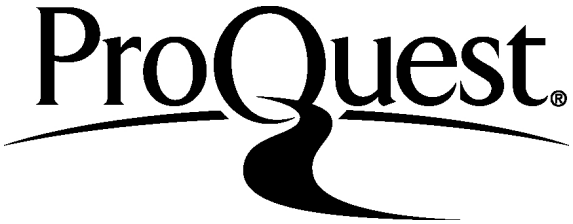
NA,K-β – A MASTER CHAPERONE FOR ION TRANSPORTERS AND ADHESION MOLECULES

by

Sona Lakshme Balasubramaniam

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

Summer 2015



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ABSTRACT

Cell membranes are enriched with a variety of proteins that play various roles in maintaining cell architecture and function. Structural and functional integrity of epithelial cells are maintained by synergistic interaction between adherent and tight junctions. Extensive studies have been carried out to understand the architecture of these complexes and the signal transducers that aid in maintaining the morphology of epithelia. Na,K-ATPase is a very well established membrane protein involved in maintaining ion homeostasis by mediating [Na^+] efflux and formation of tight junctions. It is a hetero-oligomer with a catalytic α-subunit (Na,K-α) and a regulatory β-subunit (Na,K-β). Historically, Na,K-β was identified as a chaperone for the transport and stabilization of its cognate partner Na,K-α (Geering, 1997). Na,K-ATPase expression governs intracellular Na^+ and Ca^{2+} concentration thus assisting the formation and maintenance of tight junctions, governing epithelial polarity (Rajasekaran et al., 2001b). Further studies have reported that Na,K-β functions synergistically with well-known cell adhesion molecule, E-cadherin regulating epithelial architecture. Moreover Na,K-β plays an important role in cell-cell adhesion and suppression of cell motility (Barwe et al., 2005; Rajasekaran et al., 2001b; Shoshani et al., 2005; Tokhtaeva et al., 2011; Vagin et al., 2006). In my study, I explore the molecular basis of the functional synergism between E-cadherin and

Na,K-β, and provide evidence that Na,K-β associates with E-cadherin and regulates its membrane localization, acting as a chaperone (Chapter 2).

Recent studies have demonstrated that Na,K-β is a binding partner essential for membrane localization of other ion transporters apart from Na,K-α. 1) Na,K-β interacts and targets the transport of large conductance Ca^{2+} activated K^{+} channels (BKCa) to specific regions of plasma membrane (Jha and Dryer, 2009) and also 2) Na,K-β regulates Na,K,2Cl co-transporter (NKCC2) channel exocytosis and membrane expression (Carmosino et al., 2014). Though Na,K-α is known to functionally couple with Na^{+}, Ca^{2+} exchanger 1 (NCX1), the role of Na,K-β in the regulation of membrane expression of NCX1 remained unexplored. My study focused on elucidating the role of Na,K-β in governing NCX1 expression. The results indicate that Na,K-β mediates membrane trafficking of NCX1 that is required for suppression of Ca^{2+}-dependent epithelial cell migration (Chapter 3) (Balasubramaniam et al., 2015b).

Taken together these studies suggest that in addition to its auxiliary role in aiding the trafficking of these proteins, Na,K-β also stabilizes their membrane expression. The stabilization of these proteins at the membrane is essential for their steady-state expression and function. These interesting observations suggest the collaboration of ion transporters and adhesion proteins regulate the epithelial phenotype. Loss of Na,K-β expression has been associated with EMT that leads to cancer progression and renal fibrosis (Rajasekaran et al., 1999; Rajasekaran et al.,

2010). My study indicates that the loss of Na,K-β involves the changes in the expression and function of other membrane proteins, in addition to having a direct correlation between expression and cancer prognosis. Thus it can be inferred that during the loss of Na,K-β in pre-neoplastic lesions, a plethora of events are unleashed by downregulation of adhesion molecules and ion transporters, culminating in cancer.

Chapter 1

INTRODUCTION

1.1 Epithelial Architecture

Epithelium is the first lining of the surfaces such as skin, internal organs and cavities (lumens) throughout the body. The epithelial cells act as a barrier in the renal, digestive, and reproductive systems. These cells are densely packed with less intercellular space and run as a continuous layer. This property of the epithelium is important for selective absorption of ions, regulating paracellular movement and secretion. Cell junctions constituting of protein complexes form contacts between the epithelial cells. These cell junction proteins mediate interaction between neighboring cells and cell-basement membrane (Overton et al., 1981). The cell junctions are classified into tight junctions (TJ), adherent junctions (AJ), desmosomes, gap junctions and hemidesmosomes.

Epithelial cells function as barrier to the free movement of ions and particles from filtering units and blood, restricting their transport through either transcellular or paracellular pathways. The transcellular transport through the cells is mediated by ion transporters, pumps, carriers and channels that enable the passage of solutes through the cell from the apical side of the cell facing the external milieu or the lumen of a duct the basement membrane that connects it to the blood stream. The paracellular

movement through the epithelial layer of cells occurs via selective diffusion of solutes through intercellular space between adjacent epithelial cells (Figure 1.1).

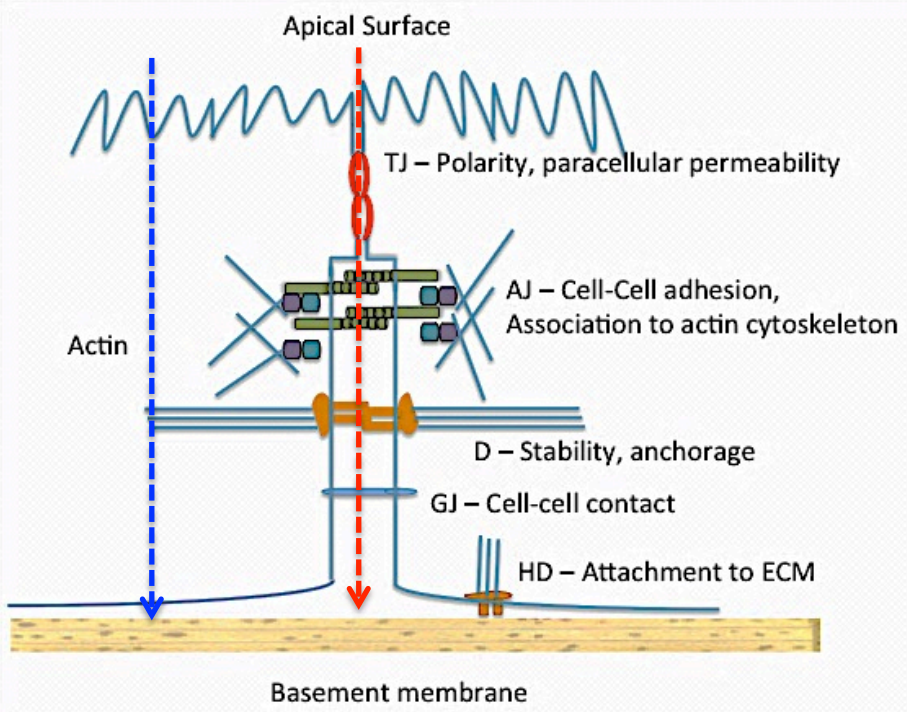


Figure 1.1. Epithelial cell architecture: The structure of the epithelial cell, the cell - cell contact it forms with adjacent cells and various junctions involved in maintaining the phenotype. TJ – tight junction, AJ – adherent junction, D – desmosome, GJ – gap junction, HD – hemi desmosome. The blue dotted line indicates the direction of transcellular ion transport and red dotted line represents paracellular ion diffusion.

These transport mechanisms rely heavily on the formation of a polarized phenotype by the TJs that seal apical side of the cells and enable the attainment of apico-basal polarity, thereby controlling trafficking of fluid solutes and immune cell infiltration (Stevenson et al., 1988). These junctions maintain the polarity of an

epithelial cell by preventing the lateral dispersal of integral membrane proteins between the apical and the basal membranes. Moreover, this barrier facilitates ions and solutes to undergo diffusion or active transport (Gonzalez-Mariscal et al., 2007; Schneeberger and Lynch, 2004). The TJ proteins such as i) occludin, ii) zona occludens, iii) claudin, and iv) JAM-1, mediate interaction of adjacent cells and form an impermeable barrier (Fanning and Anderson, 2009; Hou et al., 2013; Lapierre, 2000; Matter and Balda, 1999). TJs and septate junctions (TJ equivalent in invertebrates) regulate paracellular ion diffusion by forming zones of apparent membrane fusions or the so called "kissing points" (Tsukita et al., 2001) between adjacent cells and polarize the epithelial cell membrane into apical and basolateral membrane domains (Cereijido et al., 1998; Gumbiner, 1987; Tepass et al., 2001); this assists in the selective passage of ions and molecules, excluding proteins and lipids. The 'tight' epithelial cells such as distal convoluted tubules of the kidney have more TJ proteins controlling the re-absorption of ions and solutes whereas 'leaky' epithelial cells such as the proximal tubule have fewer TJ proteins or complex junctions enabling increased transport of ions and solutes across the epithelium.

AJs consists of cadherins and focal adhesion proteins (Etienne-Manneville, 2011). In a polarized epithelial cell, AJs form a tight basolateral belt like border close to the TJs (Figure 1.1). The actin cytoskeleton organized parallel to the plasma membrane providing a framework for the cell is associated with AJ proteins. AJ mainly consists of two proteins, very well established cadherins and newly discovered protein, nectins. Cadherins are a large family of transmembrane glycoproteins

facilitating specific cell-cell adhesion between adjacent cells in a Ca^{2+} dependent manner (van Roy and Berx, 2008). E-cadherin is a classical cadherin and is the most studied protein of this group, it consists of five repeat domains, EC1-EC5 in the extracellular region that mediate Ca^{2+} dependent cell-cell adhesion, a single transmembrane domain, and a cytoplasmic domain. The cytoplasmic domain consists of phosphorylation sites that are important for its selective binding to different proteins such as β-catenin that associates E-cadherin to α-catenin or p120 catenin that associates to γ-catenin, thereby linking E-cadherin to actin cytoskeleton (McCrea and Park, 2007; van Roy and Berx, 2008).

Similar to AJs, a third family of protein known as desmosomes anchor the plasma membrane proteins via intermediate filaments providing mechanical strength to the cell architecture. Desmosomes consist of desmogleins and desmocollins, that associate with desmoplakin, a scaffolding protein on the cytoplasmic side and connect these proteins to the intermediary filaments (Harmon and Green, 2013) (Figure 1.1). The fourth family that is involved in formation of junctions between adjacent cells are gap junctions. Clusters of channels made up of connexin proteins, a group of 6 connexin proteins known as connexons form gap junctions. These densely packed channels provide intercellular communication regulating the cell volume and ion homeostasis (Takeuchi and Suzumura, 2014).

1.2 Epithelial to Mesenchymal Transition and Fibrosis

An important mechanism that mediates the differentiation or transformation of tight mono layered epithelial cells into a loose mesenchymal/fibroblastic phenotype is known as epithelial to mesenchymal transition (EMT). EMT is a dynamic process in embryonic development, conversion of epithelial cells to fibroblasts during fibrosis (a type of EMT) of organs and in cancer progression (Kalluri and Neilson, 2003; Rastaldi, 2006). (Figure 1.2). This process in epithelial cells involves these essential steps: (a) loss of epithelial phenotype, cell-cell adhesion, actin remodeling, (b) expression of α-smooth muscle actin (SMA), (c) disruption of extracellular matrix, (d) increased cell migration and invasion.

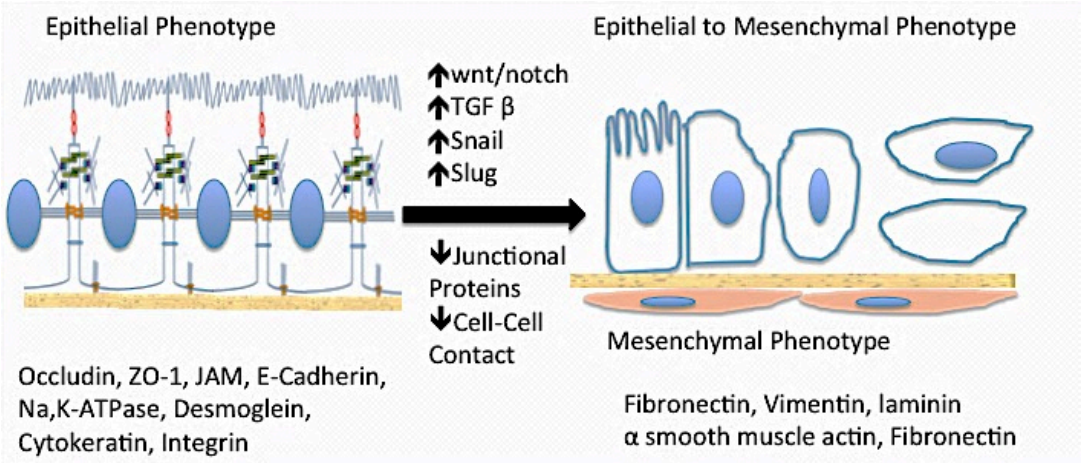


Figure 1.2. Epithelial to Mesenchymal Transition (EMT): The transition of the epithelial cells into fibroblasts involving various biochemical and molecular changes is highlighted in this representation.

