min. Finally, cells were stained with staining solution (filipin 1 mg/ml, 0.2% BSA, 0.2% fish skin gelatin in PBS) in the dark at room temperature for 30 min. Samples were washed three times with PBS and mounted onto slides. Filipin signals were viewed by a fluorescence microscope with a UV filter (excitation: 360 nm; emission: 440 nm).

Phenotypic Analysis

For cholesterol metabolism study, following a 4-h fast, mice were euthanized with sodium pentobarbital (150 mg/kg). The animal was weighed before the abdominal cavity was opened, and whole liver organ was carefully dissected and weighed. The liver weight/body weight ratio was calculated. Subsequently, liver tissues were immediately frozen in liquid nitrogen and stored at -80℃ for cholesterol assay and Western blot analysis. Meanwhile, blood was drawn from the inferior vena
cava, stored on ice for 20-30 min, and centrifuged at 2800 x g for 10 min. The supernatant (plasma) was transferred to a fresh Eppendorf tube and sent to the University Medical Center immediately for plasma lipid profile analysis.

For hypertension study, mice were euthanized as mentioned above. The animal was weighed before the abdominal cavity was opened, and whole heart and kidney were carefully dissected and immediately frozen in liquid nitrogen. Then the organs were stored at -80°C for Western blot analysis.

\textbf{3H-ouabain Binding Assay}

The number of ouabain binding sites in cultured cells was assayed by the protocol described previously (Liang et al., 2007). Briefly, cells were seeded into 12-well plates, cultured to 90% confluence, and then serum starved overnight. Cells were then rinsed, incubated in K⁺-free Kreb’s solution (NaCl 142.4 mM; CaCl₂ 2.8 mM; NaH₂PO₄ 0.6 mM; MgSO₄ 1.2 mM; dextrose 10 mM; Tris 15 mM; pH 7.4) in the presence of 20 µM monensin for 10 min, and then exposed to 2 µM ³H-ouabain for 15 min at 37°C. Monensin was added to clamp intracellular Na⁺ and to prevent recycling of the Na/K-ATPase from the intracellular pool to the plasma membrane. At the end of incubation, cells were washed four times with ice-cold K⁺-free Kreb’s solution, solubilized in 0.1 N NaOH/0.2% SDS, and measured by a scintillation counter. The binding data were normalized by the protein content in corresponding dishes.
Semi-quantitative RT-PCR

Cells were seeded in 6-cm culture dish. After treatment, cells were washed with PBS and total RNA were extracted by TRIzol reagent (Invitrogen). DNA contaminants were removed by DNase digestion at 65°C for 10 min. Then mRNA were converted to cDNA via reverse transcription by OmniScript Kit (Qiagen, Germany). Afterwards, a pair of PCR primers (sense primer: 5’-TGTGAAGAACTTGGAGGCTGTGGA-3’ and anti-sense primer: 5’-ACAGCTGGTACTTGTTGGTGAGT-3’) were used for PCR amplification of pig α1 cDNA. Another pair of PCR primers (sense primer: 5’-ACAGTTTCTGGAGCTGGTCCAAA-3’ and anti-sense primer: 5’-AGCTTCCCATTCTCAGCTTTGACT-3’) were used for PCR amplification of pig glyceraldehydes 3-phosphate dehydrogenase (GAPDH) cDNA as internal control. 30 cycles were picked for semi-quantitative RT-PCR as it was within the linear amplification range as tested before. The PCR products were run in 1% agarose gel with ethidium bromide (0.4 µg/ml). DNA gel results were recorded by a digital camera and quantified by Image J software.

Purification of Na/K-ATPase from Pig Kidneys, and GST-Fused Proteins

Pig kidney Na/K-ATPase was purified as described (Jorgensen, 1974). GST-fused proteins were expressed in Escherichia coli BL21 (Invitrogen) and subsequently purified using glutathione beads (Invitrogen). Soluble GST-fused proteins were eluted from the glutathione beads with elution buffer (10 mM reduced
glutathione, 0.1% Triton X-100, 50 mM Tris-HCl, pH 8.0). The eluted solution was dialyzed in the buffer containing 0.1% Triton X-100, 50 mM Tris-HCl (pH 8.0) to remove glutathione.

**Fluorescence Assay for NBD-cholesterol Binding to Purified Na/K-ATPase or GST-fused Proteins**

The NBD-cholesterol fluorescence binding assay was performed according to the protocol described (Petrescu et al., 2001). NBD-cholesterol was incubated with purified proteins in 96-well plates at room temperature for ~10 min. For detection of the fluorescence from NBD-cholesterol, it was excited at 473 nm and emission was scanned at 530 nm by a spectrofluorometer. For detection of FRET signals, the amino acid tryptophan was excited as 295 nm and emission of NBD-cholesterol was detected at 530 nm.

**Measurement of Systolic Blood Pressure in Mice**

Systolic blood pressure levels were determined in conscious mice by noninvasive tail-cuff system. All mice were subjected to one week daily training to get used to the tail-cuff system before formal blood pressure measurement. All mice were tail-cuffed and placed in the warm chamber for at least 10 min to calm down before recording. During the recording, mice were closely monitored to avoid the artificial value from mouse tail movement. Each mouse was subjected to three measurements separated by 5-second intervals. Measurements with SD of more than
30 mmHg for systolic blood pressure levels were not accepted.

**Isolation of early endosomes from the mice kidneys**

Endosomes were isolated on a floatation gradient using the method described before (Chibalin et al., 1998) with minor modifications. Briefly, cells were homogenized in homogenization buffer (sucrose 250 mM, imidazole 3 mM, pH 7.4) with protease inhibitors (phenylmethylsulphonyl fluoride, NaF, 0.5 mM EDTA) and gently homogenized (15-20 strokes) to minimize damage of the endosomes using a Dounce homogenizer. Samples were subjected to centrifugation at 3000 rpm, 4 min for 10 min. The postnuclear supernatant was adjusted to 40.6% sucrose using a stock solution (62% sucrose, 3 mM imidazole, pH 7.4). The final volume was about 1 ml, and loaded at the bottom of a SW centrifuge tube. A gradient consisting of three layers was then loaded sequentially (2 ml of 16% sucrose in D$_2$O, 3 mM imidazole pH 7.4; 2 ml of 10% sucrose in D$_2$O, 3 mM imidazole pH 7.4; 1 ml of homogenization buffer). The gradient was centrifuged at 35,000 rpm for 75 min at 4°C in a SW41Ti rotor. The early endosomal fractions were collected at the homogenization buffer and 10% sucrose interface. The endosomal preparation was analyzed on SDS-PAGE and subjected to Western blot analysis.

**$^{22}$Na$^+$ transcellular transport assay**

$^{22}$Na$^+$ transcellular *in vitro* transport assay was performed as described previously (Liu et al., 2002). The cells were grown to confluence on the 12-mm polycarbonate
transwell culture filter inserts and cultured for seven days. Medium was replaced daily.

To measure transcellular $^{22}\text{Na}^+$ transport, the upper compartment of a filter insert was filled with 700 µl DMEM containing 15 mM $^{22}\text{Na}^+$ (1 µCi/ml), and the lower compartment was filled with 1 ml DMEM. Aliquots were removed at intervals as indicated from the lower compartment for scintillation counting. Each assay was done in the presence and absence of 100 nM ouabain for indicated time.

Data Analysis

Data are given as mean±S.E. Statistical analysis was performed using the Student’s $t$ test and significance was accepted at $p<0.05$. 
RESULTS

Part I: Regulation of Cholesterol Distribution by the Na/K-ATPase

Knockdown of the Na/K-ATPase α1 Redistributes Both Cav-1 and Cholesterol

Thanks to the work of my labmates, we have previously established several Na/K-ATPase knockdown and rescued cell lines via siRNA-based techniques (Liang et al., 2006). As shown in TABLE 2, the P-11 cell line is derived from LLC-PK1 cells that were transfected with an empty vector so that the expression of the Na/K-ATPase α1 is intact. So the P-11 cells serves as control cells to the other LLC-PK1 derived

TABLE 2. Sodium pump expression level in different LLC-PK1 derived cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Na⁺/K⁺-ATPase α1 Subunit Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-11</td>
<td>100</td>
</tr>
<tr>
<td>A4-11</td>
<td>40</td>
</tr>
<tr>
<td>PY-17</td>
<td>10</td>
</tr>
<tr>
<td>AAC-19</td>
<td>100</td>
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</table>

negative control cell

knockdown cell

rescued cell
cells in the following studies. On the other hand, the A4-11 cell line is a Na/K-ATPase α1 knockdown cell line with ~40% of the α1 expression compared to control cells. Moreover, PY-17 cells show further decrease of the α1 expression to only 10% of the control level. Finally, a rat α1 gene was introduced into the PY-17 cells and the α1 level is rescued to control level in AAC-19 cells.

Our previous study has shown that the Na/K-ATPase α1 was concentrated in caveolae and interacted dynamically with Cav-1 via NT in LLC-PK1 cells (Wang et al., 2004). Moreover, knockdown of the α1 increased the mobility of Cav-1, resulting in decreases in the number of caveolae and the amount of Cav-1 in the plasma membrane (Cai et al., 2008). Since cholesterol plays an important role in regulation of the mobility of Cav-1 and the formation of caveolae, we reasoned that knockdown of the α1 might also alter the membrane distribution of cholesterol. To test this hypothesis, cell lysates were fractionated using a detergent-free and carbonate-based density gradient fractionation procedure (Liu et al., 2003; Wang et al., 2004). The low density fractions 4/5 prepared from the control cells contained about 50% of Cav-1 and the Na/K-ATPase α1 and was taken as caveolin-enriched caveolar fraction (FIGURE 4A). Consistent with what was reported in the literature (Sampson et al., 2004), this caveolar fraction also contained more than 50% of total cellular cholesterol (FIGURE 4B). When Cav-1, the Na/K-ATPase α1 and cholesterol content were measured in this fraction prepared from different cell lines, we found that the Na/K-ATPase knockdown caused an α1 amount-dependent decrease in both Cav-1 and cholesterol (FIGURE 4C). While cholesterol in A4-11 cells showed a 20%
reduction in comparison to that in control cells, a 35% decrease was recorded in PY-17 cells. Moreover, when PY-17 cells were rescued by knocking in a rat α1, we found that expression of rat α1 was sufficient to restore both Cav-1 and cholesterol content (FIGURE 4C). Finally, when total cholesterol was measured in different cell lines, we found no different among these cell lines (FIGURE 4D). These findings suggest that the Na/K-ATPase plays an important role in maintaining the plasma membrane pool of both Cav-1 and cholesterol in LLC-PK1 cells and that knockdown of the Na/K-ATPase likely redistributes cholesterol from the plasma membrane to other cellular compartments.

Knockdown of the Na/K-ATPase α1 Redistributes Cholesterol from the Plasma Membrane to the Cytosolic Compartments

To further confirm the above observation, we investigated cellular distribution of cholesterol using a fluorescence cholesterol probe, filipin, in both control (P-11) and the knockdown (PY-17) cells. In P-11 cells, a majority of filipin signals (which labels non-esterified cholesterol) were detected in the plasma membrane. Weak signals were also detected in the peri-nuclear region and in the form of intracellular vesicles as pointed by arrows (FIGURE 5A). These findings are consistent with what has been reported in the literature (Lange et al., 1989). When compared, PY-17 cells exhibited weaker plasma membrane filipin signals. This is in accordance with the results depicted in FIGURE 4. Concomitantly, increases in filipin labeling were noted in the peri-nuclear region in PY-17 cells. Moreover, PY-17 cells contained many
vesicles that were strongly labeled with filipin (as pointed by the arrows) (FIGURE 5B). Taken together, the above studies indicate that knockdown of the Na/K-ATPase redistributed cholesterol from the plasma membrane to intracellular vesicles as well as the peri-nuclear region.

To test whether cholesterol is redistributed from light caveolar fraction (fraction 4/5) to higher density membrane fractions, we fractionated the cell lysates as in FIGURE 4. Afterwards, the caveolar fraction (4/5) and non-caveolar fractions (6 to 11) were diluted and centrifuged at 100,000g x 60 min. The membrane pellets were collected and subjected to cholesterol measurement. Interestingly, no changes in cholesterol content were detected in the combined non-caveolar fractions in the α1 knockdown cells (data not shown). The findings indicate that the plasma membrane cholesterol must be redistributed to cellular compartments (e.g., vesicles) that are too light to be pelleted down by 100,000g x 60 min. To further test this postulate, we separated the cell lysates from the control and Na/K-ATPase knockdown cells into two fractions, namely the crude membrane pellet and the cytosol fraction obtained after 100,000g x 60 min centrifugation. When cholesterol was measured in these two fractions, we detected a decrease in cholesterol in the membrane fraction (FIGURE 6A) and a concomitant increase in the cytosol cholesterol (FIGURE 6B) in cell lysates prepared from the knockdown cells. As expected, the differences in cholesterol distribution among these cells became more obvious when ratios of cytosol over membrane cholesterol were calculated and compared, especially in the case of A4-11 cells (FIGURE 6C). Consistent with the findings depicted in FIGURE 4, knocking in
a rat α1 was sufficient to restore the cytosol and membrane cholesterol to a level comparable to that in the control P-11 cells (FIGURE 6). Taken together, these studies provided further support to the notion that knockdown of cellular Na/K-ATPase redistributed cholesterol from the plasma membrane to other cellular compartments. The findings also demonstrated that this centrifugation analysis can be used to detect the Na/K-ATPase-mediated cholesterol redistribution.

The Interaction between Na/K-ATPase and Cav-1, but Not Src, Is Involved in Control of Plasma Membrane Cholesterol Distribution

The Na/K-ATPase interacts and keeps Src in an inactive state and knockdown of the Na/K-ATPase increases basal Src activity and consequently the activity of ERK1/2 (Liang et al., 2006). It is known that Src and ERK1/2 play an important role in regulation of cholesterol synthesis (Abidi et al., 2005; Sun et al., 2005). Moreover, Src is involved in regulation of Cav-1 trafficking (Cai et al., 2008; Pelkmans and Zerial, 2005). Therefore, we tested whether inhibition of Src by PP2 would restore the normal cellular distribution of cholesterol. As previously reported, exposure of PY-17 cells to 1μM PP2 for 2 hours was sufficient to restore cellular Src and reduce the mobility of Cav-1 vesicle (Cai et al., 2008). However, it failed to change intracellular cholesterol distribution in either control P-11 cells or PY-17 cells when the cytosol/membrane ratios were calculated and compared (FIGURE 7A).

It is known that Cav-1 directly binds cholesterol and affects cholesterol trafficking. Because the Na/K-ATPase knockdown changes cellular distribution of
Cav-1, we reasoned that the interaction between the Na/K-ATPase and Cav-1 could be important for cholesterol trafficking to the plasma membrane. To test this postulation, we first determined the effect of Cav-1 knockdown on intracellular cholesterol distribution. Cav-1 knockdown cell line C2-9 was generated as previously described using siRNA (Wang et al., 2004). As depicted in FIGURE 7B, these cells express ~20% of Cav-1 in comparison to that in control P-11 cells. Interestingly, knockdown of Cav-1, like Na/K-ATPase knockdown, significantly increased the cytosol/membrane ratio of cholesterol (FIGURE 7C). In accordance, filipin staining showed an accumulation of cytosolic cholesterol vesicles with a concomitant decrease in the plasma membrane cholesterol in C2-9 cells (FIGURE 5C).

To further test the role of Na/K-ATPase/Cav-1 interaction, we repeated the above experiments in PY-17 cells rescued by a caveolin-binding motif mutant (mCBM) rat α1. The α1 subunit contains a highly conserved caveolin-binding motif at the NT. The mCBM rat α1 was generated by mutating two of the aromatic amino acid residues in the binding motif to Ala (F97A and F100A). We showed previously that these mutations abolished the interaction between the Na/K-ATPase and Cav-1 (Cai et al., 2008). As depicted in FIGURE 5, expression of mCBM rat α1 (FIGURE 5E), unlike the expression of wild type rat α1 (FIGURE 5D), failed to reduce the vesicular cholesterol staining. Consistently, it also did not restore the cytosol/membrane cholesterol ratio (FIGURE 7C).

To seek further support and test whether the NT of α1 can function as a dominant negative mutant, we transfected LLC-PK1 cells with a YFP-tagged NT
(amino acids 1-160) of α1 (NT-YFP) and generated a stable cell line (Chen et al., 2008). Confocal imaging analyses revealed that the expressed NT-YFP resided mainly in the intracellular compartments (FIGURE 8A). Interestingly, expression of NT-YFP led to accumulation of the Cav-1 protein in the peri-nuclear regions. Furthermore, filipin staining showed that expression of NT-YFP was sufficient to alter the cholesterol distribution as did by Na/K-ATPase knockdown (FIGURE 8B and 8C). Consistently, it also increased cytosol/membrane ratio of cholesterol (FIGURE 8D).

Expression of a Pump-null α1D371N Mutant Is Capable of Restoring Intracellular Cholesterol Distribution in the Knockdown Cells

To probe whether the pumping function of Na/K-ATPase is required for the regulation of cholesterol distribution, we transfected PY-17 cells with a pump-null mutant (YFP-α1D371N). We showed previously that expression of this mutant was sufficient to restore the plasma membrane distribution of Cav-1 (Cai et al., 2008). Consistently, we found that like YFP-α1, expression of YFP-α1D371N mutant in PY-17 decreased the cytosol cholesterol and concomitantly increased the membrane cholesterol, resulting in significant reduction in the ratio of cytosol/membrane cholesterol (FIGURE 9A and 9B). It is important to note that these measurements were made in transiently transfected PY-17 cells because we could not generate stable cell lines that express YFP-α1D371N. To seek further evidence, we transfected TCN23-19 cells with the same mutant construct and then stained transfected cells with filipin. Like PY-17 cells, TCN23-19 cells express less than 10% of Na/K-ATPase
(Liang et al., 2006). Unlike PY-17 cells, these cells do not express GFP, making it easier to conduct imaging analyses of YFP-α1D371N distribution. As shown in FIGURE 9C, the plasma membrane expression of the pump-null D371N mutant was able to increase the filipin signal in the plasma membrane. However, because of low transfection efficiency, this increase in the plasma membrane cholesterol appeared to be modest. Taken together, these findings indicate that the Na/K-ATPase can regulate cholesterol distribution independent from its pumping function.

**Downregulation of Na/K-ATPase α1 Subunit in Mice Leads to Hepatic Cholesterol Redistribution**

The data from LLC-PK1 cells indicated that the Na/K-ATPase α1 interacts with Cav-1 and plays an important role in regulating intracellular cholesterol distribution. To further test the physiological relevance of the above findings, we determined whether reduction of the α1 expression in α1+/− mice could alter cellular cholesterol distribution. Since liver plays an essential role in the cholesterol metabolism, here we focused on liver tissue samples from α1+/+ and α1+/− mice. As illustrated in FIGURE 10A, Western blot analysis showed a 30% decrease in α1 expression in the α1+/− livers, which was consistent with previous report on other tissues (James et al., 1999). When cholesterol was measured, we observed that reduction of the α1 expression in liver significantly increased the cytosol/membrane ratio of cholesterol (FIGURE 10B), which is consistent with the data obtained in cultured cells (FIGURE 6).
To be sure that reduction in cellular amount of Na/K-ATPase alters interaction among the Na/K-ATPase and its partners in vivo, we measured cellular Src and ERK activity as well as Cav-1 distribution. As shown in FIGURE 11A, we detected a significant increase in cellular Src and ERK1/2 activity in α1+/− liver samples. Moreover, knockdown of the Na/K-ATPase significantly redistributed Cav-1 from fraction 4/5 to high density fractions in liver tissues as detected in cultured cells (FIGURE 11B) (Cai et al., 2008).

Taken together, the above in vitro and in vivo studies indicate that the Na/K-ATPase plays a role in regulation of cellular cholesterol distribution. Knockdown of the Na/K-ATPase could reduce the plasma membrane pool of cholesterol, resulting in a concomitant increase in cholesterol in the “cytosol”.

**Reduction of Cellular Na/K-ATPase Activates SREBP2, Resulting in an Increase in Cellular HMG-CoA Reductase and LDL Receptor in Vivo**

It is known that the ER cholesterol is highly regulated by the plasma membrane cholesterol (Lange et al., 1999). Therefore, it is conceivable that the Na/K-ATPase knockdown-induced reduction in the plasma membrane cholesterol could alter the ER cholesterol and then the activity of SREBP2, a cholesterol-sensitive transcription factor (Horton et al., 1998). These studies were performed on liver samples from both α1+/+ and α1+/− mice since our preliminary study failed to detect SREBP2 using the commercially available antibodies in LLC-PK1 cells. As depicted in FIGURE 12A, we observed a 40% increase in the amount of active SREBP2 in
liver samples from the \( \alpha_{1}^{-/-} \) mice, supporting the notion that knockdown of the \( \alpha_{1} \) may be sufficient to alter the ER cholesterol sensing process. To seek additional support, we measured the amount of HMG-CoA reductase in the liver samples. As shown in FIGURE 12B, a 100% increase in HMG-CoA reductase was recorded in \( \alpha_{1}^{-/-} \) mouse liver. Increases in the amount of HMG-CoA reductase could be the result of two ER cholesterol-mediated regulations. While a decrease in the ER cholesterol reduces the degradation of HMG-CoA reductase (Goldstein et al., 2006), activation of the SREBP pathway increases the expression of the enzyme. Thus, to determine whether increases in the active form of SREBP2 stimulate the expression of SREBP2-responsible genes, we also measured cellular amount of LDL receptor. Consistently, there was also a 40% increase in cellular LDL receptor in livers from \( \alpha_{1}^{-/-} \) mice (FIGURE 12C).

### TABLE 3. Downregulation of \( \alpha_{1} \) affects liver cholesterol content and plasma lipid profile

<table>
<thead>
<tr>
<th></th>
<th>( \alpha_{1}^{-/+} )</th>
<th>( \alpha_{1}^{-/-} )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>cholesterol/unit of protein (%) ( \text{of WT} )</strong></td>
<td>100±2</td>
<td>115±5*</td>
</tr>
<tr>
<td><strong>plasma cholesterol (mg/dL)</strong></td>
<td>120±11.1</td>
<td>81.7±25.8†</td>
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* \( p<0.05 \) compared to \( \alpha_{1}^{+/+} \).
† \( p<0.01 \) compared to \( \alpha_{1}^{+/+} \).

A Reduction in Cellular Na/K-ATPase Is Sufficient to Change Liver Cholesterol Metabolism and Affect Plasma Lipid Profile

To further explore the significance of Na/K-ATPase in cholesterol metabolism, we tested whether increases in cellular HMG-CoA reductase and LDL receptor affect total cellular cholesterol. As depicted in TABLE 3, we found a 15% increase in total
cellular cholesterol in livers from α1+/− mice. Consistently, when plasma cholesterol was measured, plasma cholesterol content was significantly decreased in the α1+/− mice. Moreover, the liver from the α1+/− mice appeared to be larger (FIGURE 12D). Taken together, these data indicate that reduction of the Na/K-ATPase redistributes hepatic cholesterol, which subsequently alters cholesterol metabolism in vivo.