Interestingly during development and organogenesis, renal tubules are derived from metanephric mesoderm through a highly regulated mesenchymal to epithelial transition (MET) (Horster et al., 1999). And recent studies suggest that more than one third of renal fibrosis originate from tubular epithelia by reversal or re-transformation to its developmental origin (Kalluri and Neilson, 2003). i.e majority of the renal fibrosis is caused due to transformation of these renal tubular epithelial cells back into fibroblasts. In fibrosis, the fibroblasts are activated that leads to copious amounts of ECM secretion. These transformations have been correlated with deteriorating renal function and end-stage renal failure. So an area of immense interest with clinical implications is MET, where the EM laying transformed cells can be reverted to the original state or regulated to reduce the amount of matrix protein they synthesize. Various studies have explored the mechanisms that trigger EMT, such as expression and secretion of growth factors TGF-β, EGF, IGF-II, and FGF-2, activation of their respective receptor signaling pathways. These receptors are coupled with various kinases that activate downstream signaling cascade upon ligand binding (Fan et al., 1999; Morali et al., 2001; Okada et al., 1997; Strutz et al., 2002). An important event occurring during EMT is the expression of the transcription factor, snail that binds to E-boxes present in the promoter region and represses the synthesis of TJ proteins claudin, occludin and AJ protein, cell-cell adhesion molecule E-cadherin; this causes increase in TJ permeability (Carrozzino et al., 2005).

Along with a number of junctional proteins, Na,K-ATPase (sodium potassium adenosine triphosphate), an ion transporter involved in regulation of ion homeostasis

is downregulated during EMT (Rajasekaran et al., 1999; Rajasekaran et al., 2010). Snail has been shown to bind to any or all five potential E-boxes in the promoter region of the β-subunit of Na,K-ATPase, and is involved in its down-regulation in carcinoma cells (Espineda et al., 2004). Studies have reported a drastic reduction in Na,K-ATPase subunits in addition to TJ and AJ proteins in renal fibrotic tissues obtained from patients with diabetic nephropathy (Rajasekaran et al., 2010) (Figure 1.2).

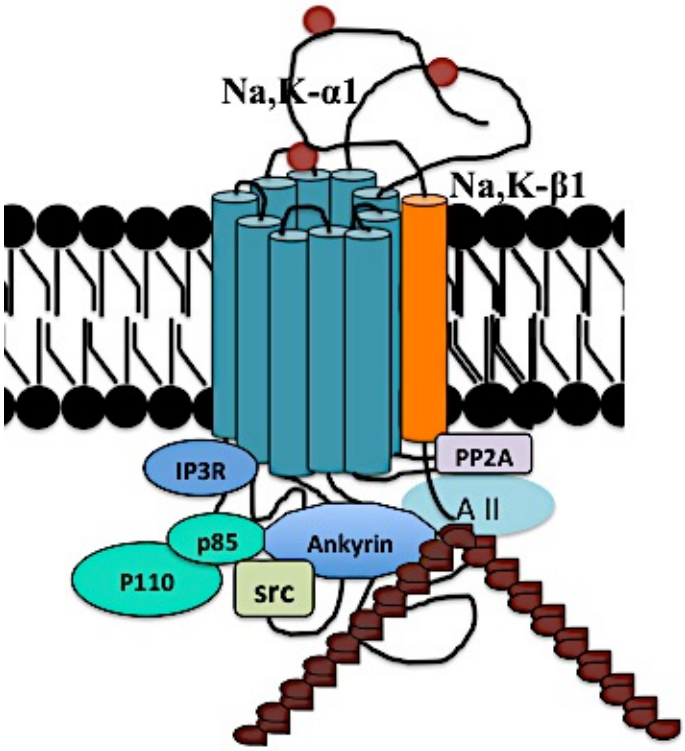


Figure 1.3. Na,K-ATPase: Na,K-α is a 110 kDa catalytic protein with 10 membrane spanning α helixes that act as the sodium pump and enable the exchange of Na^+ and K^+ ions across the epithelia. The Na,K-α has sites for the ion and ATP binding in addition to hormone binding site. Na,K-β is 55kDa transmembrane regulatory protein with a single transmembrane helix, a large extracellular domain and a short cytoplasmic tail. These subunits associate with a number of cytoplasmic proteins that anchor it to the actin cytoskeleton.

1.3 Na,K-ATPase Subunit Components and Functions

Na,K-ATPase is a highly conserved integral membrane hetero-oligomeric protein with a 110 kDa catalytic α-subunit (Na,K-α) (Baker et al., 1969; Skou, 1963), acting as the pump with ATP phosphorylation and ion binding sites, a 55 kDa regulatory β-subunit (Na,K-β), that is essential for the proficient synthesis (Ackermann and Geering, 1990), trafficking, and membrane insertion of Na,K-α (Figure 1.3). The crystal structure of Na,K-α indicates that the sodium pump consists of 10 transmembrane spanning segments. There are four known tissue specific isoforms of Na,K-α (α1- α4) (Martin-Vasallo et al., 1989b; Sanchez et al., 2006; Shull et al., 1986; Shull et al., 1985; Shull and Lingrel, 1987; Young et al., 1987) and 3 isoforms of Na,K-β (β1- β3) (Blanco and Mercer, 1998; Martin-Vasallo et al., 1989a; Martin-Vasallo et al., 2000; Mobasheri et al., 2000).

1.3.1 **Na,K-α Subunit**

Na,K-α1 isoform is ubiquitously expressed in all types of cells, and the isoform expressed in kidney and lungs, whereas Na,K-α2 and α3 isoforms are predominantly found in cardiac, skeletal, and neuronal tissues in addition to Na,K-α1 (Herrera et al., 1987; Lingrel, 1992); these isoforms are encoded by different genes. The recently recognized isoform Na,K-α4 found in testis, has been known to regulate sperm motility (Sanchez et al., 2006) .

1.3.2 **Na,K-β Subunit**

Na,K-β1 is ubiquitously expressed in all cells whereas the isoform Na,K-β2, also known as adhesion molecule on glia (AMOG) is expressed in neurons and Na,K-β3 is expressed during the developmental stages in the nervous system (Lingrel, 1992). Na,K-β is a single transmembrane glycoprotein with a large extracellular domain and a short cytoplasmic tail. Na,K-β subunit plays an important role in the biogenesis of Na,K-α and enhances its rate of synthesis, half-life, protein trafficking and membrane stabilization (Chow and Forte, 1995; Geering, 1990; Rajasekaran et al., 2004). Na,K-α synthesized in the absence of Na,K-β is rapidly degraded in the ER (Ueno et al., 1997). Many studies have reported the sites of interaction between these two proteins involving all three domains of Na,K-β, the cytoplasmic NH2-terminal domain, the transmembrane domain and the COOH-terminus extracellular domain (Barwe et al., 2007; Shoshani et al., 2005; Tokhtaeva et al., 2011; Vagin et al., 2012).

Many studies in the early to late 2000's have shown that Na,K-β play an important role in processes related to cell polarity, cell motility, and oncogenic transformation apart from EMT (Barwe et al., 2005; Contreras et al., 1999; Espineda et al., 2003; Espineda et al., 2004; Inge et al., 2008b; Rajasekaran et al., 2003a; Rajasekaran et al., 1999; Rajasekaran et al., 2003b; Rajasekaran et al., 2001a; Rajasekaran and Rajasekaran, 2009; Shoshani et al., 2005; Violette et al., 2006). Studies have shown that various types of poorly differentiated carcinoma cells expressed low levels of Na,K-β (Rajasekaran et al., 1999) and AJ protein, E-cadherin (Wijnhoven et al., 2000), indicating the loss of cell–cell contact between the epithelial cells (Rajasekaran et al., 2001b).

1.4 Model System For the Study

S. H. Madin and N. B. Darby derived Madin–Darby canine kidney cells (MDCK) cells from distal convoluted tubule of canine kidney in 1958. This cell line exhibits epithelial morphology and has been widely used an epithelial model system since 1960's. MDCK cells are tightly packed into sheets resembling *in vivo* epithelia, so MDCK cells are utilized to study the role of membrane proteins involved in regulation of epithelial characteristics such as cell-cell adhesion, epithelial polarity and cell signaling. MDCK virally transformed with Moloney sarcoma virus (MSV-MDCK) are highly invasive and express low levels of E-cadherin and Na,K-β; expression of either E-cadherin or Na,K-β alone does not restore the epithelial phenotype or polarity in these cells. However, co-expression of E-cadherin and Na,K-β induces polarity in these cells by assisting the formation of tight junctions and desmosomes accompanied with reduced cell motility (Rajasekaran et al., 2001b). Studies have also highlighted the importance of Na,K-β in blastocyst formation, regulation of $[Na^+]_i$ homeostasis and localization of TJ proteins during pre-implantation stage of development (Madan et al., 2007).

In our study MDCK cells are used as the renal epithelial model system to explore the moonlighting functions of Na,K-β. Unlike previous studies which utilized MSV-MDCK, our study tested the direct role of Na,K-β by knockdown with shRNA specific for Na,K-β (β-KD) in MDCK cells. Further I also replenished β-KD cells with Na,K-β over-expressing vector with silent mutations in the shRNA recognition site.

The β-KD/R cells are important in determining the specificity and reversibility of the effects observed due to reduction in Na,K-β expression.

1.4.1 **Sodium Potassium Homeostasis by Na,K-ATPase**

Na,K-α, the sodium pump, is an important transmembrane protein that has Na^+, K^+ and ATP binding sites; this enables the exchange of 3 Na^+ in exchange for the influx of one K^+ mediated by ATP hydrolysis against the concentration gradient. This is pivotal in regulating osmotic equilibrium, membrane potential and gradient for the Na^+ dependent ion transporters; this function governs the excitation contraction coupling in the heart, Na^+ and water reabsorption in the kidney, and release of neurotransmitters in the synaptic junctions. Concisely, Na,K-ATPase is critical in maintaining the dynamics of ion balance and volume of the cell. Studies have shown that structural association of Na,K-β with Na,K-α within their transmembrane domains is involved in the regulation of Na,K-ATPase enzyme activity (Geering, 2008). This decrease in $[Na^+]_i$ provides the membrane potential for Na^+ dependent transport proteins, such as Na, Ca exchanger (NCX1) that enables the restoration of the sodium effluxed by Na,K-ATPase, Na-glutamate transporter etc.

1.4.2 **Na,K-ATPase regulation of intracellular Ca^{2+} homeostasis**

Ca^{2+} is an important and highly versatile second messenger-signaling pathway that is essential in the regulation of various cellular processes such as re-arrangement

of actin cytoskeleton, cell motility, proliferation, and apoptosis. Consequentially, tightly regulated mechanisms govern Ca^{2+} signaling, chiefly by exchangers and ion channels. In most absorptive cells, the activity of Na,K-ATPase is closely coupled with NCX1 function on the membrane (Figure 1.4). For example, the efflux of the $3Na^{+}$ provides the concentration gradient for NCX1, which enables the influx of 3 Na^{+} and efflux of Ca^{2+} (Baker et al., 1967).

Ouabain facilitated positive inotropy is mediated by inhibition of the activity of Na,K-ATPase, leading to an elevation in the intracellular Na^{+} level. This inhibits the transport activity of NCX1 resulting in elevated Ca^{2+} and subsequent increased contractility of the heart (Nigam et al., 1992; Stuart et al., 1994). This is similar to the mechanism of action of the digitalis drugs used in treatment of congestive heart failure. NCX1 is one of the predominant proteins involved in Ca^{2+} extrusion in heart and skeletal muscle (Goldhaber and Philipson, 2013), as well as distal convoluted tubules and connecting tubules of nephrons (Matsuda et al., 1997). Na,K-ATPase is known to regulate NCX1 dependent Ca^{2+} entry by functional and physical coupling (Figure 1.4) (Moore et al., 1993).