Part II: Regulation of Na/K-ATPase α1 Membrane Trafficking by Cholesterol

Depletion of Plasma Membrane Cholesterol Results in Downregulation of the Na/K-ATPase α1 in LLC-PK1 Cells

According to the literature, cellular cholesterol level is regulated via a negative-feedback mechanism (Brown and Goldstein, 2009). Low plasma membrane cholesterol level leads to activation of the SREBP pathway that restores cellular cholesterol content. The data from Part I indicate that the Na/K-ATPase α1 plays a role in regulation of cholesterol level in the plasma membrane. Therefore, if α1 is one component of the plasma membrane cholesterol sensing machinery, we could anticipate that the Na/K-ATPase binds cholesterol directly. We further contend that change in cellular cholesterol may affect cellular distribution of Na/K-ATPase. To test our proposition, we did a series of experiments shown below. Firstly, we treated the

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1 My labmate, Dr. Xin Li has contributed equally to this part of the data.
cells with a cholesterol depletion drug, methyl β-cyclodextrin (Mβ-CD). Because of its high affinity for cholesterol, Mβ-CD is able to specifically extract cholesterol from the plasma membrane, which dramatically lowers cell surface cholesterol pool (Kilsdonk et al., 1995; Rodal et al., 1999). Moreover, previous work from our lab demonstrated that treatment of LLC-PK1 cells with 10mM Mβ-CD for 30~60 min at 37°C significantly lowered plasma membrane cholesterol pool (Wang et al., 2004). Therefore, in this study we started with the same condition. After Mβ-CD treatment to deplete cellular cholesterol, we washed the drug off and replenished cellular cholesterol by incubating the cells in serum-free culture medium. Then, at different time points we collected cell lysates and checked for proteins and cholesterol level. As shown in FIGURE 13A, depletion of cellular cholesterol by Mβ-CD resulted in ~40% decrease in α1 level. Interestingly, Mβ-CD treatment led to similar decrease in cellular cholesterol level (FIGURE 13B). 6 hours after cell recovery, both α1 and cholesterol levels remained at similar low levels suggesting it took longer time for the cells to recover. However, 24 hours after cell recovery both α1 and cholesterol levels returned to control level (FIGURE 13A and 13B). It should be noted that alterations in both α1 and cholesterol levels showed similar patterns during cholesterol depletion and repletion, which indicated that cellular α1 level was positively related to cholesterol level. Moreover, the cholesterol effect on α1 expression was not a general effect on all the membrane proteins because expression of another plasma membrane protein, insulin receptor, was unchanged during cholesterol depletion and repletion (FIGURE 13A and 13B). To further confirm the result, we performed α1 immunostaining after
cholesterol depletion by Mβ-CD. Consistent with the Western blot data, we detected lower α1 signals from the plasma membrane in Mβ-CD treated cells. Moreover, we observed many intracellular α1 signals in Mβ-CD treated cells suggesting that cholesterol depletion led to α1 redistribution to intracellular compartments (FIGURE 13C).

The data above indicated that acute cholesterol depletion downregulated plasma membrane α1 level. To test whether chronic cholesterol depletion had the similar effect, we next cultured the cells in either normal culture medium (DMEM plus 10% FBS) as control, serum-free medium (DMEM only) or lipoprotein-free medium (DMEM plus lipoprotein-deficient FBS) for 48 hours. Our preliminary study revealed that while normal culture medium contained high level of lipoproteins, which served as exogenous cholesterol source for the cells, both serum-free and lipoprotein-free media were devoid of lipoproteins (data not shown). As expected, culturing the cells in both cholesterol-depleted media resulted in reduction in cellular cholesterol level (FIGURE 14A). Accordingly, α1 level was decreased in cells cultured in the cholesterol-depleted media (FIGURE 14B). Furthermore, both α1 and cholesterol level showed similar percentage of reduction in lipoprotein-free medium suggesting again that α1 level was positively correlated to cholesterol level.

To further establish that it was the cell surface cholesterol pool that regulated plasma membrane α1, we treated the cells with an intracellular cholesterol trafficking inhibitor, U18666A. The U compound is an amphiphile that disrupts intracellular cholesterol trafficking between internal membranes and cell surface, and causes
accumulation of cholesterol within late endosomes/lysosomes (Lange et al., 1998). Consistent with the literature, treatment of LLC-PK1 cells with U18666A led to the redistribution of free cholesterol from the plasma membrane to intracellular compartments (FIGURE 15A). Accordingly, α1 signals were reduced in the plasma membrane but increased in the intracellular compartments, correlated with the pattern of cholesterol distribution (FIGURE 15A). Moreover, these effects of U18666A on cellular Na/K-ATPase were time and dose-dependent (FIGURE 15B and 15C). Similar to cholesterol depletion, the effect of U compound on α1 expression appeared not to be a general effect of all plasma membrane proteins as insulin receptor content remained undisturbed in U18666A–treated cells (FIGURE 15C). Finally, to verify that U18666A downregulated cell surface α1, we did ³H-ouabain binding assay. The result confirmed that reduction of plasma membrane cholesterol led to downregulation of cell surface α1 (FIGURE 15D). Taken together, the data strongly indicated that α1 protein level was regulated by the plasma membrane cholesterol level.

Depletion of Plasma Membrane Cholesterol Leads to Endocytosis of the Na/K-ATPase α1

The above findings demonstrated that plasma membrane cholesterol depletion downregulated Na/K-ATPase α1. It is of interest to reveal the underlying mechanism. One possibility is that it decreased α1 synthesis. To test this possibility, we extracted

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² This experiment was mainly conducted by my labmate, Dr. Jiang Tian.
total mRNA from control and U18666A-treated cells and conducted a semi-quantitative PCR analysis. As shown in FIGURE 16A, α1 mRNA level showed no difference compared to control, which suggested that plasma membrane cholesterol depletion may not affect α1 de novo synthesis. Another possibility is that it disrupted normal trafficking of the newly synthesized α1 from ER to the plasma membrane. So they got stuck in certain intracellular compartments since we observed more intracellular α1 signals from Mβ-CD and U18666A-treated cells (FIGURE 13C and 15A). To test this possibility, we treated the cells with a protein synthesis inhibitor, cycloheximide, before adding U18666A to the cells. As shown in FIGURE 16B, blockage of protein de novo synthesis by cycloheximide did not prevent U18666A-induced α1 redistribution to the intracellular compartments. It suggested that U18666A induced internalization of the cell surface α1.

As cholesterol was also accumulated within the cells (FIGURE 15A), we next checked whether α1 and cholesterol stuck in the same cellular compartment. Because the protocol for cholesterol staining is different from that for α1 protein staining, in order to look at both cholesterol and α1 in the same view we transfected LLC-PK1 cells with YFP-α1 followed by U18666A treatment and cholesterol staining with filipin. The results clearly demonstrated that most of the internalized α1 colocalized with free cholesterol after U18666A treatment (FIGURE 16C, arrows pointing to the colocalization dots).

According to the literature, U18666A treatment leads to free cholesterol

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3 This experiment was conducted by my labmate, Qiqi Ye.
accumulation in late endosomes/lysosomes (Huynh et al., 2008; Lange et al., 2002). Therefore, colocalization of free cholesterol with α1 in U18666A-treated LLC-PK1 cells suggested that α1 was endocytosed and accumulated within late endosomes/lysosomes together with cholesterol. To test this proposition, we transiently transfected LLC-PK1 cells with RFP tagged Rab7 (RFP-Rab7), a late endosomes/lysosomes marker (Zhang et al., 2009), before we treated the cells with U18666A. As shown in FIGURE 16D, in non-treated control cells α1 mostly resided in the plasma membrane and no colocalization between α1 and Rab7 was detected. However, after U18666A treatment intensive intracellular α1 dots appeared, most of which clearly colocalized with Rab7 signal indicating that α1 accumulated within late endosomes/lysosomes. Taken together, depletion of plasma membrane cholesterol by blocking its intracellular trafficking in LLC-PK1 cells not only induced endocytosis of α1 but also resulted in accumulation of α1 together with cholesterol in the late endosomes/lysosomes.

The Na/K-ATPase α1 Is able to Interact With Cholesterol Directly in Vitro via the N-terminal Cholesterol-binding Motif

So far, we have demonstrated that the Na/K-ATPase α1 and cholesterol reciprocally regulate membrane trafficking of each other. Besides, cholesterol is able to modulate activity of the Na/K-ATPase. Therefore, it is tempting to speculate that the two molecules may interact directly. To test this proposition, we did the following experiments. First, we acquired purified Na/K-ATPase within membrane fragments
from pig kidney outer medullar using a well-established method (Jorgensen, 1974). To make sure the method worked efficiently, we run the purified pig kidney enzyme samples (PKE) on the SDS-PAGE and stained the gel with Coomassie Blue solution. As shown in FIGURE 17A, two bands with the corresponding sizes of \( \alpha_1 \) and \( \beta_1 \) were detected in the gel. Further Western blot analysis confirmed that the upper band represented \( \alpha_1 \) subunit and bottom band \( \beta_1 \) subunit (data not shown). Next, because the purified membrane fragments contained large amount of cholesterol, which may already saturated binding sites on the Na/K-ATPase, we extracted most of the cholesterol content by M\( \beta \)-CD (FIGURE 17B).

To study the binding between the Na/K-ATPase and cholesterol, we used a cholesterol analogue, NBD-cholesterol (FIGURE 17C), for the binding assay. NBD-cholesterol was used for the study of protein-cholesterol interaction before (Petrescu et al., 2001). Unlike cholesterol, NBD-cholesterol is able to emit fluorescence signals that peak at \( \sim 530\text{nm} \) when excited at 473nm. In the aqueous solution, its fluorescence is largely quenched. However, when it binds to a protein and exposed to a hydrophobic environment, the fluorescence signals will increase. Moreover, it has much higher critical micelle concentration (~650nM) than that of the cholesterol (30nM) so that higher concentration of the NBD-cholesterol can be used during the binding assay. As we incubated 200nM PKE with different doses of NBD-cholesterol and measured NBD fluorescence signal, we observed a NBD-cholesterol dose-dependent saturation curve (FIGURE 17D). The Kd value is 100nM~200nM. To further confirmed what we observed represented lipid-protein
interaction instead of lipid-lipid interaction, we measured fluorescence resonance energy transfer (FRET) signals. When NBD-cholesterol binds to a protein, the tryptophan amino acid can be excited at 295nm and emits at ~350nm, which overlaps with the excitation spectral of the NBD-cholesterol (Petrescu et al., 2001). As expected, after incubating PKE with NBD-cholesterol and exciting Trp at 295nm, we detected a NBD-cholesterol dose-dependent saturation curve of the FRET signals at 530nm (FIGURE 17E). To further confirm our results, we incubated 62.5nM NBD-cholesterol with different concentrations of PKE. Consistent with the previous experiment, incubation of NBD-cholesterol with PKE produced PKE dose-dependent saturation curve of both NBD signals (FIGURE 17F) and FRET signals (FIGURE 17G). Taken together, our observations indicated that purified Na/K-ATPase is able to directly interact with NBD-cholesterol \textit{in vitro}.

To further dissect the cholesterol-binding site in the Na/K-ATPase, we searched the literature and discovered that a cholesterol recognition/interaction amino acid sequence and consensus pattern (CRAC) was identified in most of the known cholesterol-binding proteins (Li and Papadopoulos, 1998). Following studies further revealed that CRAC was responsible for cholesterol-binding and mutating the key amino acid Tyr in the middle of CRAC completely abolished cholesterol-binding affinity (Jamin et al., 2005). Via database search, we found that there is one CRAC within NT of the Na/K-ATPase $\alpha_1$ subunit. To test whether NT was responsible for Na/K-ATPase-cholesterol interaction, we added a glutathione-S-transferase (GST) tag to NT peptide and purified GST-NT via GST pull-down assay (FIGURE 18A). Next,
we did NBD-cholesterol binding assay as did in FIGURE 16. As shown in FIGURE 18B and 18C, while GST itself induced no FRET signal, GST-NT induced dose-dependent saturation FRET signal curve suggesting NT contained cholesterol-binding affinity. Furthermore, we mutated the amino acid Tyr53 to Ser53 in the middle of CRAC and performed the same NBD-cholesterol binding assay. Interestingly, the mutant NT-Y53S displayed no cholesterol-binding affinity. This indicated that CRAC in the NT was responsible for cholesterol-binding and Tyr53 was a key amino acid within CRAC.