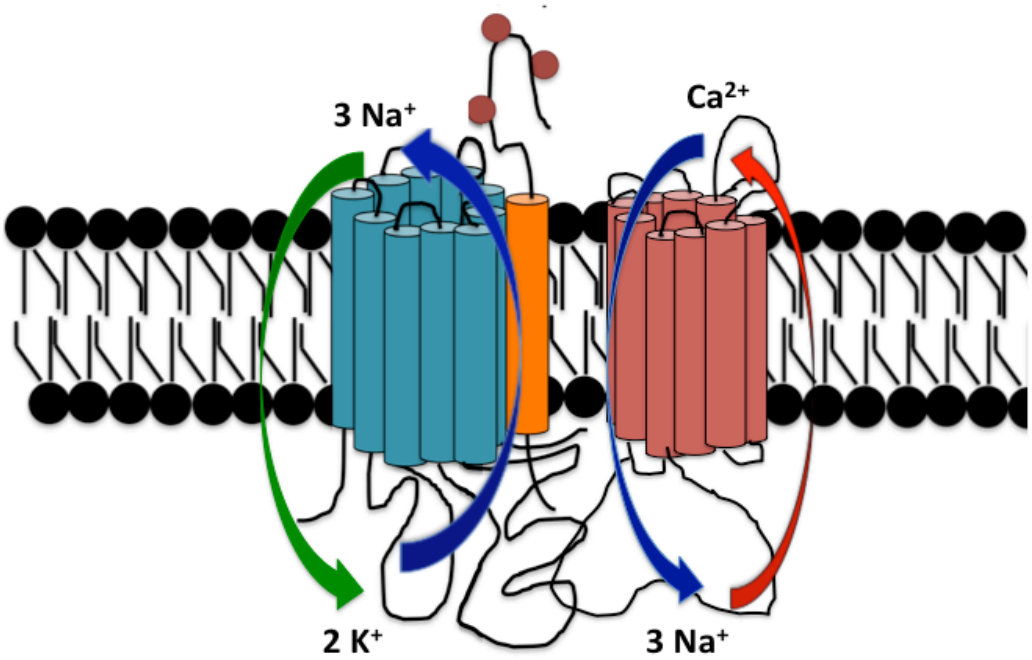


Figure 1.4. Functional association between Na,K-ATPase and NCX: Na,K-ATPase and NCX1 proteins are in close proximity on the membrane and mediate ion homeostasis by regulating the transport of Na^+, K^+ and Ca^{2+} ions across the epithelia.

1.4.3 Tight Junction Formation and Maintenance by Na,K-ATPase

The regulation of ion homeostasis by the sodium pump has been well documented since its discovery in 1957 (1991; Gibson and Harris, 1968; Hayslett et al., 1973; Heinz and Hoffman, 1965; Jorgensen and Skou, 1971; Skou and Esmann, 1992). Since the early 90's the involvement of Na,K-ATPase has been explored for its roles in initiation of epithelial polarity, its action as hormone receptor and in its regulation of actin dynamics, cell movement and signal transduction (Cereijido et al., 2012; Rajasekaran et al., 2005; Rajasekaran et al., 2001a).

TJs are essential in maintaining epithelial cell polarity, as described in section 1.1 (Schneeberger and Lynch, 2004). Interestingly, inhibition of the Na,K-ATPase pump activity by ouabain treatment in epithelial cells prevented the formation of TJs, increased cellular permeability and disrupted existing TJ and septate junctions (SJ), the invertebrate TJ equivalent (Paul et al., 2003; Rajasekaran et al., 2003a; Rajasekaran and Rajasekaran, 2003; Rajasekaran et al., 2003c; Rajasekaran et al., 2001a). Similarly, treatment with Na^+ ionophore gramicidin or low extracellular K^+ also caused defect in the formation of TJ suggesting the crucial role of Na,K-ATPase in regulating intracellular Na^+ homeostasis, which is vital in maintenance of the epithelial phenotype (Rajasekaran et al., 2001a).

In normal epithelial cells localization of the Na,K-ATPase in the basolateral side results in diffusion of Na^+ ions into the intercellular space; the Na^+ ions move towards the capillaries as the TJs seal the apical side in epithelial cells directing the movement of ions into the bloodstream for reabsorption (Cereijido et al., 2012). Na,K-ATPase is linked to the scaffolding protein ankyrin-B, which anchors it to the cytoskeletal proteins, this association facilitates maintenance of TJ structure (Devarajan et al., 1994), whereas PP2A a protein phosphatase that dephosphorylates occludin, binds to and governs the stability of TJ complex (Rajasekaran et al., 2007); Ouabain mediated increase in $[Ca^{2+}]_i$, has been reported to interfere with the biogenesis of TJs (Nigam et al., 1992; Stuart et al., 1994). These studies indicate that the Ca^{2+} fluctuations induced by Na,K-ATPase activity regulate stability of the TJs. Detailed reports emphasize that inhibition of Na,K-ATPase activity with ouabain at

concentration of 10-100nM increased the tightness of junction i.e., the distance between the membranes in the TJ region (Liu et al., 2000) but at concentrations ≥ 200nM, ouabain prevented TJ formation, increasing the permeability (Rajasekaran et al., 2001a) and cell detachment (Contreras et al., 2004; Contreras et al., 1999).

1.4.4 Na,K-ATPase as a Member of the Epithelial Junctional Complex

Epithelial cell architecture is structured by junctional proteins that undergo trans dimerization i.e adjacent cell-to-cell interaction and cis dimerization i.e interaction between proteins of same cell (Hartsock and Nelson, 2008). Trans and cis-dimerization of membrane proteins enable association with anchoring proteins, which attach cell adhesion molecules to the cytoskeleton; these interactions also initiate a junction associated signaling cascade. Density gradient centrifugation has shown that Na,K-ATPase co-distributed with TJ proteins occludin, ZO-1, ZO-2, protein phosphatase 2A (PP2A) (Vogelmann and Nelson, 2005) and AJ protein E-cadherin, in association with scaffolding and anchoring proteins (ankyrin, annexin2) (Barwe et al., 2005; Rajasekaran and Rajasekaran, 2009; Vogelmann and Nelson, 2005). Na,K-β binds to annexin II, a Ca^{2+} dependent scaffolding protein that binds to actin cytoskeleton (Barwe et al., 2005). Increasing evidence supports the presence of Na,K-ATPase in TJs and AJs (Liu et al., 2011; Rajasekaran et al., 2007; Vogelmann and Nelson, 2005). The localization of Na,K-ATPase in the basolateral membrane, is required for maintenance of apico-basal polarity and vectorial transport of ions to facilitate lumen formation during organogenesis. This has been demonstrated in 3-

dimensional cultures of MDCK cells in collagen, zebrafish gut, *Drosophila* trachea and mouse blastocysts (Bagnat et al., 2007; Paul et al., 2003; Violette et al., 2006). These studies increased the appreciation for the role of Na,K-ATPase mediated ion homeostasis in TJ formation and organogenesis.

1.5 **Pump Independent Functions**

1.5.1 **Cell-Cell Adhesion**

During the early phase of the identification of ion homeostasis independent functions of Na,K-ATPase, the other possible roles of Na,K-β were also explored, but to lesser extent. Recognition that the adhesion molecule on glia (AMOG) is a homologue of the Na,K-β (β2) (Gloor et al., 1990) indicated that other isoforms of Na,K-β may function similarly and that Na,K-ATPase subunits may have additional functions unrelated to pumping ions. Na,K-β functions synergistically with E-cadherin, to restore epithelial phenotype of virally transformed cells (as described above) Over-expression of the Na,K-β and E-cadherin in these cells increased their resistance to detergent extraction, assisted in the formation of cell-cell contacts and restored epithelial phenotype, which was not observed with E-cadherin alone (Rajasekaran et al., 2001b). The molecular basis of this functional synergism remained unexplored for the last decade and forms the basis of the first part of my study (Chapter 2).

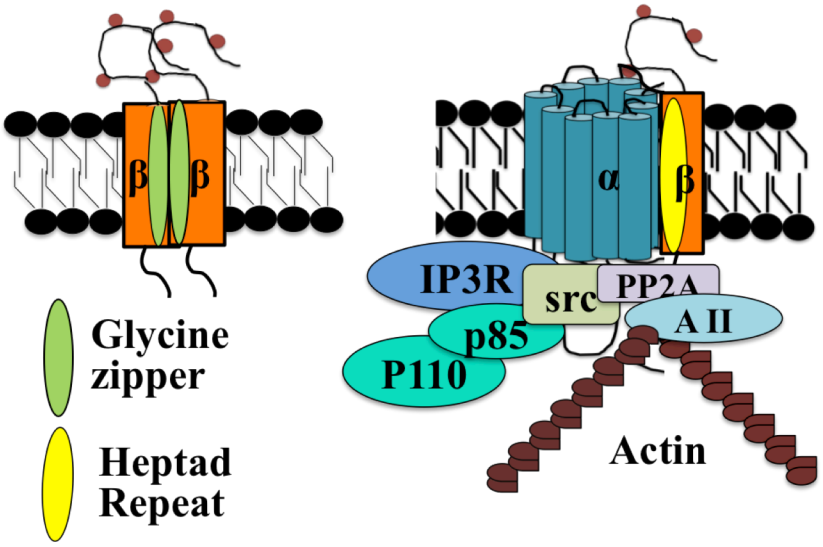


Figure 1.5 Protein-protein interactions mediated by Na,K-ATPase subunits: The glycine zipper motif (shown in green in the homo-dimerization panel) and N-glycan domains (shown as three red circles in extracellular region) of Na,K-β mediates Na,K-β/Na,K-β interaction involved in cell adhesion. The heptad repeat motif of Na,K-β that mediates Na,K-α/Na,K-β interaction is shown in orange in the hetero-dimerization (Na,K-α/β) cartoon. Na,K-α interaction with Src, IP3R and p85 subunit of PI3-K is shown along with Na,K-β association with annexin II and PP2A.

Further reports showed that specific amino acid residues on opposite faces of the transmembrane domain of Na,K-β that mediate i) homo-oligomerization and intercellular adhesion via the glycine zipper motif on one face and ii) hetero-oligomerization with Na,K-α via the heptad repeat motif on the other face of the transmembrane domain, thereby regulating pump activity (Barwe et al., 2007). Cells expressing mutant Na,K-β that fail to hetero-oligomerize with Na,K-α were used to determine the contribution of ion transport activity in additional cellular functions governed by Na,K-ATPase. In addition Na,K-β facilitates trans homo-oligomerization

by N-glycans in the extracellular domain (Barwe et al., 2007; Shoshani et al., 2005; Tokhtaeva et al., 2011; Vagin et al., 2006) (Figure 1.5). However cell adhesion was unaltered in the heptad repeat mutant of Na,K-β, but had reduced Na,K-ATPase pump activity, confirming that Na,K-β induced cell adhesiveness was independent of Na,K-ATPase activity (Barwe et al., 2007).

All these imply that Na,K-β is an important component of adhesional complex mediating cell-cell adhesion. In addition, Na,K-α and Na,K-β hetero-dimer are expressed as a part of the junctional complex and anchored to the actin cytoskeletal network by scaffolding proteins ankyrin and annexin, respectively (Barwe et al., 2007; Rajasekaran et al., 2008; Vagin et al., 2012). These associations of Na,K-ATPase are important in the formation and maintenance of epithelial polarity in mammalian cells, independent of its pump activity.

1.5.2 **Cell Signaling**

In the presence of ouabain, the Na,K-ATPase associates with Src kinase to transactivate the Epidermal Growth Factor Receptor (EGFR), which recruits a number of signalosome components to the membrane including PhospholipaseC-γ (PLC-γ1) and p85 subunit of PI3-K (Barwe et al., 2005; Haas et al., 2002; Nunbhakdi-Craig et al., 2002; Pedemonte et al., 2005; Xie et al., 2013; Yuan et al., 2005). Na,K-ATPase also functions as a signaling scaffold for protein kinase C (PKC), PP2A and Inositol-3-phosphate receptor (IP3R) (Gable et al., 2014; Haas et al., 2002; Pedemonte et al., 2005; Xie et al., 2013; Yuan et al., 2005). At concentrations that do not inhibit Na,K-

ATPase activity completely i.e 10-100nM, ouabain binds to Na,K-α and activates signaling pathways involved in proliferation and differentiation (Kaplan, 1978; Liu et al., 2000; Nguyen et al., 2007; Xie et al., 2013). Knockdown of Na,K-β also activated PI3-K/ERK signaling, which is associated with increased proliferation, reduced contact inhibition and disruption of the polarized phenotype in epithelial cells (Barwe et al., 2012). Na,K-ATPase pump activity was likely not involved in these functions, since the heptad repeat mutant of Na,K-β with compromised Na,K-ATPase function did not activate PI3-K/ERK signaling or disrupt epithelial polarity.

Similar to the re-expression of E-cadherin in invasive MSV-MDCK cells, over-expression of Na,K-β in MSV-MDCK cells suppressed motility and invasion (Rajasekaran et al., 2001b), while knockdown of Na,K-β increased migration and reduced contact inhibition (Balasubramaniam et al., 2015b; Barwe et al., 2012; Huynh et al., 2015), confirming a role for the Na,K-β in the regulation of cell migration. This migration was dependent on protein interactions between Na,K-ATPase subunits with PI3-K and annexin II, but independent of pump function. Our recent study showed that activation of PI3-K/ERK signaling and increased migration in Na,K-β knockdown cells was Ca^{2+}-dependent (Balasubramaniam et al., 2015b). The increase in $[Ca^{2+}]_i$ was mediated by the reduction of functional NCX1, a major regulator of Ca^{2+} flux in renal epithelial cells.