**The CRAC of Na/K-ATPase α1 Is Essential for cholesterol to regulate α1 Endocytosis**

The data in the previous section demonstrated that the NT of Na/K-ATPase α1 was capable of interacting with cholesterol via CRAC in vitro. Thus, it is of interest to explore whether the CRAC plays a role in plasma membrane cholesterol depletion-induced α1 endocytosis. Firstly, we treated wild-type rat α1 rescued-cells, AAC-19, with U18666A and stained for both α1 and cholesterol. As shown in FIGURE 19A, the wild-type rat α1 in the LLC-PK1 cells behaved like endogenous pig α1 (FIGURE 15A). Depletion of plasma membrane cholesterol led to endocytosis of α1 in a dose-dependent manner. Next, we mutated the essential Tyr55 in the rat α1 CRAC (corresponds to the Tyr53 in pig α1) to Ser55 by a site-directed mutagenesis and generated the stable mutant rat α1-rescued cell line (PY-17-Y55S). Once the cell line was established, we did the same experiment as we did on AAC-19 cells.
Interestingly, contrarily to the wild-type rat α1, the Y55S mutant rat α1 showed no obvious endocytosis in response to U18666A treatment (FIGURE 19B). Thus, it appeared that cholesterol-regulated α1 membrane trafficking was dependent on the cholesterol-α1 interaction.

**Part III NT Expression in Mice Leads to Salt-sensitive Hypertension**

Previous studies from our lab have demonstrated that the Na/K-ATPase is not only an ion transporter but also a receptor and signal transducer. CTS such as ouabain can specifically bind to the Na/K-ATPase and activate protein kinase cascades without changing intracellular Na\(^+\) and K\(^+\) concentrations (Haas et al., 2002; Liu et al., 2000; Wang et al., 2004). It was further shown that the signaling Na/K-ATPase mainly resides in caveolae and the formation of the signaling complex involved interaction between the Na/K-ATPase α1 and Cav-1 as well as Src (Tian et al., 2006; Wang et al., 2004). Like other signal transduction processes, binding of the extracellular CTS signal triggered endocytosis of the receptor complex, which required the presence of Cav-1 (Liu et al., 2005). Interestingly, CTS signal also downregulated NHE3 expression and reduced its content in the apical membrane in LLC-PK1 cells (Oweis et al., 2006). Knockdown of Cav-1 in LLC-PK1 cells attenuated CTS effect on NHE3 expression. Thus, the trafficking of the Na/K-ATPase and NHE3 may be coupled via
CTS signaling in renal proximal tubules, which affects renal sodium reabsorption and handling (Liu and Shapiro, 2007). Because renal sodium handling is important for long-term blood pressure regulation, in this part of the study, we focused on whether and how the signaling Na/K-ATPase affected blood pressure.

Knockout of Cav-1 Leads to Salt-sensitive Hypertension in Mice

Since the presence of Cav-1 is important for receptor function and trafficking of the Na/K-ATPase, knockout of Cav-1 may inhibit the receptor function and impair the endocytosis of the Na/K-ATPase in response to CTS signal, which affect blood pressure in vivo. We tested this hypothesis by giving control or Cav-1 KO mice either normal salt diet (0.5% NaCl) or high salt diet (4% NaCl) and measured systolic blood pressure (SBP) by tail-cuff system at different time points. As shown in FIGURE 20A, when the mice were maintained on normal salt diet, the SBP were similar between control and Cav-1 KO groups (~110mmHg at 0 week time point). However, starting from the first week after high salt diet, the Cav-1 KO mice showed significantly higher SBP than control mice (140mmHg v.s. 120mmHg) and stayed at that level as long as they were fed on the high salt diet. To confirm that the Cav-1 KO mice were salt-sensitive, we fed control or Cav-1 KO mice diet with different amount of NaCl (0.5%, 4.0%, and 8.0% to control and 0.5%, 1.0%, 2.0%, 4.0%, and 8.0% to Cav-1 KO) and measured SBP a week after they were on the specific diet. As shown in FIGURE 20B, the NaCl intake v.s. SBP curve shifted to the right for Cav-1 KO mice indicating that those mice were more sensitive to high salt loading. These results are
consistent with the notion that Cav-1 is important for the receptor function of Na/K-ATPase, which plays a role in blood pressure regulation.

**The Expression of α1 N-terminus Inhibits Ouabain-induced Reduction of Transepithelial Na\(^+\) Transport in the Monolayer of LLC-PK1 Cells**

The previous results from Cav-1 KO mice prompted us to further examine the signaling Na/K-ATPase in blood pressure control mechanism. However, we realized that Cav-1 KO mouse model may not be a good model for this purpose because Cav-1 protein has been involved in various signaling events and knockout of Cav-1 in vivo could generate complicated responses. In review of our previous data, we noticed that the signaling Na/K-ATPase relies on the NT to interact with its signaling partners (e.g. PI3K, IP3 receptor, Cav-1 and cholesterol). Expression of NT in the cells may compete with the endogenous Na/K-ATPase for binding to these signaling proteins and disrupt the function of signaling complex. Consistent with the above notion, NT expression in the LLC-PK1 cells redistributed both Cav-1 and cholesterol as shown in FIGURE 8. Moreover, we found that expression of NT in the LLC-PK1 cells did not alter the pumping function of the Na/K-ATPase (data not shown). Thus, we have found a potential way to specifically disrupt the signaling Na/K-ATPase without affecting the pumping function. To test whether NT expression affected CTS-induced endocytosis of both α1 and NHE3 (Liu and Shapiro, 2007), we treated control LLC-PK1 cells and NT-YFP expressing LLC-PK1 cells (NT-YFP) with ouabain and
checked early endosome α1 and NHE3 amount. As shown in FIGURE 21A, ouabain increased both α1 and NHE3 in the early endosomes in control cells as expected. On the other hand, ouabain-induced endocytosis of α1 and NHE3 was significantly attenuated in NT-YFP cells. This was in agreement with our hypothesis that NT expression interrupts CTS signaling that controls α1 and NHE3 trafficking. Downregulation of α1 at basolateral membranes and NHE3 at apical membranes could lead to reduction of Na⁺ reabsorption. Therefore, we next did 22Na⁺ transcellular transport assay on CTS-treated cells. Consistent with our hypothesis, ouabain reduced 22Na⁺ transport from apical side to basolateral aspect of LLC-PK1 monolayers. In contrast, this ouabain-induced reduction was significantly attenuated in NT-YFP expressing cells (FIGURE 21B).

**Generation of NT-expressing Transgenic Mice**

Our previous *in vitro* experiments in the polarized renal epithelial cells demonstrated that NT expression impaired CTS-induced endocytosis of α1 and NHE3 and reduction of Na⁺ transcellular transport. It indicated that the signaling Na/K-ATPase may play a role in renal sodium handling. To test this proposition *in vivo*, we generated three lines of NT-YFP mice (NT mice). First, we inserted the gene encoding the N-terminus 160 amino acids of rat α1 after a CMV promoter, which drives the expression of its downstream gene constitutively (FIGURE 22A). To facilitate detection of our target gene, we included the gene encoding YFP tag after

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4 Early endosome isolation and Na⁺ transcellular transport assay were conducted by Dr. Jiang Liu
NT gene. Thus, the DNA construct, once incorporated into the mice genome, may produce NT-YFP fusion protein in vivo. Once the DNA construct was generated and confirmed by DNA sequencing, we sent it to the University of Michigan Tansgenic Animal Model Core and the transgenic mice were produced by DNA microinjection method (Brinster et al., 1985). After screening for the NT-YFP positive mice, we have established three lines of NT-YFP mice (104, 119, 129). As shown in FIGURE 22B, PCR analysis indicated the presence of NT gene in the three lines. To make sure the NT-YFP protein was expressed in vivo, we sacrificed the mice and collected different organs such as heart, kidney and liver and did Western blot analysis, which confirmed the expression of NT-YFP in all three organs (shown in the FIGURE 22B bottom panel was the Western result from kidney tissues, the data from other tissues were not displayed). As the 104 line displayed the highest renal NT-YFP level among different NT mouse lines, we used 104 line as NT mice for the following studies unless indicated otherwise.

**Renal NT Expression Leads to Salt-sensitive Hypertension**

When we fed the NT-YFP mice and their litter mates with normal salt diet (0.5% NaCl), the SBP showed no difference (~110 mmHg) between the two groups (FIGURE 23A, 0 week point). As we challenged them with high salt diet (4.0% NaCl), SBP increased in both groups. However, the SBP increase pattern were dramatically different. While the control mice only showed a moderate SBP increase (10 mmHg) in ~2 weeks, the NT mice displayed an acute SBP increase (~50 mmHg) during the same
period (FIGURE 23A). Moreover, as long as the mice were maintained on the high salt diet, the SBP for both groups stayed constant (120 mmHg for control mice and 160 mmHg for NT-YFP mice) up to 16 weeks when they were sacrificed. This suggests that NT-YFP expression in vivo altered long-term blood pressure regulation in response to high salt loading. Moreover, this was in agreement with the data from Cav-1 KO mice (FIGURE 20) and supported the hypothesis that the signaling Na/K-ATPase plays a role in long-term blood pressure regulation.

To establish the correlation between renal NT-YFP expression and hypertension in response to high salt loading, we next fed control and different lines of NT mice with high salt diet and measured SBP. As shown in FIGURE 23B, the three lines of NT-YFP mice increased their SBP to different levels in response to high salt loading. The 104 line showed the highest increase of SBP while the 119 line behaved like control mice. Furthermore, the 129 line displayed SBP (~140 mmHg) between 104 and 119 lines. As we performed statistical correlation test on SBP after one week of high salt loading and relative renal NT-YFP expression level, the result demonstrated significant correlation between the two parameters (FIGURE 23C). Thus, our findings indicated that renal NT-YFP expression resulted in salt-sensitive hypertension in mice.

**NT-YFP Expression Does Not Alter Ions Balance during High Salt Loading but Induces Higher Level of MBG Excretion**

To further explore the mechanism underlying the NT-YFP-induced
salt-sensitive hypertension, we analyzed various metabolic parameters. These analyses were performed in mice that were housed individually in metabolic cages. As shown in TABLE 4, the body weight and food consumption in the four groups showed no obvious difference. Moreover, water consumption during high salt loading showed no difference among control and NT-YFP groups. These data suggest that NT expression did not change the eating and drinking habit of mice. As we collected mice urine, high salt loading dramatically increased the urine volume as expected, but no difference was detected between control and NT-YFP groups. When we measured sodium and potassium level, high salt loading significantly increased sodium excretion while only moderate increase of potassium excretion was observed (FIGURE 24A). However, comparing the data from control and NT-YFP groups, we could not find obvious difference. Furthermore, plasma sodium and potassium ion levels were similar between control and NT-YFP groups (FIGURE 24B). Therefore, it is unlikely that NT-YFP expression altered sodium or potassium balance in response to high salt loading.

The endogenous CTS such as marinobufagenin (MBG) was previously discovered to be involved in salt-induced high blood pressure (Fedorova et al., 2002). To explore whether MBG was related to NT-YFP-induced salt-sensitive hypertension, we measured urine MBG excretion level. As shown in FIGURE 24C, high salt loading led to increased urine MBG excretion in control mice, which is consistent with the previous report that urine MBG excretion increased in response to high salt loading (Fedorova et al., 2002). Interestingly, while MBG excretion levels were
similar between control and NT-YFP groups either on normal salt diet or one day after high salt loading, two days after, MBG excretion in NT-YFP group almost doubled that in control group. It was also correlated to the further increase in SBP two days after high salt loading (FIGURE 23B). It further suggested that an endogenous Na/K-ATPase ligand may serve as a natriuretic hormone that is excreted in response to high salt loading. Once it binds to the renal Na/K-ATPase \( \alpha_1 \), the signaling \( \alpha_1 \) is activated and initiated the endocytosis of both \( \alpha_1 \) and NHE3, which subsequently reduces \( \text{Na}^+ \) transcellular transport. By this way, sodium excretion is increased to balance the high salt intake. However, NT-YFP expression inhibits the activation of signaling Na/K-ATPase. So the body has to excrete more endogenous ligands to maintain high salt excretion.
FIGURE 4. Knockdown of the Na/K-ATPase α1 reduces both Cav-1 and cholesterol in caveolar fractions. (A). Cell lysates from P-11 and PY-17 cells were subjected to sucrose gradient fractionation. Caveolin-enriched fractions (4/5) together with fractions 6–11 were taken for Western blot analysis of Na/K-ATPase α1 and caveolin-1. A representative Western blot of five independent experiments is shown. (B). Cell lysates from P-11 cells were fractionated as in panel A and all 12 fractions were assayed for cholesterol. The percentage of the cholesterol amount in each fraction was calculated.
FIGURE 4. Knockdown of the Na/K-ATPase α1 reduces both Cave-1 and cholesterol in caveolar fractions. (C). A representative Western blot of three experiments showing the amount of Na/K-ATPase α1 and caveolin-1 in caveolin-enriched fractions (4/5). Cholesterol content was measured from caveolin-enriched fractions. **p<0.01 compared to P-11 control cells, n=5. (D). Cholesterol content was measured from total cell lysates, no difference was detected, n=3.
FIGURE 5. Effects of alterations in Na/K-ATPase or Cav-1 on cellular cholesterol distribution. (A), (B), (C), (D) and (E) shows filipin staining of free cholesterol from P-11, PY-17, C2-9, AAC-19 and mCBM cells, respectively. Arrows are pointing to the intracellular vesicular filipin signals. Scale bar: 20µm.
FIGURE 6. Effects of changes in Na/K-ATPase amount on cytosol and membrane cholesterol content. Cytosol and membrane fractions were prepared from cell lysates as described. Cholesterol was measured from membrane fractions (A) and the cytosol fractions (B). The ratio between cytosol and membrane cholesterol is also shown (C). *p<0.05 or **p<0.01 compared to P-11 control cells, n=5.
FIGURE 7. The interaction between the Na/K-ATPase α1 and Cav-1 is important for proper cholesterol distribution. (A). P-11 and PY-17 cells were treated with 1μM PP2, a src inhibitor for 2 hours. Afterwards, cell lysates were fractionated into cytosol and membrane fractions and then subjected to cholesterol measurement. Data are mean ± SE, n=4. (B). Cav-1 protein level was assayed in P-11 and C2-9 cells by Western blot. (C). Cell lysates were processed as in Panel A and measured for cholesterol. The cholesterol ratio was calculated. *p<0.05 or **p<0.01 compared to P-11 control cells, n=3.
FIGURE 8. Overexpression of NT of Na/K-ATPase α1 redistributes cellular cholesterol. (A). A typical confocal image showing the cellular distribution of NT-YFP (bottom left) and the endogenous Cav-1 protein in the LLC-PK1 cells (top right) or in the NT-YFP cells (bottom right). (B) and (C). Filipin staining of cellular cholesterol from control P-11 and NT-YFP-expressing LLC-PK1 cells. Scale bar: 20µm. (D). Cell lysates were processed as described in FIGURE 5, the cholesterol ratio was calculated. **p<0.01 compared to P-11 control cells, n=3.
A Vector-transfected PY-17
YFP-α1D371N-transfected PY-17

Relative cholesterol content
% of control
cytosol membrane cytosol/membrane

B
Vector-transfected PY-17
YFP-α1D371N-transfected PY-17

Relative cholesterol content
% of control
cytosol membrane cytosol/membrane

**
*
**
**
FIGURE 9. Effects of the expression of α1D371N mutant on cellular cholesterol distribution. Relative cholesterol content was assayed and calculated in the cytosol and membrane fractions from (A). PY-17 cells and AAC-19 cells, which is wild-type α1-transfected PY-17 cells and (B). the vector-transfected and the pump-null D371N transfected PY-17 cells. *p<0.05 or **p<0.01 compared to control cells, n=6. (C). The representative confocal images of 3 separate experiments showing (left panel) YFP-D371N mutant signal, (middle panel) filipin signal and (right panel) merged image from the pump-null D371N transfected TCN cells. Arrows indicate the plasma membrane signals. Scale bar: 20µm.
FIGURE 10. Downregulation of Na/K-ATPase α1 in mice liver leads to cholesterol redistribution. (A). Western blot analysis showed the downregulation of α1 level in the α1^{+/-} mice liver. n=6. (B). Liver samples from both α1^{+/+} and α1^{+/−} mice were processed as in FIGURE 5. Cholesterol from cytosol and membrane fractions was measured and ratio was calculated. **p<0.01 compared to α1^{+/+}, n=6.
**A**

- **Src-pY418**
  - α1⁺/+ vs. α1⁻/-

- **Src**

- **Src-pY418**
  - Relative level (% of α1⁺⁺)

- **pERK1/2**
  - α1⁺/+ vs. α1⁻/-

- **ERK1/2**

- **pERK1/2**
  - Relative level (% of α1⁺⁺)
FIGURE 11. Downregulation of Na/K-ATPase α1 in mice liver leads to the activation of src and ERK1/2 and redistribution of Cav-1. (A). Western blot analysis showed the increase in active src (src-pY418) and pERK1/2 in α1 +/- mice liver samples. n=6. (B). Liver samples from both α1 +/- and α1 +/− mice were processed as in FIGURE 3. Caveolin-enriched fractions (4/5) together with fractions 6~11 were taken for Western blot analysis of Cav-1. A representative Western blot from three independent experiments is shown. The percentage of signals from caveolin-enriched fractions against total signals is shown below. *p<0.01 compared to α1 +/-.
FIGURE 12. SREBP2 pathway is activated in α1+/− mice liver. (A), (B), and (C). Samples from both α1+/+ and α1+/− mice livers were analyzed by Western blot analysis for the active form of SREBP2, HMG-CoA reductase and LDL receptor, respectively. A representative Western blot is shown and quantitative data are collected from 5 α1+/+ and 5 α1+/− mice livers. D. The liver weight/body weight ratio was calculated from 4 α1+/+ and 7 α1+/− mice livers. *p<0.05, **p<0.01 compared to α1+/+. 
A

**Na/K-ATPase α1**

**Insulin-receptor**

**α-tubulin**
FIGURE 13. Acute cellular cholesterol depletion downregulates the Na/K-ATPase. Cellular cholesterol was depleted by treating LLC-PK1 cells with 10mM Mβ-CD for 1 hour. Then cells were allowed to recover in DMEM with 10% fetal bovine serum for 0 hour, 6 hours and 24 hours. (A). Top panel, Western blot analyses showed protein levels of α1, insulin receptor and α-tubulin at different time points. Bottom panel, quantitative results of the Western blot data. (B). Total cellular cholesterol contents were measured in different time points. (C). Typical confocal images were taken on control and MβCD treated cells immunostained with α1. Scale bar: 20µm. *p<0.05, **p<0.01 compared to control, n=3.
FIGURE 14. Chronic cellular cholesterol depletion downregulates the Na/K-ATPase. LLC-PK1 cells were cultured in DMEM with 10% fetal bovine serum as control medium. Chronic cellular cholesterol depletion was achieved by culturing the cells in either serum-free DMEM or lipoprotein-free DMEM for 48 hours. (A). Total cellular cholesterol contents were measured in the cells treated with different medium. (B). Left panel. Western blot analyses showed protein levels of α1 and α-tubulin. Right panel, quantitative results of the Western blot data. *p<0.05 compared to control, n=3.
FIGURE 15. Disruption of intracellular cholesterol trafficking by U18666A downregulates the Na/K-ATPase. (A). LLC-PK1 cells were treated with U18666A for 48 hours and stained for either cholesterol or α1. Scale bar: 20µm. (B). Western blot analyses showed α1 level was downregulated by U18666A in a dose-dependent manner while insulin receptor was unchanged. α-tubulin served as loading control. (C). LLC-PK1 cells were treated with U18666A (10µg/ml) for different time. Western blot analyses showed α1 level was downregulated by U18666A in a time-dependent manner while insulin receptor and α-tubulin showed no difference. (D). 3H-ouabain binding demonstrated that cell surface α1 is decreased by U18666A treatment in a time-dependent manner. *p<0.05 compared to control, n=3.
A

Control   U18666A

α1

GAPDH

α1/GAPDH (% of control)

control   U18666A

B

CHX

U18666A

+ 0µg/ml  + 5µg/ml  + 10µg/ml

α1

C

YFP-α1  cholesterol  merge

Control

U18666A
FIGURE 16. Disruption of intracellular cholesterol trafficking by U18666A leads to endocytosis of the Na/K-ATPase. (A). Left panel, LLC-PK1 cells were treated with U18666A (10µg/ml) for 48 hours and total mRNA were extracted from non-treated control cells and U compound-treated cells. Then semi-quantitative PCR for α1 and GAPDH (loading control) was performed. A typical DNA gel result of PCR products of α1 and GAPDH is shown. Right panel, quantitative results of the DNA gel. (B). LLC-PK1 cells were first treated with the protein synthesis inhibitor, cycloheximide (50µg/ml) for 1 hour before adding different doses of U18666A. Then the cells were incubated with both drugs for one more hour before fixation and immunostaining for α1. Typical confocal images from 3 separate experiments were shown. (C). LLC-PK1 cells were transiently transfected with YFP-α1 for 24 hours. After that, the cells were treated with U18666A (5µg/ml) for additional 24 hours before fixation. Cholesterol staining with filipin and immunostaining of α1 were performed. Typical fluorescence images of TFP-α1 (left), cholesterol (middle) together with merged images (right) were shown. (D). LLC-PK1 cells were transiently transfected with RFP-Rab7, a late endosome marker for 24 hours. Then the cells were treated with U18666A (5µg/ml) for additional 24 hours before fixation. Immunostaining of α1 was performed. Typical confocal images of α1 (left), RFP-Rab7 (middle) together with merged figures were shown. Scale bar: 20µm.
Na/K ATPase \( \alpha_1 \)  
Na/K ATPase \( \beta_1 \)  
1: protein marker  
2 & 3: purified PKE