Studies carried out in the last decade have emphasized loss of Na,K-β in failing human heart, progression of carcinoma, and diabetic nephropathy accompanied by

activation of Akt and ERK signaling pathways (Rajasekaran et al., 1999; Rajasekaran et al., 2005; Rajasekaran et al., 2010; Schwinger et al., 1999). Over-expression of Na,K-β in highly invasive, MSV-MDCK cells aided in trans-epithelial transport, restoration of the membrane polarity, suppression of motility and invasion, and tumor growth (Barwe et al., 2005; Inge et al., 2008b; Rajasekaran et al., 2001b). Na,K-β has been shown to play a role in suppression of cell motility by localizing to the lamellipodia in association with annexin II, a Ca^{2+} dependent scaffolding protein (Barwe et al., 2005). MDCK cells with Na,K-β knockdown (β-KD) exhibited an increase in phosphorylation of PI3-K and ERK signaling pathway (Barwe et al., 2012). This activation of PI3-K and ERK in β-KD cells of motility is not just through protein dependent function. My study aims at understanding the mechanism behind activation of these signaling pathways, details provided in chapter 3 and (Balasubramaniam et al., 2015b).

1.5.3 **Molecular Chaperone**

Apart from Na,K-α, Na,K-β has been shown to regulate membrane expression of other ion transporters. It has also been shown that Na,K-β interacts and targets membrane localization of a large conductance of Ca^{2+} activated K^{+} channels (BKCa) to specific regions of plasma membrane This plays an important role in regulation of intracellular Ca^{2+} concentration (Jha and Dryer, 2009) and the reduction in BKCa expression due to the absence of Na,K-β leads to significantly reduced membrane potential. The change in the membrane potential due to altered BKCa activity has been

reported in increased cancer cell migration and invasion (Wondergem and Bartley, 2009), these studies indicate that Na,K-β chaperons BKCa that is involved in suppression of cancer invasion and progression.

Further studies have also shown that Na,K-β also regulates Na,K,2Cl Co-transporter2 (NKCC2) channel exocytosis and membrane expression that facilitates NKCC2 dependent electrolyte transport (Carmosino et al., 2014). NKCC2 is an important player in the regulation of Na and Cl reabsorption in thick ascending limb thereby playing an important role in blood pressure homeostasis. This further substantiates the role of Na,K-β in regulation of other ion transporters. Mice with cardiac-specific knockout (KO) of Na,K-β failed to respond to ouabain, as ouabain response needs NCX1 as described earlier. These KO mice were ouabain insensitive attributing to the specific reduction in NCX1 protein, without any change in other major Ca^{2+} handling proteins, such as plasma membrane Ca^{2+} ATPase (PMCA) or sarcoplasmic Ca^{2+} ATPase (SERCA) (Barwe et al., 2009).

My study concentrated on understanding the basis of the regulation of NCX1 by Na,K-β, whether Na,K-β chaperones NCX1 to increase its expression similar to Na,K-α, BKCa, NKCC2 and governs $[Ca^{2+}]_i$ concentration, which is examined in chapter 3. My study focused on establishing the role of Na,K-β as a molecular chaperone responsible for membrane trafficking of proteins important in epithelial cells, i) adhesion molecule E-cadherin and ii) ion transporter NCX1. Further, the study

explores the signaling pathways activated in response to reduced Na,K-β expression and its consequences.

Chapter 2

MOLECULAR INTERACTION BETWEEN NA,K-β AND E-CADHERIN REPRESSES β-CATENIN TRANSCRIPTIONAL ACTIVITY

2.1 Introduction

E-cadherin, a type I transmembrane protein with a large extracellular domain, is involved in Ca^{2+} dependent cell-cell adhesion, (Takeichi, 1977). E-cadherin was first identified as a cell adhesion molecule and over time has been thoroughly characterized both in normal and in pathological conditions (van Roy and Berx, 2008). These adhesion molecules undergo homophilic interactions within same cells via cis-interaction through interaction between the transmembrane domain, as well as between adjacent cells via trans-interaction through association of extracellular domains (Nagafuchi et al., 1987). In addition to the extracellular domain, which plays an important role in cell-cell adhesion, another vital component of E-cadherin is its cytoplasmic domain. The cytoplasmic tail of E-cadherin is associated to the actin cytoskeleton through a family of catenin proteins (Ozawa and Kemler, 1998; van Roy and Berx, 2008).

β-catenin associates to the distal end of E-cadherin cytoplasmic domain and links E-cadherin to the actin cytoskeleton, causing clustering of this protein at adherens junctions (AJ) and stabilizing cell-cell adhesion (Hirano et al., 1992). The AJ

integrity depends on the association of the E-cadherin/β-catenin complex. β-catenin has 3 structural domains: a) the amino terminal consists of GSK3 phosphorylation site that is involved in its ubiquitination, b) the carboxyl terminal contains a transcription activation domain and a PDZ domain that enables β-catenin interaction with other proteins, and c) a central domain containing twelve armadillo repeats; that regulates β-catenin activity, its nuclear translocation and mediates transcription of targeted genes via direct association with T-cell factor/lymphocyte enhancer factor-1 (TCF/LEF1) transcription factors.

In the absence of wnt stimulus, a destruction complex containing axin, adenomatous polyposis coli (APC) and GSK3 phosphorylates β-catenin. Phosphorylation of β-catenin near the amino terminus, helps in recognition by E3-ubiquitin ligase β-TrCP, that causes ubiquitination and subsequent proteasomal degradation of β-catenin (Luo and Lin, 2004; Stamos and Weis, 2013). In the presence of wnt stimulus, the destruction complex does not phosphorylate β-catenin, this prevents its ubiquitination and following proteosomal degradation. In non-canonical wnt signaling E-cadherin is destabilized on the membrane, this causes disassembly of the adherens junctions, decreasing cell adhesion strength and increased cell migration. β-catenin from the AJ is internalized to the cytoplasm and translocated into the nucleus (Hulsken et al., 1994). Furthermore, β-catenin that is translocated into the nucleus activates TCF/LEF dependent transcription of specific target genes during embryo development (Luo and Lin, 2004) similar to classical wnt/β-catenin signaling

pathway. Research over the past few decades have highlighted the involvement of β-catenin signaling in events associated with initiation of a number of malignancies, such as melanoma, medulloblastoma, colon, hepatocellular, ovarian, endometrial, and prostate cancer (Morin, 1999). Studies have reported activation of the β-catenin pathway in these cancers with upregulation of transcriptional targets cyclin D1, Axin, GSK3-β, Groucho, Conductin, and TCF (Chiurillo, 2015; Polakis, 2000).

MSV-MDCK cells lack cell junctions and are fibroblastic in phenotype, hence these cells are used as a model system to study the effects of loss of junctional proteins. MSV-MDCK cells express low levels of E-cadherin, in addition to reduced expression of Na,K-β, another cell adhesion molecule. Na,K-ATPase is a trans-membrane protein important in maintaining a sodium gradient across the membrane. It is an important regulator of Na^+ and K^+ homeostasis in the cell. It is a hetero trimeric protein with a catalytic Na,K-α subunit acting as the pump, a regulatory Na,K-β subunit acting as a chaperone for the pump, and another regulatory Na,K-γ subunit expressed in certain tissue types. The regulation of ion homeostasis by the sodium pump has been well documented in the past decades. Other roles of Na,K-ATPase in regulating tight junctions, actin dynamics, cell movement and signaling are highlighted in a review (Rajasekaran et al., 2005). Inhibition of Na,K-ATPase pump activity resulted in cell detachment (Contreras et al., 2004; Contreras et al., 1999), prevented tight junction formation and increased the permeability in MDCK cells (Rajasekaran et al., 2001a). Further studies have shown that over-expression of Na,K-β

in MSV-MDCK cells aids in the maintenance of epithelial polarity (Rajasekaran et al., 2001b), suppression of motility (Barwe et al., 2005), and potential tumor suppression role (Inge et al., 2008b).

Overexpression of E-cadherin alone was not sufficient to re-establish cell-cell adhesion, revert the cells to epithelial phenotype, induce polarity or reduce invasiveness in MSV-MDCK cells. However, co-expression of E-cadherin and Na,K-β in these cells induced MET, restoring epithelial phenotype, suppressing cell motility and invasiveness (Rajasekaran et al., 2001b). This was enabled by the formation of junctional complexes suggesting that there is a functional relationship between these two proteins. Moreover other studies have also shown that Na,K-β and E-cadherin are drastically reduced in invasive carcinoma cells (MSV-MDCK) and the expression of both these proteins are repressed by the transcription factor snail during EMT (Espineda et al., 2004; Rajasekaran et al., 2010). Since, Na,K-β and E-cadherin exhibited a synergistic interaction, my study was carried out to understand the molecular basis of this interaction.

I aimed to establish the molecular basis of the functional synergism between Na,K-β and E-cadherin. Co-immunoprecipitation indicated that Na,K-β, β-catenin and E-cadherin associate in a complex, to test the possibility of a direct binding of Na,K-β and β-catenin an *in vitro* binding assay was carried out. β-catenin has a number of binding partners, but the most important interacting partners are E-cadherin (membrane stabilization) and TCF (nuclear localization), which occurs at the armadillo (arm) domain. Radioactive labeling of Arm domain of β-catenin and

subsequent *in vitro* pull-down with GST bound cytoplasmic tail of Na,K-β indicates that these two proteins associate directly.

During reduction of Na,K-β in β-KD cells (generated as discussed below in materials and methods), β-catenin nuclear localization occurred. This was associated with an increase in the β-catenin transcriptional activity and increase in the transcription of downstream target of β-catenin signaling pathway, Axin 2. Since the dissociation of β-catenin from E-cadherin is known to destabilize its membrane localization, the surface expression of E-cadherin was evaluated. Cell surface biotinylation and detergent extraction assay indicated the membrane expression and stability of E-cadherin association to the actin cytoskeleton is compromised in β-KD cells. This could possibly be due to the reduced anchorage to E-cadherin at the adherent junction due to the loss of its interaction partner Na,K-β or an increase in the rate of internalization of E-cadherin thereby reducing the surface expression and association to the cytoskeleton. These results suggest that the presence of Na,K-β causes suppression of β-catenin transcriptional activity through the maintenance of the adherens junction formation.

2.2 Materials and Methods

2.2.1 Cell Lines and Maintenance

MDCK, MSV-MDCK, PC3 cell lines were obtained from American Type Culture Collection (Rockville, MD) and grown in DMEM with 10% fetal bovine serum, 2 mM/L L-glutamine, 25 U/mL penicillin, and 25 µg/mL streptomycin. The stable knockdown of Na,K-β1 and the rescue clones were generated, as previously described (Barwe et al., 2012). Stable knockdown of Na,K-β1 (β-KD) and Annexin II (AII KD) was achieved by transfecting pSilencer 5.1-U6 Retro vector (Ambion, TX) containing siRNA targeting specific sequence into MDCK cells. The full-length dog Na,K-β1 cDNA harboring silent mutations within the shRNA recognition site to make it shRNA resistant was first constructed as described in (Barwe et al., 2012). Transfection was done by electroporation using the Amaxa Nucleofector (Lonza, Walkersville, MD), following the manufacturer's instructions. Cells were maintained in a selection medium with 10 µg/mL puromycin and 500 µg/mL neomycin, respectively. Stable clones expressing the transfected constructs were utilized for the experiments.

2.2.2 Antibodies

Monoclonal antibodies against Na,K-β1 (M7-PB-E9) and Na,K-α1 (M17-P5-F11) were from ThermoFisher Scientific Inc. (Waltham, MA). Rat E-cadherin

antibody (Decma1) and mouse β-actin antibody was purchased from Sigma-Aldrich® (St. Louis, MO). Mouse β-catenin antibody was purchased from BD Biosciences® (San Diego, CA) and α-catenin antibody was purchased from Chemicon® (Temecula, CA). The mouse, rabbit, rat total or mouse/rabbit light chain horseradish peroxidase conjugated secondary antibodies were obtained from Cell Signaling Technology® (Lexington, KY).

2.2.3 **Immunoblot Analysis and Co-immunoprecipitation**

Total protein cell lysates were prepared in a lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM ß-glycerolphosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mg/ml of antipapain, leupeptin, and pepstatin). 50 or 100 μg of cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Blots were blocked in 5% nonfat milk in Tris buffered saline with 0.1% Tween 20 (TBST) at room temperature (RT) for 1h. Primary antibodies were diluted in either 5% bovine serum albumin (BSA) or nonfat milk in TBST and incubated overnight at 4°C. Secondary antibodies were diluted in 5% nonfat milk in TBST at RT for 1 h, and blots were developed with chemiluminescent lightning system according to the manufacturer's recommendations, ECL or ECL Prime (GE Healthcare).

For co-immunoprecipitation, cell lysates were prepared as described above for immunoblotting and pre-cleared with Protein A Mag Sepharose (GE Biosciences®).

Antibodies were pre-coupled to the mag beads with rabbit anti-mouse antibody (RAM) for 4 hours and incubated overnight with 1000 μg of total protein lysate. The beads were washed and separated by SDS-PAGE and the samples were subjected to immunoblotting as described above.

2.2.4 **qRT-PCR Analysis**

RNA was obtained by Trizol extraction method and cDNA was prepared prior to setting up the qPCR reaction by iScript™ cDNA Synthesis Kit (Bio-Rad) as per manufacturer's instructions. The cDNA was amplified via real-time polymerase chain reaction using the SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: (a) Na,K-β1 (forward: TTACCCTTACTACGGCAA GCTCCT, reverse: TTCAGTGTCCATGGTGAGG TTGGT), (b) E-Cad (forward: TTCCTCCCAATACATCTCCCTTCACAGCAG, reverse: CGAAGAAACAGCA AGAGCAGCAGAATCAGA), (c) Cyclin D1 (forward: AGGAGCAGAAGTG CGAGGAG, reverse: CACATCTGTGG CACAGAGCG), (d) Axin2 (forward: GGACA AATGCGTGGATACCT, reverse: TGCTTGGAGACAATGCTGTT), (e) GAPDH (forward: GCTGTCCAACCAC ATCTCCTC, reverse: TGGGGCCGAAGA TCCTGTT).

Quantitative PCR was performed in a 384 well plate on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Samples were assayed in triplicates and RNA levels were calculated by relative quantification (RQ) normalizing the samples to the endogenous control GAPDH. Graphs represent the average of the relative mean

expression level (RQ value) of three different experiments. The error bars represent the standard error of the RQ value (GraphPad Software).

2.2.5 Reporter Assay – TOPFLASH/FOPFLASH Assay

MDCK, β-KD and β-KD/R cells were cultured on 24 well plates for 24 hours and then the cells were transfected with 0.2 μg reporter plasmid (TOPFLASH or FOPFLASH) and 0.2 ng internal control pRennila., After 24 hours the transfected cells were tested for luciferase activity using Dual-Glo luciferase assay kit (Promega, Madison, WI) with fire-fly and luciferase activity in VICTOR X multi-label plate reader (Perkin Elmer, Cambridge, MA) . The transfection efficiency was normalized across the cell lines by renilla luciferase activity as an internal control. The graph is representative of an average of three independent experiments, as shown in GraphPad Software.

2.2.6 Cell Surface Biotinylation

The cell surfaces of sub-confluent monolayers were labeled on ice with 0.5 μg/ml membrane-impermeable EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) in TEA buffer (150 mM NaCl, 10 mM triethanolamine pH 9, 1 mM CaCl2, 1 mM MgCl2) and quenched with ammonium chloride (50 mM NH_4Cl in PBS, 0.1 mM CaCl2, 1 mM MgCl2). The cells were then lysed in 300 μl of lysis buffer, same as the buffer used for immunoblotting. Lysates were incubated with 30 μl Ultralink

streptavidin beads (Pierce) and rotated for 16 h at 4° C. The pulled down beads were washed with the lysis buffer and immunoblotted.

2.2.7 **Endocytosis Biotinylation**

For internalization experiment the EZ-Link Sulfo-NHS-SS-Biotin (Pierce) was used to biotinylate cell surface proteins at 4°C, as described. Control plates were retained on ice and experimental plates were incubated at 37°C for 30 or 60 minutes. Once the incubation was done the cells were transferred back to 4°C, one control dish was reduced, and the second dish was not reduced, thereby serving as 0 and 100% biotinylation references, along with the 30 and 60 min, with reducing solution (250 mM of glutathione (Sigma, St. Louis, MO), 10mM NaCl, 250 mM NaOH, 10% FBS. The free sulfhydryl groups of biotin were quenched in 5mg/ml iodoacetamide (Sigma) in 5% BSA solution. Then the cells were lysed and treated as described for the cell surface biotinylation experiment.

2.2.8 **Triton X-100 Extraction**

Confluent monolayers were extracted with 200 µl of extraction buffer (50 mM NaCl, 10 mM piperazine-N, N^1 bis (2-ethanesulfonic acid) pH 6.8, 3 mM MgCl2, 0.5% Triton X-100, 300 mM sucrose, 1 mM PMSF, 100 U/ml DNase) for 10 min at 4°C. The lysates were centrifuged at 4°C for 30 min at 14,000 rpm in a microfuge and the pellet was suspended in 200 µl sample buffer. Same volumes of each pellet and supernatant fraction were immunoblotted, as described above.

2.2.9 **Immunofluorescence and Confocal Microscopy**

The confluent monolayer of cells was fixed with either ice cold methanol (−20°C) or 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 20 mins. Primary antibodies were diluted in 1% BSA in PBS and incubated overnight at 4°C, followed by Alexa-488™, Alexa-546™ conjugated secondary antibodies and TO-PRO®-3-Iodide (Life Technologies) for nuclear staining. The coverslips were affixed on glass slides with ProLong gold antifade reagent (Life Technologies). Confocal images were captured in Leica TCS SP5 (Leica microsystems).

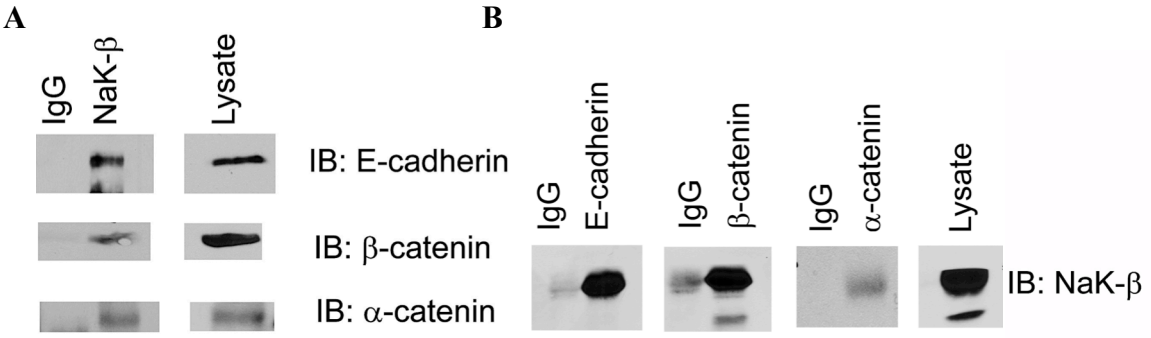
2.2.10 **GST Pull-down and Immunoprecipitation**

The cytoplasmic tail of Na,K-β from amino acid 1-35 (β CD) was cloned in pGEX-5X vector (Invitrogen). The GST, GST β-CD constructs were transformed in *Escherichia coli* (BL-21) and induced with 0.25mM IPTG (isopropylthiogalactoside) for 4 h. The bacterial cells were centrifuged and reconstituted in the lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM $MgCl_2$, 250 g/ml lysozyme, 1mM PMSF, and 10 g/ml each of antipain, leupeptin, and pepstatin) and then sonicated. The lysates were centrifuged at 13,000 rpm at 4°C for 15 min. The supernatant was rotated with glutathione-coupled agarose beads (Amersham Biosciences®, Kingsport, TN) for 1h at 4°C. The glutathione beads coupled with protein were washed and the bound fusion protein was assessed using coomassie-stained SDS gels. MDCK cells were lysed and incubated with 1mg of GST or GST-fusion proteins conjugated beads in separate

tubes, overnight at 4°C. The beads were washed with the same lysis buffer and immunoblotted as above.

2.2.11 *In vitro* Binding Assay

TnT® SP6 Quick Coupled Transcription/Translation System was purchased from Promega. The linearized PCS2+MT plasmid containing the β-catenin armadillo domain was incubated with the rabbit reticulocyte from the kit and S^{35} methionine



(Perkin Elmer) was used to radioactively label the newly synthesized protein; then the solution was incubated with Glutathione beads, GST, GST-βCD or GST-Annexin II. Bound proteins were resolved by SDS-PAGE and analyzed by autoradiography, developed in a Phosphor screen and quantitated in Typhoon imager from GE life sciences.

Figure 2.1. Interaction of Na,K-β with E-cadherin and other E-cadherin associated proteins: (A) Co-immunoprecipitation of 1mg of MDCK lysate with Na,K-β antibody and immunoblotting for E-cadherin, β-catenin and α-catenin suggest that Na,K-β is in a complex with E-cadherin, β-catenin and α-catenin; the total lysate from input is used as a control. (B) This panel shows the reverse immunoprecipitation of Na,K-β with E-cadherin, β-catenin and α-catenin antibodies. These results indicate that these proteins either exist in a complex or might be directly interacting.

2.3 Results

2.3.1 Na,K-β Complexes with E-cadherin and Associated Catenins

To test whether the functional synergism between Na,K-β and E-cadherin involves molecular interactions between these two proteins, we performed a co-immunoprecipitation analysis using MDCK cells. Immunoprecipitates with anti-Na,K-β antibody (M17-P5-F11; targeted against the extracellular domain of Na,K-β) showed the presence of E-cadherin, β-catenin and α-catenin (Figure 2.1A). Conversely, immunoprecipitation using anti-E-cadherin, β-catenin or α-catenin antibodies pulled down Na,K-β (Figure 2.1B).

The stoichiometry of the interacting proteins that form the complex was not determined, but based on the immunoprecipitation the involvement of α-catenin and β-catenin in the complex formation was established. A HRP-linked secondary antibody specifically detecting the light chain of antibody was used to avoid detection of the antibody heavy chain band which is similar in size to the fully glycosylated Na,K-β. Taken together, these results suggest that Na,K-β is in a complex with E-cadherin and its associated catenins.

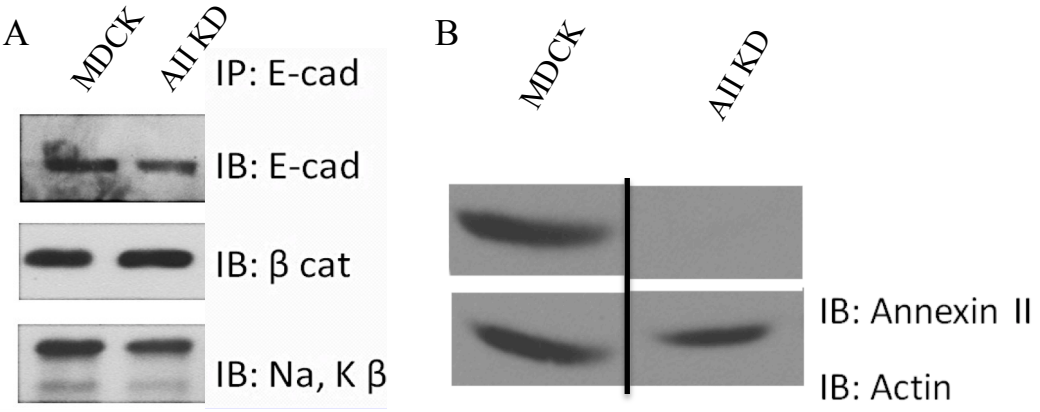


Figure 2.2. Interaction of Na,K-β with E-cad in the absence of Annexin II: A) Immuno-precipitation of E-cadherin in MDCK, Annexin II KD cell lysates. Immunoblotting of the proteins pulled down with E-cadherin antibodies demonstrate that Na,K-β interacts with E-cadherin even in the absence of Annexin II. B) Immunoblot of cell lysates from MDCK and Annexin II KD cells indicate undetectable level of Annexin II in AII KD cells.

2.3.2 Annexin II and α-catenin Are Dispensable For the Complex Formation of Na,K-β with E-cadherin

Na,K-β associates with the actin cytoskeleton via interaction of its cytoplasmic domain with annexin II, which is a F-actin binding protein (Barwe et al., 2005). Therefore, to test the involvement of annexin II in the complex formation between Na,K-β and E-cadherin, MDCK cells with specific knockdown of annexin II (AII-KD) were utilized. Na,K-β co-immunoprecipitated with E-cadherin in AII-KD

cells similar to MDCK cells. This data indicates that annexin II is dispensable for the complex formation (Figure 2.2A). Endogenous levels of annexin II and β-actin (as loading control) in MDCK and A-II KD cell lysates are shown in Figure 2.2B.

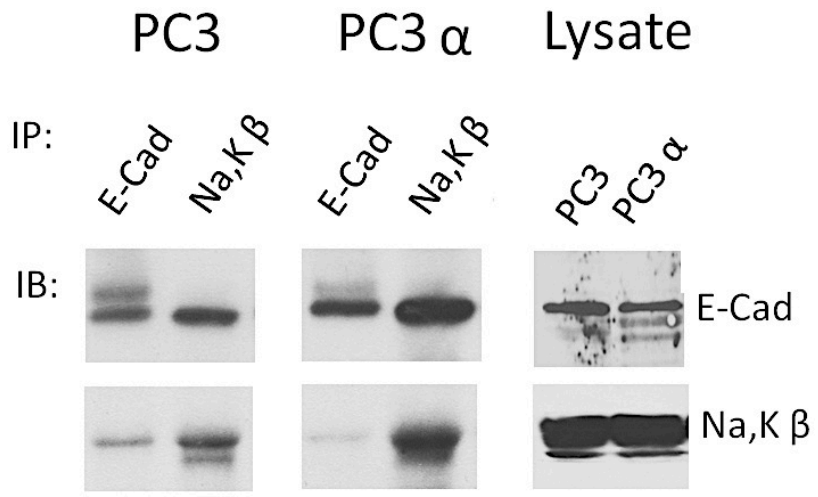


Figure 2.3. Interaction of Na,K-β with E-cad in the absence of α-catenin: Co-immunoprecipitation of PC3 (devoid of α-catenin) and PC3 α (α-catenin replenished) cells with Na,K-β and E-cadherin antibodies demonstrate that Na,K-β interacts with E-cadherin even in the absence of α-catenin. Panel 1 indicates the co-immunoprecipitation of E-cadherin and Na,K-β in PC3 cells and the panel 2 shows the reverse IP. The panel 3 is the input from the IP lysates that has been used as a loading control to indicate that even in the absence of α-catenin there is no significant difference in the expression of Na,K-β and E-cadherin in these cells.