A

B

cholesterol content of PKE

control  |  MJβ-CD

μg cholesterol/mg protein

C

D

NBD-cholesterol

E

Fluorescence intensity at 520nm with excitation at 425nm

Fluorescence intensity at 520nm with excitation at 425nm

NBD-CH (nM)
FIGURE 17. Purified pig kidney Na/K-ATPase (PKE) is capable of interacting with cholesterol in vitro. (A). Pig kidney Na/K-ATPase was purified as described in “MATERIALS AND METHODS”. The purified samples were run on the SDS-PAGE gel and only α1 and β1 bands were detected. (B). The purified PKE was incubated with 10 mM Mβ-CD at 37°C for 1 hour. Cholesterol content within the non-treated control and Mβ-CD-treated samples were measured. (C). Schematic drawing of the structure of cholesterol and NBD-cholesterol. The red circles denote the only difference between the two molecules at position C25. (D). 200 nM PKE was mixed with different concentrations of NBD-cholesterol in 96-well plates. NBD signals were detected by excitation at 473 nm and emission at 530 nm. A typical dose-dependent saturation curve of NBD signals is shown. (E). Experiment conditions were the same as in C. The FRET signals between the amino acid tryptophan and NBD-cholesterol were collected by excitation at 295 nm and emission at 530 nm. A typical dose-dependent saturation curve of FRET signals is shown. (F). 62.5 nM NBD-cholesterol was mixed with different concentrations of PKE in 96-well plates. NBD signals were collected as in C. A typical PKE dose-dependent saturation curve of NBD signals is shown. (G). Experiment conditions were the same as in E. The FRET signals were detected as in D. A typical dose-dependent saturation curve of FRET signals is shown.
FIGURE 18. Purified GST tagged NT of the pig Na/K-ATPase α1 is capable of interacting with cholesterol via its cholesterol-interaction motif in vitro. (A). SDS-PAGE gel showed purified protein samples after GST pull-down assay. (B). 100nM of the purified peptide samples were mixed with different concentrations of NBD-cholesterol in 96-well plates. FRET signals between tryptophan and NBD-cholesterol were detected by excitation at 295nm and emission at 530nm. GST tagged NT peptide displayed significant FRET signals while the GST itself as negative control and GST tagged Y53S mutant NT peptide showed no FRET signal. n=3. (C). 125nM of NBD-cholesterol were mixed with different concentration of GST or GST tagged peptide. FRET signals were measured as in B. Only GST-NT displayed significant FRET signals. n=3.
FIGURE 19. U18666A induces endocytosis of wild-type rat α1 but not Y55S mutant rat α1 in LLC-PK1 cells. (A). Wild-type rat α1 rescued-PY17 cells were treated with different concentrations of U18666A. α1 immunostaining and cholesterol staining by filipin were performed as in FIGURE 14. Typical confocal images of α1 immunostaining and fluorescence images of cholesterol staining were shown. (B). Y55S mutant rat α1 rescued-PY17 cells were treated with different concentrations of U18666A. α1 immunostaining and cholesterol staining by filipin were performed as in FIGURE 14. Typical confocal images of α1 immunostaining and fluorescence images of cholesterol staining were shown.
FIGURE 20. Cav-1 knockout leads to salt-sensitive hypertension in mice. (A). Control and Cav-1 KO mice were maintained on normal salt diet (0.5% NaCl) before switching to high salt diet (4.0% NaCl) at 0 week time point. Systolic blood pressure was measured at week 0, 1, 2, 4, 8 and 16. n=8. (B). Control and Cav-1 KO mice were fed with diet with different concentration of NaCl for one week before measuring the systolic blood pressure. The NaCl intake (Y axis) v.s. S B P ( X a x i s ) c u r v e s w e r e d r a w n. n = 8.
FIGURE 21. NT expression in LLC-PK1 cells inhibits ouabain-induced reduction of Na⁺ transcellular transport. (A). LLC-PK1 cells or NT overexpressing LLC-PK1 cells (NT-YFP) were treated with or without 100nM ouabain for 1 hour. Cell lysates were collected and early endosomes were isolated. Western blot analyses for α1 and NHE3 level were performed. Typical Western results from 4 separate experiments were shown. (B). LLC-PK1(control) or NT-YFP (NT) cells were grown to confluence on the 12-mm polycarbonate transwell culture filter inserts. Then the cells were treated with or without 100nM ouabain for indicated time. ²²Na⁺ transcellular transport assay was performed as described in “MATERIALS AND METHODS”. n=4. *p<0.05, **p<0.01.
FIGURE 22. Generation of NT-expressing transgenic mice. (A). Schematic drawing of the DNA construct for NT transgenic mice. The gene encoding for the first 160 amino acids of rat Na/K-ATPase α1 was placed after CMV promoter and followed by enhanced YFP gene and SV40 poly A tail. (B). Three different transgenic lines of NT mice (104, 119, 129) were generated. Upper panel, mice genomes were isolated from control and NT mice tails and subjected to PCR analysis for the presence of NT construct. PCR products were run on 1% agarose gel and the arrow denotes the NT fragment amplified by PCR. Lower panel, kidneys from control and NT mice were lysed and homogenized. Western blot analyses showed different renal NT-YFP expression levels in different transgenic lines.
FIGURE 23. Renal NT expression in mice led to salt-sensitive hypertension. (A). Control and 104 line NT mice were maintained on normal salt diet (0.5% NaCl) before switching to high salt diet (4.0% NaCl). Systolic blood pressure was measured at week 0, 1, 2, 4, 8 and 16. n=8. (B). Control and three different lines of NT mice were given high salt diet and systolic blood pressure was measured at day 0, 2 and 7. n=6–7. (C). Relative renal NT expression level is highly correlated with systolic blood pressure after one week high salt diet in control and NT mice.
TABLE 4. Metabolic parameters of mice high salt loading study

<table>
<thead>
<tr>
<th>metabolic cage study</th>
<th>normal salt diet (0.5% NaCl)</th>
<th>high salt diet (4% NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control (n=8)</td>
<td>NT (n=8)</td>
</tr>
<tr>
<td>body weight (g)</td>
<td>27.17±1.53</td>
<td>28.01±3.13</td>
</tr>
<tr>
<td>food consumption (g)</td>
<td>11.42±0.70</td>
<td>12.86±1.23</td>
</tr>
<tr>
<td>water consumption (ml/day)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>urine volume (ml/day)</td>
<td>3.2±0.4</td>
<td>3.6±0.6</td>
</tr>
</tbody>
</table>

Data are mean ± SE.
*p<0.01 between normal salt group and high salt group
NA: not available
FIGURE 24. NT expression in mice does not change ion excretion in response to high salt loading but leads to higher MBG excretion level. (A). Control or NT mice on normal salt or high salt diet for one week and 24-hour urine samples were collected on day 2. Na⁺ or K⁺ level in the urine was measured and calculated. (B). 4 months after high salt diet, mice were sacrificed and plasma samples were collected. Plasma Na⁺, K⁺ levels were measured. (C). Urine MBG excretion was measured and calculated. n=8. *p<0.05, **p<0.01.
FIGURE 25. A simple flow chart showing the relationship among the Na/K-ATPase, cholesterol and caveolin-1 based on the data from previous studies and current project.
DISCUSSION

Recent studies have produced accumulating evidence that the Na/K-ATPase is more than an ion transporter. It can behave as a signal transducer in response to its specific CTS ligand like ouabain (Xie and Askari, 2002) and regulate localization and functions of other proteins like Src (Tian et al., 2006; Wang et al., 2004), IP3 receptor (Chen et al., 2008), and Cav-1 (Cai et al., 2008). In LLC-PK1 cells, a non-pumping pool of the Na/K-ATPase has been demonstrated, which mainly resides in the special lipid-enriched membrane domain called caveolae and directly interacts with proteins like Src and Cav-1 (Liang et al., 2007; Tian et al., 2006; Wang et al., 2004). Interestingly, Cav-1 protein, which interacts and is regulated by the Na/K-ATPase, is known to be involved in cellular cholesterol homeostasis, which suggests that the Na/K-ATPase may also play a role in regulation of cholesterol. Therefore, this study has explored the involvement of the Na/K-ATPase in cellular cholesterol regulation and revealed two important findings. First, via regulation of Cav-1 membrane trafficking, the Na/K-ATPase also controls intracellular cholesterol transport between inner membranes and plasma membrane and influences cholesterol metabolism in vivo. Second, plasma membrane cholesterol also regulates cell surface Na/K-ATPase amount, which is dependent on interaction between the CRAC domain of Na/K-ATPase α1 subunit and cholesterol. The two findings together have suggested that the Na/K-ATPase α1 may serve as a plasma membrane cholesterol sensor and be involved in sensing and regulation of the cellular cholesterol level. Moreover, the regulation of cell surface Na/K-ATPase by cholesterol implies that cholesterol may
modulate kinase activities and signaling events through the signaling Na/K-ATPase. This work is significant because it is the first time to demonstrate a link between the Na/K-ATPase and cholesterol network, which may provide new clues on membrane cholesterol trafficking and metabolism. Also, it warrants further investigation of the function of the Na/K-ATPase in cholesterol trafficking-related disorders like atherosclerosis and NPC disease.

In addition to the study on cholesterol and the Na/K-ATPase, this research project also explored the receptor function of the Na/K-ATPase in long-term blood pressure regulation in two mouse models. Our results indicates that endogenous CTS signaling may couple trafficking of both Na/K-ATPase and NHE3 in the renal proximal tubule cells, which modulates renal sodium reabsorption and contributes to the regulation of blood pressure. It paves the way for the further study on the underlying molecular mechanism to advance our knowledge on renal sodium handling and long-term blood pressure regulation.

Regulation of Intracellular Cholesterol Distribution by the Na/K-ATPase

We have recently suggested that there is a large pool of non-pumping Na/K-ATPase in LLC-PK1 cells (Liang et al., 2007). It appears that a large portion of the non-pumping Na/K-ATPase resides in caveolae and dynamically interacts with various proteins, including Cav-1 and Src (Wang et al., 2004). Moreover, we found that reduction of this pool of Na/K-ATPase mobilized Cav-1, resulting in a decrease in plasma membrane Cav-1 and a concomitant accumulation of Cav-1 in intracellular
vesicles (Cai et al., 2008). Here we have further established that knockdown of cellular
Na/K-ATPase also reduced the amount of cholesterol in the plasma membrane. Filipin
labeling indicated that the plasma membrane cholesterol was redistributed to
intracellular compartments in the knockdown cells (FIGURE 5B). When total cell
lysates were separated into membrane and cytosol pools, we found that the membrane
cholesterol was redistributed to the cytosol fractions in the α1 knockdown cells.
Moreover, this redistribution effect was correlated to the amount of cellular
Na/K-ATPase (FIGURE 6). This increase was reversible. Expression of an exogenous
rat α1 restored not only cellular Na/K-ATPase, but also cholesterol distribution in
PY-17 cells (FIGURE 6). Furthermore, it appears that the effect of Na/K-ATPase on
cellular cholesterol distribution is independent of its pumping activity because
expression of a pump-null mutant α1 (D371N) was capable of restoring membrane
distribution of cholesterol in the knockdown PY-17 cells (FIGURE 9). Consistently,
we found that A4-11 cells exhibit similar pumping activity as that of PY-17 cells, but
much less change in cholesterol distribution (FIGURE 6) (Liang et al., 2007). Finally,
similar changes were detected in livers from α1+/− mice (FIGURE 10 and 11). Knockdown of the Na/K-ATPase α1 not only increased basal Src and ERK1/2 activity,
but also altered Cav-1 and cholesterol distribution.

The Interaction between Na/K-ATPase and Cav-1 Is Important for Maintaining
the Plasma Membrane Pool of Cholesterol

Na/K-ATPase is known to interact with Cav-1 and Src directly (Wang et al.,
2004) and these interactions play a pivotal role in regulation of Cav-1 trafficking (Cai et al., 2008). Moreover, it is known that Cav-1 directly binds cholesterol in a 1:1 ratio (Murata et al., 1995) and that Cav-1 regulates cholesterol trafficking from and to the plasma membrane (Uittenbogaard et al., 2002; Uittenbogaard et al., 1998). Finally, it was reported that expression of a caveolin dominant-negative mutant led to accumulation of cholesterol intracellularly (Pol et al., 2001). Thus, the Na/K-ATPase knockdown could reduce Cav-1 and consequently cholesterol in the plasma membrane and redistribute both to the intracellular compartment. The notion appears to be supported by our new findings presented in FIGURE 5-11. First, knockdown of Cav-1 was sufficient to alter cholesterol distribution as did the Na/K-ATPase knockdown. Second, expression of a wild-type rat α1, but not a CBM mutant rat α1, could restore cholesterol distribution in PY-17 cells. Finally, overexpression of the N-terminus of α1 subunit that contains the caveolin-binding motif was equally effective in redistributing cholesterol from the plasma membrane to intracellular vesicles. Considering a previous report showing that caveolin protein transported cholesterol directly from ER to caveolae (Smart et al., 1996), a plausible senario is that the caveolar Na/K-ATPase serves as a docking site for caveolin membrane trafficking and plays a role in maintaining caveolin membrane localization. Knockdown of the Na/K-ATPase reduces trafficking of Cav-1 to caveolae and redistributes these proteins to intracellular compartments. Accordingly, redistribution of Cav-1 affects trafficking of ER cholesterol to the plasma membrane and they are also stuck in the little cytosolic structures such as lipid bodies.
However, an alternative should be considered. As we demonstrated in FIGURE 17 and 18, the Na/K-ATPase α1 contains a CRAC motif within NT, which is responsible for α1-cholesterol interaction, it is plausible that the interaction between the two also plays a role in regulation of cholesterol distribution. Nevertheless, it should be pointed out that plasma membrane cholesterol amount far more exceeds the amount of any protein, it is quite unlikely that one to one interaction between the Na/K-ATPase α1 and cholesterol can significantly alter the cellular cholesterol distribution. On the other hand, the CRAC motif of α1 may contribute to the cholesterol trafficking from the cholesterol carrier protein such as Cav-1 to the plasma membrane. That both CRAC motif and the caveolin-binding motif reside within NT of α1 suggests cholesterol could be passed from Cav-1 to α1 when Cav-1 attaches to α1 and then α1 transports the cholesterol molecule from NT to the plasma membrane. If this is so, the α1-Cav-1 cholesterol transport model will be similar to the recently demonstrated NPC1-NPC2 cholesterol transport model in the late endosome membrane (Kwon et al., 2009).