Similarly, E-cadherin is tightly linked to the actin cytoskeleton via its interaction with α-catenin (Desai et al., 2013). To test whether α-catenin mediates the interaction between Na,K-β and E-cadherin, I utilized prostate cancer cell lines PC3 and PC3-α described in detail previously (Inge et al., 2008a). PC3 cells are devoid of α-catenin, while PC3-α cells express α-catenin. Na,K-β and E-cadherin co-

immunoprecipitated with each other regardless of the presence of α-catenin (Figure 2.3).

2.3.3 Na,K-β Cytoplasmic Tail is Essential for Its Complex Formation with E-cadherin

To determine the domain of Na,K-β involved in complex formation with E-cadherin, I utilized MSV-MDCK cells described previously by (Simard and Nabi, 1996). These cells have reduced levels of Na,K-β and E-cadherin protein. The interaction between Na,K-β and E-cadherin was readily detected by co-immunoprecipitation in lysates from MSV-β-E-cad cells (Figure 2.4A). However, E-cadherin was not detectable in Na,K-β immunoprecipitates in MSV-βΔCD-E-cad cells expressing a cytoplasmic domain deletion mutant of Na,K-β (Figure 2.4A), suggesting that the interaction between Na,K-β and E-cadherin requires the cytoplasmic domain of Na,K-β. Furthermore, β-catenin binding to Na,K-β was also abrogated in MSV-βΔCD-E-cad cells, raising the possibility that the interaction between Na,K-β and E-cadherin is mediated via β-catenin. To confirm the interaction of β-catenin with the cytoplasmic domain of Na,K-β, a GST pull-down assay using purified GST-tagged cytoplasmic domain of Na,K-β and MDCK cell lysates was used. I observed that β-catenin was pulled down by Na,K-β-CD-GST, but not by GST beads alone (Figure 2.4B), suggesting that the Na,K-β cytoplasmic domain associates with β-catenin. I tested whether β-catenin binding to Na,K-β required E-cadherin.

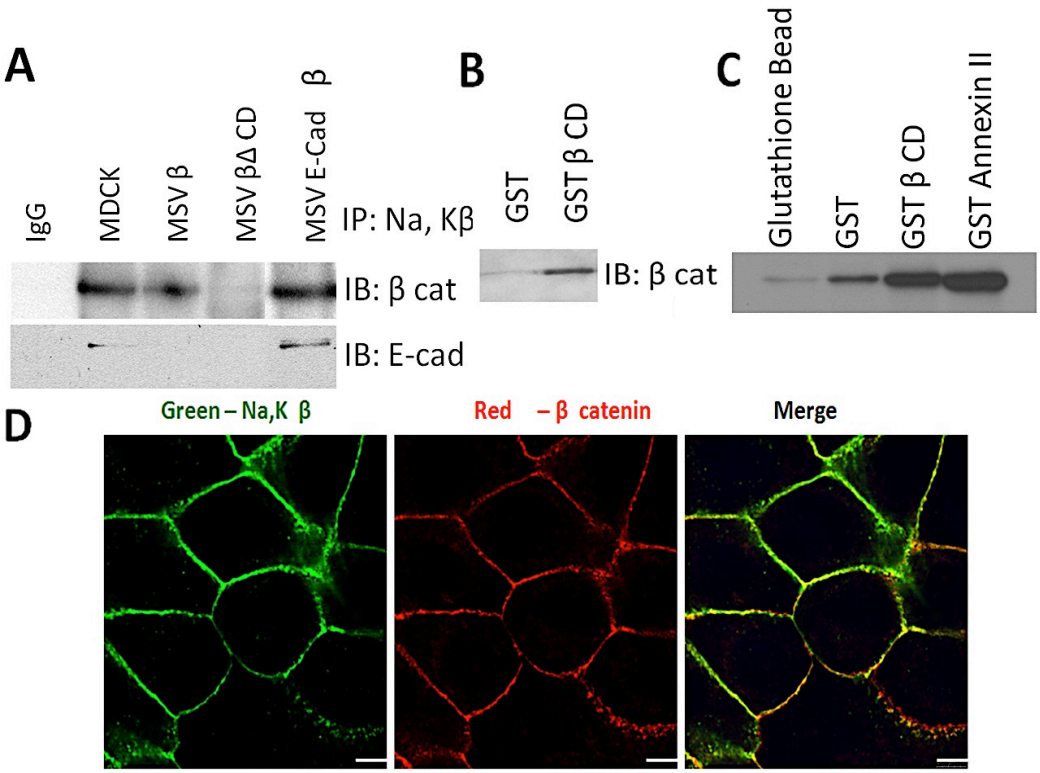


Figure 2.4. Interaction of Na,K-β with β-catenin requires the cytoplasmic tail of β subunit: (A) The co-immunoprecipitation of β-catenin and E-cadherin with Na,K-β antibody MDCK, MSV β (β replenished cells), MSV β ΔCD (replenished with β cytoplasmic deletion), and MSV E-cad β (both E-cad and β replenished cells) lysates. (B) Immunoprecipitation of GST and GST β CD with 1mg of MDCK lysate overnight and blotting for β-catenin; a band at desired molecular weight indicated that the cytoplasmic tail of Na,K-β associates with β-catenin. (C) *In vitro* binding assay of myc tagged β-catenin with GST tagged cytoplasmic tail of Na,K-β demonstrates that arm domain of β-catenin directly interacts with the cytoplasmic tail of Na,K-β. (D) This panel shows the co-localization of Na,K-β (green) and β-catenin (red) at the membrane in MDCK cells.

Na,K-β co-immunoprecipitated β-catenin not only in MDCK cells (presence of E-cadherin) but also in MSV-β cells (absence of E-cadherin) (Figure 2.4A). This data suggested that β-catenin binding to Na,K-β was independent of E-cadherin. This does

not eliminate a direct interaction between the cytoplasmic tail of Na,K-β and E-cadherin which was not tested in this study.

2.3.4 **Na,K-β Cytoplasmic Tail Directly Associates with β-catenin**

The twelve arm repeats of the armadillo domain of β-catenin has been shown by crystallographic structure analysis to form a super helix with extended, positively charged groove. Moreover, β-catenin utilizes this elongated binding surface to interact with negatively charged ligands like cadherins, the Axin/APC degradation complex, and the LEF/TCF transcription factors (Daugherty and Gottardi, 2007). Since armadillo domain of β-catenin has a number of interaction partners and might likely mediate the interaction between Na,K-β and β-catenin, I performed an *in vitro* binding assay where in vitro synthesized myc tagged armadillo domain of β-catenin and Na,K-β-CD-GST coupled to glutathione beads were incubated together. Glutathione beads alone and GST coupled glutathione beads were used as control. Annexin II-GST was used as a positive control, because it has been reported in another study that annexin II associates with β-catenin (Heyraud et al., 2008).

Following incubation, the beads were washed, resolved by SDS-PAGE and the presence of the radioactive β-catenin band was examined by fluorography. β-catenin was detected in GST tagged Na,K-β-CD and annexin II-GST glutathione beads suggesting that the arm domain of β-catenin directly associates with the cytoplasmic tail of Na,K-β (Figure 2.4C). Taken together, the interaction between Na,K-β and E-

cadherin occurs via β-catenin binding to the cytoplasmic domains. Immunostaining of Na,K-β and β-catenin indicated that these two proteins co-localize in the membrane (Figure 2.4D).

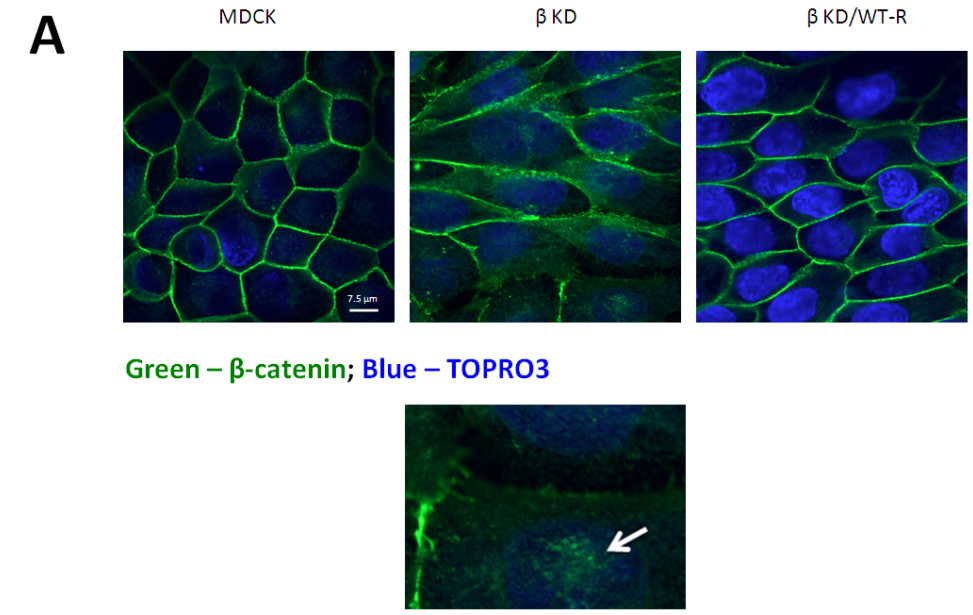
2.3.5 Nuclear Localization, Increased Transcriptional Activity and Reduced Ubiquitination of β-catenin in Na,K-β Knockdown Cells

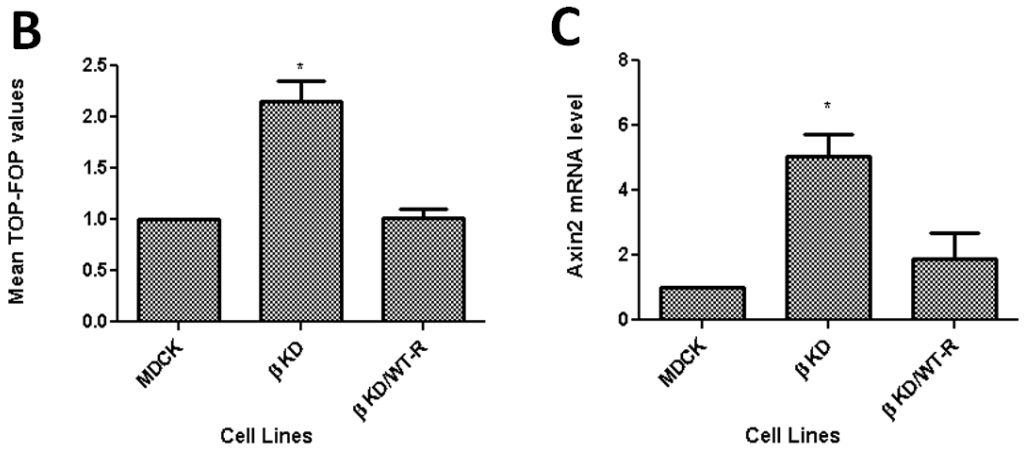
Immunofluroscence staining in Figure 2.4D indicates that Na,K-β and β-catenin are co-localized, to understand the impact of the loss of interaction between Na,K-β and β-catenin on the membrane localization of β-catenin, immuno-staining of β-catenin was performed in parental MDCK cells, MDCK cells with stable knockdown of Na,K-β (β-KD) and β-KD cells overexpressing a shRNA-resistant mutant of Na,K-β (β-KD/WT-R) as described in the materials and methods. Immuno-staining of β-catenin in the presence of TO-PRO3 (a nuclear stain) revealed the presence of β-catenin in the nucleus in β-KD cells. However, no such localization was observed in MDCK or β-KD/WT-R cells indicating that specific knockdown of Na,K-β mediates β-catenin localization into the nucleus (Figure 2.5A).

β-catenin is not only involved in cell adhesion as a binding partner of E-cadherin, but it also functions as a transcriptional activator (Nollet et al., 1996). In the presence of Wnt ligand/signals, β-catenin is phosphorylated at tyrosine residue 142, which stabilizes β-catenin and allows evasion from proteasomal degradation (Daugherty and Gottardi, 2007).

So to test whether this increase in nuclear localization of β-catenin in β-KD cells stimulates β-catenin transcriptional activity, I utilized the β-catenin/LEF/TCF reporter TOPflash/FOPflash assay. In this system, the luciferase activity produced by the binding of β-catenin to specific promoter (TOPflash) and a scrambled promoter (FOPflash) were assayed. Renilla luciferase was co-transfected and its luciferase activity was assayed to serve as a control for transfection efficiency. I observed that the luciferase activity produced by the activation of the TOPflash reporter after subtracting the FOPflash background luciferase activity and following normalization with renilla luciferase activity was 2-fold higher in β-KD cells as compared to MDCK and β-KD/WT-R cells (Figure 2.5B).