Combined with our previous findings that the Na/K-ATPase α1 regulates membrane trafficking of Cav-1 (Cai et al., 2008), our new discoveries in this study have revealed that membrane localization of the caveolae structure components, Cav-1 and cholesterol, are regulated by the Na/K-ATPase α1. This raises the possibility that the Na/K-ATPase α1 is not only a caveolae resident but also indispensable for the formation of caveolae. Binding of the Na/K-ATPase α1 to Cav-1 may be the key events during generation of caveolae. As ouabain has been shown to
regulate the interaction between Na/K-ATPase α1 and Cav-1 (Wang et al., 2004), if the above notion can be confirmed, that means ouabain not only triggers the Na/K-ATPase-mediated signal transduction but also affects other caveolae-based signal transductions by modulating the caveolae structure. This issue will be further explored in the future study.

**Regulation of Cholesterol Sensing by the Na/K-ATPase in Vivo**

Liver plays an essential role in cholesterol metabolism. It is one of the major organs for *de novo* cholesterol biosynthesis and is the predominant site for LDL uptake via LDL receptor-mediated endocytosis, which is a major factor for lowering the plasma LDL level (Brown and Goldstein, 1986). Generally, hepatic cholesterol level is tightly regulated by the cellular cholesterol sensing mechanism. Lowering the ER cholesterol will reduce the binding of cholesterol to SCAP, which will activate the transcription factor SREBP2 and prolong the half life of HMG-CoA reductase (Brown and Goldstein, 1999). Activation of SREBP2 can also increase the transcription of HMG-CoA reductase and LDL receptor. It is known that most of cellular cholesterol resides in the plasma membrane and that the ER cholesterol is highly sensitive to the plasma membrane cholesterol (Lange et al., 1999). Thus, it is conceivable that lowering the plasma membrane cholesterol by knockdown of the Na/K-ATPase may abolish the cholesterol transport from the plasma membrane to the ER, resulting in a reduction in the ER cholesterol and subsequent activation of SREBP2. Consistently, our results showed an activation of the SREBP2 pathway in α1+/− mouse livers. This
notion is further supported by increases in both HMG-CoA reductase and LDL receptor (FIGURE 12). Moreover, the decrease in the plasma cholesterol level along with the increase of the hepatic cholesterol content in $\alpha_1^{+/-}$ mice corroborate the notion that hepatic LDL receptor activity is increased (TABLE 3). Taken together, these results indicate that the Na/K-ATPase is involved in cellular cholesterol sensing and regulation. Downregulation of the Na/K-ATPase affects the cellular cholesterol balance and alters cholesterol metabolism.

**Regulation of Cell Surface Na/K-ATPase by the Plasma Membrane Cholesterol**

Cellular cholesterol is tightly regulated via a negative-feedback mechanism (Brown and Goldstein, 2009). That means that the cholesterol regulators should be able to respond to the alteration of the cellular cholesterol level. Consistent with the notion that the Na/K-ATPase is a component of the plasma membrane cholesterol sensing machinery, reduction of the plasma membrane cholesterol content decreased the cell surface Na/K-ATPase. For instance, either acute or chronic depletion of cellular cholesterol led to downregulation of the Na/K-ATPase (FIGURE 13-15). Moreover, an intracellular cholesterol inhibitor, U18666A, which redistributed cholesterol from the plasma membrane to late endosomes/lysosomes (Lange et al., 2000) also downregulated cell surface Na/K-ATPase (FIGURE 15). As total cellular cholesterol was actually increased in the U18666A-treated cells (data not shown), these results clearly indicate that it is the plasma membrane cholesterol content that affects the cell surface Na/K-ATPase. This cholesterol depletion effect appeared to be
specific to the Na/K-ATPase because the expression level of insulin receptor, another plasma membrane protein, did not change (FIGURE 13 and 15).

At that point, it was of interest to examine how cholesterol depletion downregulates cell surface α1. Immuno-staining revealed the redistribution of α1 from cell surface to intracellular compartments (FIGURE 13 and 15). This indicated two possible scenarios: either newly synthesized α1 failed to traffic to the cell surface or the surface α1 got endocytosed after the surface cholesterol depletion. Subsequent studies pointed to the second scenario. First, α1 transcription showed no difference between the control and U18666A–treated cells. Second, blockade of protein translation did not prevent redistribution of α1. Finally, α1 colocalized with both cholesterol and late endosome markers, Rab7, after U18666A-treatment (FIGURE 16). Taken together, these data strongly indicate that cellular cholesterol regulates cell surface α1 content. Reduction of plasma membrane cholesterol leads to endocytosis of α1.

The Na/K-ATPase α1 Directly Interacts with Cholesterol and the Cholesterol-regulated α1 Endocytosis Is Dependent on α1-cholesterol Interaction

In the literature, it is well-known that cholesterol content affects activity of the Na/K-ATPase (Claret et al., 1978; Cornelius, 1995; 2001) and even direct interaction between the Na/K-ATPase and cholesterol was suggested (Giraud et al., 1976). However, there is lack of the direct evidence of the two interacting with each other. Therefore, in this project we used a previously established FRET analysis (Petrescu et
al., 2001) to study $\alpha_1$-cholesterol interaction and demonstrated that the Na/K-ATPase $\alpha_1$ directly interacts with NBD-cholesterol, a cholesterol analog (FIGURE 17). Moreover, we discovered a cholesterol-binding motif (CRAC) within NT of $\alpha_1$, which shares the consensus amino acid pattern with many known cholesterol-binding proteins (Li and Papadopoulos, 1998) (TABLE 1. and FIGURE 18).

Interestingly, it appeared that the CRAC was important for cholesterol-regulated $\alpha_1$ endocytosis as disruption of the cholesterol-binding motif by site-directed mutagenesis rendered $\alpha_1$ insensitive to plasma membrane cholesterol depletion (FIGURE 19). Since the CRAC mutant $\alpha_1$ localized within the plasma membrane normally (FIGURE 19B), it is unlikely that $\alpha_1$-cholesterol interaction stabilizes cell surface Na/K-ATPase. Instead, cholesterol may facilitate sorting of the Na/K-ATPase into the lipid raft, which mediates endocytosis of the Na/K-ATPase. It was proposed that lipid rafts, the assemblies of sphingolipids and cholesterol, were important for protein sorting during protein trafficking (Umebayashi, 2003). Moreover, one type of lipid raft, caveolae was shown to concentrate the Na/K-ATPase (Liu et al., 2003) and mediated its endocytosis (Liu et al., 2005). Considering the results from this study, it is conceivable that $\alpha_1$-cholesterol interaction may be the key for proper sorting of the Na/K-ATPase into the lipid rafts like caveolae and subsequent endocytosis in response to various signals. Undoubtedly, this concept should be explored in the future studies.

Functionally, the regulation of cell surface Na/K-ATPase $\alpha_1$ content by cholesterol may have an impact on the downstream kinases activity. As reported
before, the Na/K-ATPase α1 interacted with Src kinase forming a signaling complex (Tian et al., 2006). Knockdown of the Na/K-ATPase α1 by siRNA technique led to an increase in the Src kinase activity as well as its downstream ERK1/2 activity in LLC-PK1 cells (Liang et al., 2006). Consistently, downregulation of the Na/K-ATPase α1 in vivo also elevated kinase activities of Src and ERK1/2 (FIGURE 11A). Therefore, the Na/K-ATPase could link cellular cholesterol level to the kinase activities (FIGURE 25). As certain symptoms like atherosclerosis involves abnormality of both cholesterol metabolism and kinase activity regulation (Razani et al., 2008), this issue deserves further study.

**The Na/K-ATPase α1 As a Potential Plasma Membrane Cholesterol Sensor**

As an essential molecule to all mammalian cells, cholesterol level is tightly regulated by the cells. The most studied cellular cholesterol regulation machinery including HMG-CoA reductase and SCAP-SREBP2 complex reside in the ER membrane and their protein abundance and activity rely on the ER cholesterol pool (Goldstein et al., 2006). However, as most of the cellular cholesterol localizes and functions in the plasma membrane and ER cholesterol constitutes only a tiny fraction of cellular cholesterol, it is conceivable that ER cholesterol pool is controlled by plasma membrane cholesterol content (Lange et al., 1999). Thus, it was proposed that a plasma membrane cholesterol sensor exist and regulate cholesterol trafficking among plasma membrane and internal membranes, especially ER (Lange and Steck, 1996).

Via this study, the Na/K-ATPase α1 stands out as a good candidate for the
plasma membrane cholesterol sensor for the following reasons. First, like cholestetol, α1 is ubiquitously expressed in all mammalian cells and mainly localizes in the plasma membrane. Second, α1 controls cholesterol trafficking among the plasma membrane and intracellular compartments by regulating membrane trafficking of Cav-1, a protein involved in intracellular cholesterol transport. Third, cholesterol can interact with α1 and depletion of the plasma membrane cholesterol downregulates its cell surface level. Fourth, reduction of α1 results in decrease in the plasma membrane cholesterol, which subsequently reduces ER cholesterol pool and activates HMG-CoA reductase and SREBP2 pathway in vivo. Thus, a typical negative-feedback cycle is established for regulation of cellular cholesterol content by α1.

The Na/K-ATPase in Atherosclerosis and NPC Disease

The discovery of involvement of the Na/K-ATPase in intracellular cholesterol trafficking and regulation could lead to new concept on the molecular mechanisms of the diseases and symptoms related to cellular cholesterol trafficking error. For example, atherosclerosis is characterized by high accumulation of the intracellular cholesterol molecules. Therefore, a defective excess cellular cholesterol removal has been proposed to be one of the molecular mechanisms that contribute to atherosclerosis (Rader et al., 2009). Interestingly, reduced expression of the Na/K-ATPase was reported in this metabolic disorder (Chen et al., 1995). Thus, although originally considered as a side effect, reduction of the Na/K-ATPase could be one of the factors that contribute to the cellular cholesterol trafficking error and
subsequent intracellular cholesterol accumulation as shown in FIGURE 4, 5 and 10. Alternatively, it has been proposed that the atherogenic processes involve such signaling events that result in increase of reactive oxygen species and MAPK activity (Razani et al., 2008), which are also downstream events of the Na/K-ATPase-mediated signal transduction process (Tian et al., 2003; Wang et al., 2004).