β-catenin that is translocated into the nucleus binds with LEF/TCF and acts as a transcription factor. I tested whether the increase in nuclear translocation of β-catenin and the activation of its transcriptional activity increases transcription of downstream targets. To address this, I tested the transcript levels of one of the downstream targets of β-catenin transcriptional activity, axin2, utilizing qRT-PCR (Figure 2.5C). The axin2 mRNA levels was 2-fold higher in β-KD cells corresponding to the increase in TOPflash luciferase activity.


A
MDCK
β KD
β KD/WT-R
7.5 μm
Green – β-catenin; Blue – TOPRO3


B
Mean TOP-FOP values
2.5
2.0
1.5
1.0
0.5
0.0
*
MDCK
β KD
β KD/WT-R
Cell Lines
C
Axin2 mRNA level
8
6
4
2
0
*
MDCK
β KD
β KD/WT-R
Cell Lines

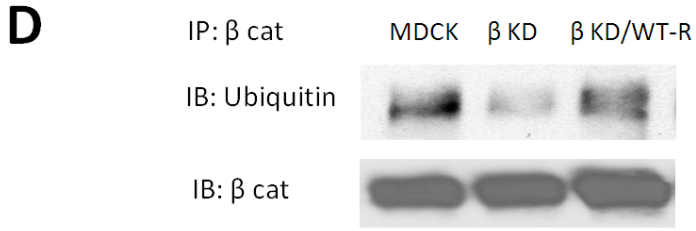

D
IP: β cat
MDCK
β KD
β KD/WT-R
IB: Ubiquitin
IB: β cat

Figure 2.5. Reduced proteosomal degradation and increased transcriptional activity of β-catenin in β-KD cells: (A) Immunofluroscence staining of β catenin in MDCK, β-KD and β-KD/R cells indicate that in β-KD cells, β catenin is reduced in the membrane and was localized in the nucleus more when compared to MDCK and β KD/WT-R cells. (B) Dual luciferase assay was utilized to determine the transcriptional activity of β catenin. The MDCK, β-KD and β-KD/WT R cells were transfected with TOP, FOP and Renilla plasmids; after 48h the luciferase substrate was added to the cell lysate and the luciferase activity was calculated. Based on those values the mean TOP-FOP values were generated ($p = 0.0279$). (C) qPCR of Axin2 mRNA level in MDCK, β-KD and β-KD/WT R cells showed that the downstream target of β catenin signaling is upregualted ($P=0.0269$). (D) Co-immunoprecipitation of β-catenin in MDCK, β-KD and β-KD/WT R cells showing the ubiquitination status of the protein. This blot shows that the ubiquitination of β catenin is reduced in the β-KD cells when compared to the MDCK or β-KD/WT R cells indicating that the loss of Na,K-β prevents β catenin from degradation and stabilizes the protein.

Unstable β-catenin in the cytoplasm is degraded by ubiquitination, so to determine the extent of β-catenin ubiquitination in MDCK, β-KD and β-KD/R cells, ubiquitin antibody was used to immunoprecipitate β-catenin. The β-catenin ubiquitination was 70% reduced in β-KD cells (Figure 2.5D), indicating that the absence of Na,K-β stabilizes β-catenin preventing it from degradation via the proteasomal degradation pathway.

2.3.6 Na,K-β is Essential For Membrane Expression of E-cadherin in Epithelial Cells

β-KD cells have 85% reduction in Na,K-β protein and transcript levels, whereas the expression of Na,K-β in β-KD/R is restored similar to MDCK levels (Figure 2.6 A).

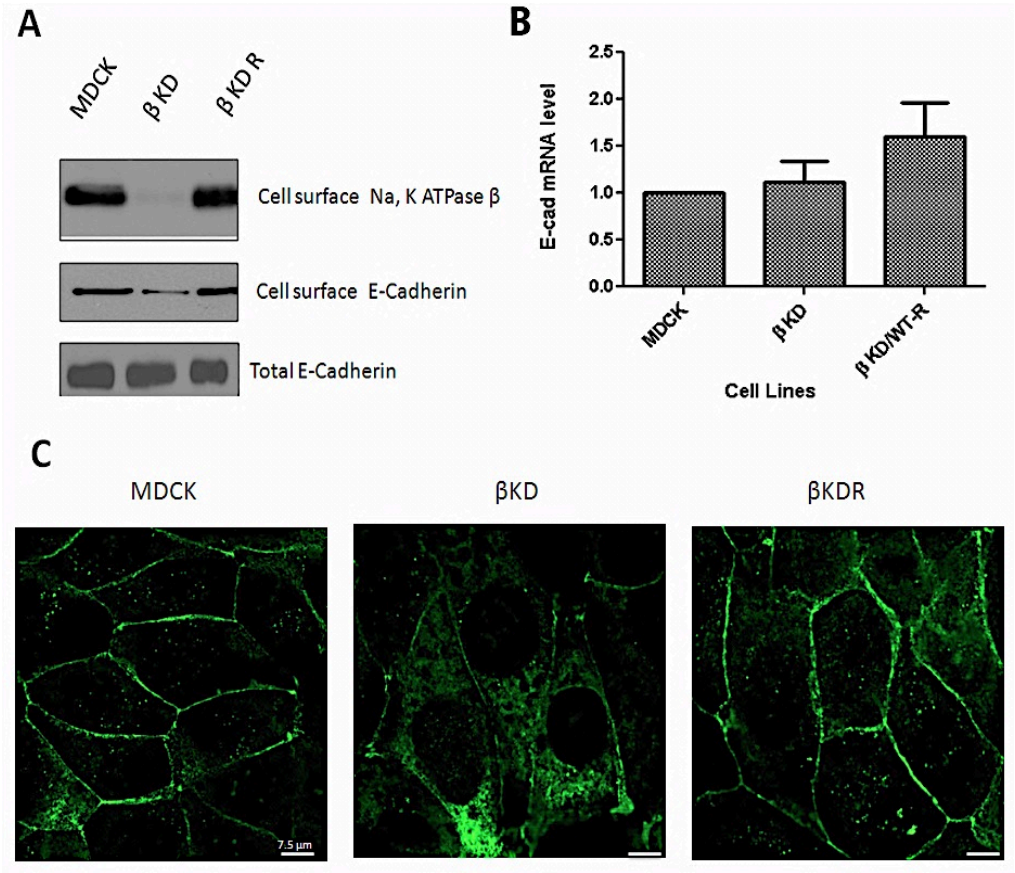


Figure 2.6. shRNA mediated knockdown of Na,K-β reduces surface expression of E-cadherin in MDCK: (A) Cell surface biotinylation of the MDCK, β-KD and β-KD/WT R cells showing the levels of surface Na,K-β and E-cadherin. The total E-cadherin indicates the amount of E-cadherin in the lysate. (B) The qRT-PCR analysis quantifies the Na,K-β and E-cadherin mRNA expression in the MDCK, β-KD and β-KD/WT R cells (p=0.011). The E-cadherin levels in these cells do not change in the presence or absence of Na,K-β. (C) The E-cadherin is localized in the cytoplasm in the β-KD whereas it is localized on the membrane in the MDCK and β-KD/WT R.

In order to understand the mechanism by which Na,K-β regulates the transcriptional activity of β-catenin, I first determined the levels of E-cadherin, which is a well-documented interacting partner of β-catenin. Also, several studies have

shown that dissociation of the adherens junctions by loss or internalization of E-cadherin can activate the non-canonical Wnt/β-catenin signaling pathway (Wang et al., 2010). I observed that the levels of E-cadherin in total cell lysates remained unchanged, however, it was very interesting to note that there was 50% reduction in the E-cadherin surface expression with the reduction in Na,K-β in cell surface biotinylation assay (Figure 2.6A). Furthermore, there was no significant difference in the mRNA levels of E-cadherin in MDCK and β-KD cells confirming that this reduced membrane expression of E-cadherin is a post-transcriptional modification (E-cadherin is not a target of β-catenin transcriptional activity, explaining the indifference in the mRNA level) (Figure 2.6B). An immunofluorescence staining for E-cadherin in MDCK, β-KD, and β-KD/WT-R also confirmed the partial intracellular localization of E-cadherin in β-KD cells compared to MDCK and β-KD/WT-R (Figure 2.6C).

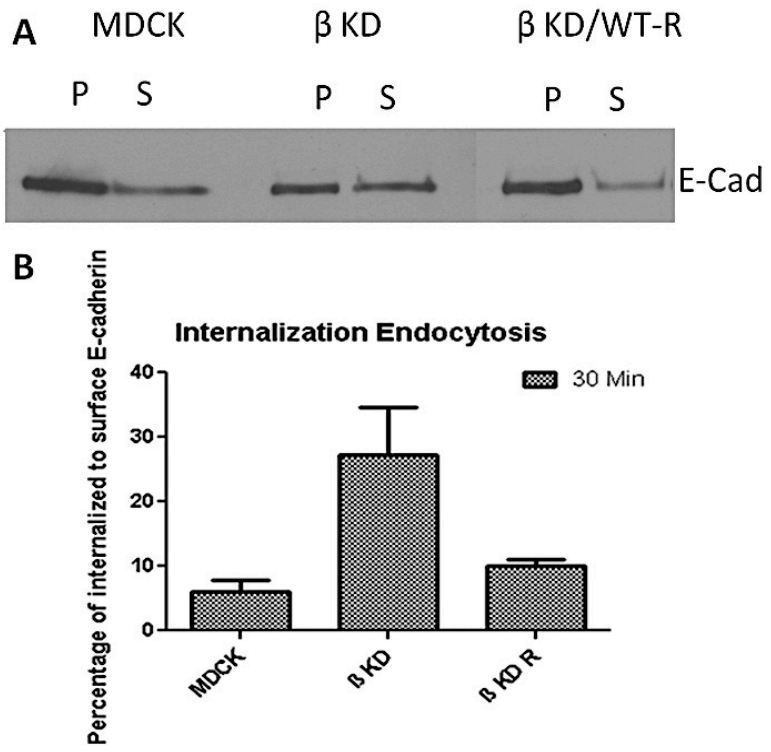


Figure 2.7. Reduced surface stability of E-cadherin in β-KD cells: (A) Detergent extraction assay of MDCK, β-KD and β-KD/WT R cells showing the levels of E-cadherin in the P-pellet and S-soluble fraction. In the β-KD cells, E-cadherin is not stably associated with actin cytoskeleton like MDCK cells, indicating an increase in the E-cadherin in the soluble fraction. (B) The Endocytosis biotinylation in MDCK, β-KD and β-KD/WT R for E-cadherin at 30 min and 60 min, when normalized to the total surface E-cadherin expression indicates an increase in the rate of internalization of E-cadherin in β-KD cells when compared with MDCK and β-KD/WT R cells.

2.3.7 Na,K-β Stabilizes the Membrane Expression of E-cadherin

To study the stability of E-cadherin in MDCK, β-KD and β-KD-R cells, detergent solubility assay was utilized. The soluble and pellet fractions following extraction with Triton X-100 were assayed in the presence of E-cadherin. In MDCK cells, E-cadherin was 2-fold more in the pellet fraction indicating a stable association

of the protein with the actin cytoskeleton. On the contrary, E-cadherin was almost same in the soluble fraction as in the pellet fraction in β-KD cells (50.98% vs 49.01%) (Figure 2.7A). In the β-KD-R cells, the conditions were similar to that of the MDCK cells, implying that the presence of Na,K-β stabilizes the membrane expression of E-cadherin (Figure 2.7A. To address the question whether this decrease in membrane stabilization increases the rate of internalization of E-cadherin, an endocytosis biotinylation was performed in MDCK, β-KD and β-KD-R cells. The cell surface E-cadherin was biotinylated and allowed to internalize for 30 min. At this point, the biotinylated E-cadherin retained on the cell surface was reduced and cells were lysed. The internalized E-cadherin remained biotinylated and was detected by immunoprecipitation by anti-E-cadherin antibody and blotting for streptavidin-HRP.

The level of biotinylated E-cadherin in a separate dish that was left unreduced at 4°C was considered 100%. The rate of internalization was calculated as percentage of surface expression that has been internalized in given time. The amount of E-cadherin internalized when compared to the total expression in β-KD cells is nearly 27.3%, which is 4.5-fold higher than the MDCK or β-KD/WT-R cells (Figure 2.7A,B). This indicates that the increase in the rate of internalization of E-cadherin in the β-KD cells can be attributed to the knockdown of Na,K-β. Taken together, the data shows that knockdown of Na,K-β increases β-catenin transcriptional activity by inducing internalization of E-cadherin.