On the other hand, NPC disease is believed to be caused by cholesterol trafficking error between late endosomes/lysosomes and the plasma membrane. After reaching late endosomes/lysosomes, the LDL-derived free cholesterol molecules have problems getting out of the organelles and accumulate within them. Interestingly, changes in cholesterol metabolism detected in \(\alpha^{+/-}\) mice share many similarities with those found in NPC\(^{-/-}\) mice such as activation of SREBP2, resulting in an increase in hepatic cholesterol and a decrease in plasma cholesterol. ((Garver et al., 2007), FIGURE 12 and TABLE 3). Furthermore, the most prominent pathology in NPC disease is the massive neurodegeneration that eventually leads to early death of the inflicted individuals. The important unanswered question is that what leads to the neuronal cell death in NPC disease. As shown in FIGURE 15, the U18666A-treatment mimicked the phenotype of the NPC disease and resulted in decreased cell surface Na/K-ATPase. Consistently, our preliminary data suggested that the Na/K-ATPase was downregulated in the fibroblast cells from the NPC patients (data not shown). Thus, it is tempting to speculate that reduction of the plasma membrane cholesterol in the disease leads to downregulation of the Na/K-ATPase, which subsequently caused
cell death as the Na/K-ATPase is known to be essential for neuronal cell survival (Wang et al., 2003).

Finally, it is of interest to mention that the Na/K-ATPase amount is decreased in other metabolic disorders such as obesity (De Luise et al., 1980) and type 2 diabetes (Djurhuus et al., 2001; Noda et al., 1990). Further investigation of \( \alpha_1 \) in cholesterol regulation will not only advance our knowledge on how cellular cholesterol is regulated but also may facilitate new drugs development with the \( \alpha_1 \) as a novel target for treatment of the metabolic diseases and NPC disease.

Expression of NT of the Na/K-ATPase \( \alpha_1 \) in Mice Kidneys Leads to Salt-sensitive Hypertension

The animal body has multiple mechanisms to control blood pressure. Nevertheless, the kidney appears to be the key for long-term blood pressure regulation by modulating body fluid volume (Guyton, 1992). One of the mechanisms to control body fluid volume by the kidney is to regulate sodium reabsorption. Since the Na/K-ATPase in the basolateral membrane provides the driving force for the renal tubule sodium reabsorption, it is of interest to examine how this process is regulated in response to high salt loading and whether the newly appreciated signaling function of the Na/K-ATPase plays a role in this process. Interestingly, as we tested this concept in two different mouse models, the results from both models indicated that the signaling Na/K-ATPase is involved in renal sodium handling and blood pressure control mechanism. First, the Cav-1 KO mice developed salt-sensitive hypertension
Second, NT-expressing mice developed salt-sensitive hypertension and the severity was correlated with the renal NT expression level (FIGURE 23). Subsequent metabolic cage study revealed that the salt-sensitive hypertension was not due to plasma expansion as salt and water metabolisms were similar between control and NT mice (TABLE 4 and FIGURE 24). Therefore, our hypothesis is that interruption of the signaling Na/K-ATPase reduces renal ability to remove excess sodium in response to high salt loading and subsequently the body further elevates the blood pressure to help remove the sodium probably via a natriuresis process.

Cardiotonic Steroids as The Potential Natriuretic Hormones by Regulating Renal Na/K-ATPase and NHE3 Trafficking

It was proposed decades ago that a rise of the endogenous Na/K-ATPase inhibitor was correlated to a renal defect in excreting sodium and hypertension (de Wardener and MacGregor, 1982). Recent studies also suggested that the α1 isoform ouabain-binding site of the Na/K-ATPase can participate in the natriuretic response to high salt loading through responding to endogenous Na/K-ATPase ligands (Loreaux et al., 2008). Moreover, the sodium pump ligands like CTS were discovered to regulate trafficking of the Na/K-ATPase and NHE3 in renal proximal tubule cells (Liu and Shapiro, 2007). Therefore, the endogenous CTS including marinobufagenin (MBG) may contribute to NT-induced salt-sensitive hypertension. Consistently, higher level of urine MBG excretion was detected in NT mice on second day of high salt loading compared to control mice (FIGURE 24D). It indicated that NT-expressing renal cells
may have a defect in terms of MBG-induced α1 and NHE3 endocytosis. So the kidney produced more CTS in NT mice to enhance the natriuretic effect in response to high salt loading. This notion was supported by in vitro Na\textsuperscript{+} transcellular transport assay. While ouabain triggered endocytosis of both α1 and NHE3 and reduced transcellular transport of Na\textsuperscript{+} in control LLC-PK1 cells, its effect on endocytosis and Na\textsuperscript{+} transcellular transport was dramatically inhibited by NT expression (FIGURE 21).

At this stage, however, it should be pointed out that it is still not clear which of the endogenous CTS (ouabain, digoxin, or MBG) plays a major role in NT-induced hypertension. All three have been implicated in blood pressure regulation and sodium homeostasis and their physiological functions are still under debate (Bagrov and Fedorova, 2005; Blaustein et al., 2009; Schoner and Scheiner-Bobis, 2007a). Generation of the NT mice has warranted further investigation of the specific signals on the Na/K-ATPase and their downstream signaling events that play important roles in long-term blood pressure regulation.

The Na/K-ATPase, a possible link among cholesterol metabolism, hypertension and cardiovascular diseases

It has been well-known that hypertension, dislipidemia and metabolic syndromes like diabetes and obesity are among the major risk factors for cardiovascular diseases (Sander and Giles, 2002). Besides, dyslipidemia are common among hypertensive patients. Studies showed that in hypertensive subjects, the prevalence of increased low-density lipoprotein is 79.2% and that of decreased
high-density lipoproteins is 22.3% (Stern et al., 2000). Lowering the plasma cholesterol level by treatment of the HMG-CoA reductase inhibitors (statins) on both animal models and human patients significantly reduced blood pressure (Tonolo et al., 2000; Wassmann et al., 2001). Therefore, it is clear that cholesterol metabolism and blood pressure regulation mechanism are closely related to each other and disfunction of these systems are the major factors that contribute to the cardiovascular diseases. The discovery of the involvement of the Na/K-ATPase in both cholesterol metabolism and blood pressure regulation has provided a potential link between the two systems. It may explain why disfunction of one of the system could have a dramatic effect on the other. Undoubtedly, further studies are required to explore this issue and may lead to a dramatic progress on our understanding of how cholesterol metabolism and blood pressure regulation interplay in the body that facilitates new and better treatments for the cardiovascular diseases.
CONCLUSIONS

1. The Na/K-ATPase α1 regulates cholesterol distribution via NT-Cav-1 interaction and affects cholesterol metabolism in vivo.

2. Plasma membrane cholesterol regulates cell surface α1 content via cholesterol-NT interaction.

3. Both knockout of Cav-1 and NT expression in mice induces salt-sensitive hypertension.
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ABBREVIATIONS

α1: Na/K-ATPase α1 subunit
ACE: angiotensin converting enzyme
AP2: adaptor protein 2
AT1: type 1 angiotensin II receptor
BSA: bovine serum albumin
Cav-1: caveolin-1
Cav-2: caveolin-2
Cav-3: caveolin-3
CBM: caveolin-binding motif
CD2: second cytosolic domain of the Na/K-ATPase α1 subunit
CD3: third cytosolic domain of the Na/K-ATPase α1 subunit
CHX: cycloheximide
CMV: cytomegalovirus
CRAC: cholesterol recognition/interaction amino acid sequence and consensus pattern
CTS: cardiotonic steroids
DMEM: Dulbecco's Modified Eagle Medium
EGFR: epidermal growth factor receptor
eNOS: endothelial Nitric Oxid synthases
ER: endoplasmic reticulum
ERK: extracellular signal-regulated kinases
FBS: fetal bovine serum
FRET: fluorescence resonance energy transfer
GAPDH: glyceraldehydes 3-phosphate dehydrogenase
GFP: green fluorescence protein
GPCR: G protein-coupled receptor
GST: glutathione-S-transferase
HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA
Insig: insulin regulated protein
IP3: inositol triphosphate
IPP: isopentenyl pyrophosphate
LDL: low density lipoprotein
MAPK: mitogen-activated protein kinase
MBG: marinobufagenin
Mes: 4-morpholineethanesulfonic acid
Mβ-CD: methyl β-cyclodextrin
NHE: sodium hydrogen exchanger
NPC: Niemann-Pick type C
NT: N-terminal cytosolic tail of the Na/K-ATPase α1 subunit
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PDGF: platelet-derived growth factor
PI3K: phosphoinositide 3-kinase
PKA: protein kinase A
PKE: pig kidney enzyme
PLC: phospholipase C
RAS: renin-angiotensin II system
RFP: red fluorescence protein
S1P: site-1 protease
S2P: site-2 protease
SBP: systolic blood pressure
SCAP: SREBP cleavage-activating protein
SCP2: sterol-carrier protein 2
SREBP: sterol regulatory element binding proteins
StAR: steroidogenic acute regulatory protein
TRPC1: transient receptor potential canonical channel 1
YFP: yellow fluorescence protein
ABSTRACT

Recent studies have ascribed many non-pumping functions to the Na/K-ATPase and it has been demonstrated to interact with a variety of proteins via its cytosolic N-terminus domain (NT), which plays an essential role for its non-pumping functions.

One of the proteins that interact with NT is caveolin-1 that is implicated in cellular cholesterol trafficking and homeostasis. Since we previously showed that the Na/K-ATPase regulated membrane trafficking of caveolin-1, in this study we have further revealed that the Na/K-ATPase is able to regulate cellular cholesterol distribution via its interaction with caveolin-1. Graded knockdown of the Na/K-ATPase leads to redistribution of cholesterol from membranes to the cytosol and this effect is independent of its pumping function. Moreover, this regulation is confirmed in α1+/− mouse liver. Functionally, the knockdown-induced redistribution appears to affect the cholesterol sensing in the endoplasmic reticulum because it activates the sterol regulatory element binding protein pathway in vivo. Interestingly, our subsequent study has demonstrated that plasma membrane cholesterol also regulates the cell surface Na/K-ATPase α1 subunit by modulating membrane trafficking of α1. Depletion of plasma membrane cholesterol leads to endocytosis of α1 and accumulation of α1 in the late endosome/lysosomes. Mechanistically, the cholesterol-regulated α1 trafficking appears to be related to cholesterol-α1 interaction at the cholesterol recognition/interaction amino acid consensus site (CRAC) within NT. Disruption of the cholesterol-α1 interaction by mutating the key amino acid in
CRAC blunts the regulation of the Na/K-ATPase trafficking by cholesterol. Thus, our studies have revealed a reciprocal regulation between the plasma membrane Na/K-ATPase and cholesterol and the Na/K-ATPase may represent a potential plasma membrane cholesterol sensor for the regulation of cellular cholesterol. Furthermore, since the Na/K-ATPase is a signaling molecule that controls kinase activities, the reciprocal regulation between the Na/K-ATPase and cholesterol may serve as an important link between kinase cascades and lipid homeostasis.

To study the physiological role of the receptor function of the Na/K-ATPase, we first revealed that Cav-1 KO mice developed salt-sensitive hypertension, which implicated the signaling Na/K-ATPase in blood pressure regulation. Furthermore, we generated NT-YFP-expressing transgenic mice and showed that renal NT-YFP expression results in salt-sensitive hypertension. Mechanistically, development of salt-sensitive hypertension is related to renal membrane \(\alpha_1\) and sodium hydrogen exchanger 3 trafficking and urine excretion of the endogenous Na/K-ATPase ligand, marinobufagenin, which may influence renal sodium reabsorption and excretion.

Taken together, this study has demonstrated that the Na/K-ATPase \(\alpha_1\) subunit plays important roles in cellular cholesterol homeostasis and is also regulated by plasma membrane cholesterol content. Moreover, \textit{in vivo} expression of NT-YFP affects \(\alpha_1\) trafficking and leads to salt-sensitive hypertension, which warrants further examination of the physiological role of the Na/K-ATPase \(\alpha_1\) in the renal and cardiovascular systems.