2.4 **Discussion**

Our previous studies have demonstrated that the cell adhesion molecules Na,K-β and E-cadherin function in a synergistic manner to mediate epithelial polarization, formation of adherens and tight junctions, and suppression of motility and invasion (Rajasekaran et al., 2001b). Although these two proteins were found to co-localize to the adherens junctions (Vagin et al., 2006), the mechanism of their synergistic action was not identified. We provide evidence for the first time that Na,K-β and E-cadherin are associated in a complex along with β-catenin. Furthermore, this association requires the cytoplasmic domain of Na,K-β and is likely mediated by the direct interaction of Na,K-β with β-catenin via the armadillo domain similar to β-catenin-E-cadherin association. We also show that knockdown of Na,K-β de-stablilized E-cadherin at the cell surface and resulted in induction of β-catenin transcriptional activity.

2.4.1 **Na,K-β Assists in the Regulation of Cell Surface Expression of E-cadherin and Its Stable Association to the Actin Cytoskeleton**

Both Na,K-β and E-cadherin mediate homophilic cell adhesion by trans homo-dimerization of extracellular domains of same types of molecules on the surfaces of adjacent cells. This interaction is further strengthened by the cis-homo-dimerization between two molecules present on a single cell surface mediated by the trans-membrane domains (van Roy and Berx, 2008). The requirement of the Na,K-β

cytoplasmic domain for the interaction between Na,K-β and E-cadherin indicates that the extracellular and trans-membrane domains are minimally involved in mediating this association. Thus, our data supports the homophilic cell adhesion scheme and indicates that the synergism in adhesion junction formation by Na,K-β and E-cadherin is mediated by the linkage of their cytoplasmic domains by β-catenin and annexin II. E-cadherin is also known to partner with another family of cell adhesion molecules, i.e. nectins, to co-operatively organize the cell-cell adherens junctions. This partnership is mediated by interactions of these two proteins via their cytoplasmic-domain associated proteins (Tachibana et al., 2000).

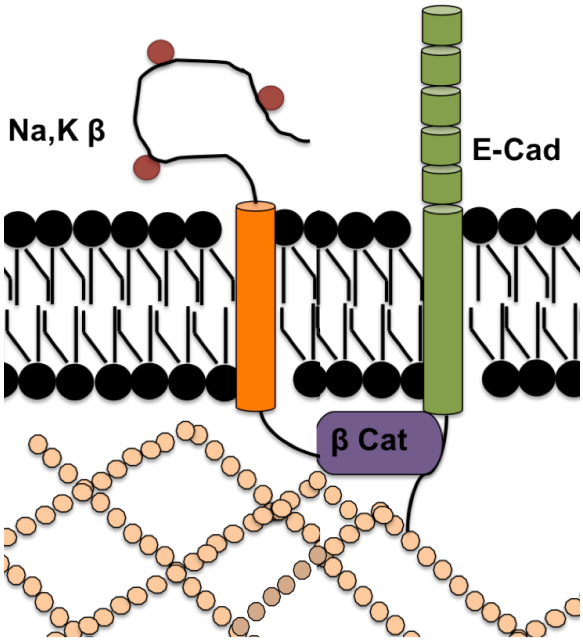


Figure 2.8. Proposed mechanism of interaction of Na,K-β and E-cadherin via β-catenin: The figure is a pictorial representation of the association of the cytoplasmic tail of Na,K-β with β-catenin that mediates the adhesional complex between Na,K-β and E-cadherin

E-cadherin is coupled to the actin cytoskeleton via β-catenin, which directly binds α-catenin that in turn associates with actin. The role of Na,K-β in strengthening the association of E-cadherin with the actin cytoskeleton was postulated

based on two studies: First, we showed earlier that expression of Na,K-β in MSV-MDCK cells reduced the solubility of E-cadherin in Triton-X 100 extractions (Rajasekaran et al., 2001b). Secondly, E-cadherin solubility was increased in the presence of a glycosylation-deficient mutant of Na,K-β with reduced cell adhesion (Vagin et al., 2008). Our observation that E-cadherin solubility is increased in β-KD cells and it could be rescued by re-expression of Na,K-β is consistent with these studies. However, the mechanism of Na,K-β mediated stabilization of E-cadherin was not known. It was proposed that this is likely via the ankyrin-spectrin cytoskeleton (Vagin et al., 2007), because both Na,K-ATPase and E-cadherin are known to be linked to the ankyrin-spectrin cytoskeleton (Devarajan et al., 1994; Kizhatil et al., 2007; Nelson and Veshnock, 1987). We showed earlier that Na,K-β is involved in the organization of the actin cytoskeleton via interactions with annexin II, which is an actin-binding protein (Barwe et al., 2005). Moreover, annexin II is involved in the formation of adherens junctions and their stable association with the actin cytoskeleton (Hansen et al., 2002; Heyraud et al., 2008; Yamada et al., 2005). Na,K-β and annexin II binds the armadillo domain of β-catenin and this domain also binds to E-cadherin suggesting that β-catenin and annexin II form a bridge that links the Na,K-β and E-cadherin together with the actin cytoskeleton for strong adhesion within the adherens junctions.

2.4.2 **Loss of Function of Na,K-β Activates β-catenin Transcriptional Activity**

β-catenin performs dual roles - as a member of the cell adhesion complex and as a transcriptional co-activator in the Wnt signaling pathway. The EMT process results in ligand-independent activation of the Wnt pathway leading to dissociation of the adherens junctions by internalization of E-cadherin that stabilizes β-catenin and aids in its nuclear translocation resulting in activation of β-catenin signaling pathway (Howard et al., 2011; Orsulic et al., 1999; Wang et al., 2010). In my study, I found that knockdown of Na,K-β resulted in nuclear localization of β-catenin, activation of transcriptional activity of β-catenin and upregulation of its downstream target axin2. Consistent with these observations, our previous study showed an increase in phospho AKT and phospho GSK3 levels associated by increased proliferation in β-KD cells (Barwe et al., 2012). Thus, it is possible that the internalization of E-cadherin in β-KD cells results in activation of Wnt pathway. However, since Na,K-β cytoplasmic domain directly binds β-catenin, it remains to be determined whether Na,K-β can directly activate Wnt signaling independent of E-cadherin localization.

2.4.3 **Conclusion**

The internalization of E-cadherin in cells with Na,K-β knockdown is likely due to the loss of stable association with the actin cytoskeleton. Other cytoskeletal proteins such as vinculin and Dia1 have been shown to regulate E-cadherin cell surface expression by a similar mechanism (Carramusa et al., 2007; Peng et al., 2010). However, to my

knowledge this is the first report showing the involvement of a cell adhesion molecule (Na,K-β) in regulating E-cadherin level on the cell surface. It has been reported earlier that knockdown of Na,K-β transforms epithelial cells into an EMT-like phenotype with a loss of contact inhibition of proliferation (Barwe et al., 2012). Furthermore, it has also been shown that during TGF-β induced EMT in kidney epithelial cells, the internalization of Na,K-β occurred prior to the disappearance of E-cadherin from the cell surface (Rajasekaran et al., 2010). Here I have shown that the surface expression of E-cadherin was reduced in β-KD cells. Taken together, these data suggest that Na,K-β is a chaperone and upstream regulator of E-cadherin mediated cell adhesion (Figure 2.8).

Chapter 3

NA,K-β INTERACTS WITH NCX1 EXPRESSION AND TARGETS NCX1 MEMBRANE LOCALIZATION

3.1 Introduction

Increase in the rate of cell migration is an important step in tumor cell invasion and metastasis. Ca^{2+} mediated signaling is crucial for directional migration, reorganization of actin cytoskeleton and alteration in cell-cell and cell-substrate attachments enabling cell movement (Monteith et al., 2012; Monteith et al., 2007; Parsons et al., 2010). Thus, changes in $[Ca^{2+}]_i$ concentrations can contribute to volatile cellular processes such as cell detachment, migration and invasion. Regulation of intracellular Ca^{2+} involves equilibrium between influx and efflux of the ions, which is in general governed by 4 groups of proteins, membrane-associated ion channels, ATPases, exchangers and binding proteins.

In renal epithelial cells, the sodium calcium exchanger 1 (NCX1), plasma membrane calcium ATPase (PMCA) and smooth endoplasmic reticulum calcium ATPase (SERCA) are the major regulators of $[Ca^{2+}]_i$ ion concentration, with NCX1 being the protein responsible for $2/3^{rd}$ of Ca^{2+} extrusion (Goldhaber and Philipson, 2013; Matsuda et al., 1997). NCX1 is also major Ca^{2+} extrusion mechanism in excitation contraction coupling in heart, skeletal and smooth muscle (Goldhaber and

Philipson, 2013; Matsuda et al., 1997). These tissues express splice variants of NCX1 but the other isoforms NCX2 and NCX3 are not expressed in these cells too (Li et al., 1994; Lytton, 2007; Nicoll et al., 1996). The splice variant NCX1.3 is expressed in the kidney and is a main Ca^{2+} regulator in distal convoluted tubules and connecting tubules of the nephron, principal regulatory sites of Ca^{2+} transport (Lee et al., 1994).

NCX1 consists of 10 α-helical transmembrane domains and large intermediate cytosolic loop 550 residues long. This large intracellular loop contains two Ca^{2+} binding domains (CBD) referred to as CBD1 and CBD2, which along with catenin like domain mediate the ion exchange activity (Hilge et al., 2006). Upon increase in intracellular Ca^{2+}, the Ca^{2+} ions bind to the Ca^{2+} binding sites though CBD1 has 7 fold higher Ca^{2+} binding affinity, increasing the electrostatic potential significantly, initiating NCX1 exchange function. In the absence of free intracellular Ca^{2+} the confirmation of CBD1 is open while CBD2 retains its structure.. NCX1 has been shown to function in forward mode i.e mediate the extrusion of one Ca^{2+} and the influx of 3 Na^{+} in one exchange movement and in reverse mode i.e the exchanger can cause an influx of the Ca^{2+} ions into the cells depending on intracellular Na^{+}, Ca^{2+}, pH, ATP and membrane potential (Reeves, 1998) (Hilge et al., 2006).

Many diseased states such as hypertension, cardiovascular disease, developmental disorders and cancer have been associated with abnormal Ca^{2+} levels (Rizzuto and Pozzan, 2003). These changes might not be the initiation step but the alteration in the intracellular Ca^{2+} concentration has been shown to play a role in proliferation, migration, invasion, and tumor progression (Monteith et al., 2012).

Consequentially, modulation of specific Ca^{2+} channels or pumps is associated with certain cancers. For example, transient receptor potential channel 8 (TRPM8) upregulation in prostate cancer (Tsavaler et al., 2001) and sarco-endoplasmic reticulum calcium transport ATPase 3 (SERCA3) downregulation in colon cancer (Brouland et al., 2005) has been reported.

Although, there is no direct evidence linking NCX1 to cancer, there are isolated studies indicating that NCX1 is involved in cell adhesion. For example, cell adhesion in prostate epithelial cells induced by stromal cell co-culture caused an upregulation of NCX1 transcript level among other genes involved in cell adhesion (Chambers et al., 2011). Furthermore, inhibition of NCX1 activity by KB-R7943 downregulated cell adhesion molecule ICAM1 and suppressed cell adhesion (Li et al., 2010).

NCX1 works in close partnership with Na,K-ATPase, by utilizing the sodium gradient generated by Na,K-ATPase to drive Ca^{2+} efflux. This functional association was validated by a lack of response to ouabain on Ca^{2+} transients in cardiomyocytes isolated from NCX1 knockout (KO) mice (6, 7). A similar insensitivity to ouabain was observed in Na,K-β KO hearts, which showed drastic reduction in NCX1 level and elevated intracellular Ca^{2+} (8), suggesting the possibility of a novel role for Na,K-β as a chaperone and regulator of NCX1.

Na,K-ATPase, also called the sodium pump, is a hetero-oligomeric protein that regulates the intracellular sodium ion homeostasis in mammalian cells. It is composed of two subunits, a catalytic α-subunit (Na,K-α) with 10 transmembrane spanning

helices that acts as the pharmacological receptor for cardiotonic steroids (CTS) such as digitalis, digoxin, ouabain, and a regulatory β-subunit (Na,K-β), important for translation, trafficking and membrane insertion of Na,K-α (Geering, 1990). The Na,K-α binds to Na^+, K^+ and ATP and aids the transport of Na^+ and K^+ ions against their electrochemical gradient with hydrolysis of one ATP molecule. Na,K-β is a type II transmembrane glycoprotein with a large extracellular domain and a small cytoplasmic tail. Na,K-α (α1- α4) and Na,K-β (β1- β3) isoforms are expressed tissue-specifically (Martin-Vasallo et al., 1989a; Martin-Vasallo et al., 1989b; Mobasheri et al., 2000; Woo et al., 2000). The subunits α1 and β1 are predominantly expressed in kidney and are referred to as Na,K-α and Na,K-β in this study. Na,K-ATPase has also been shown to function as a motility and tumor suppressor (Barwe et al., 2005; Inge et al., 2008b) and is involved in the maintenance of epithelial polarity (Rajasekaran et al., 2001b) and cell adhesion (Barwe et al., 2007; Kitamura et al., 2005). Moreover, previous study from the lab reported that knockdown of Na,K-β in MDCK cells leads to mesenchymal phenotype accompanied by increased in cell proliferation via activation of phosphatidyl inositol-3 kinase (PI3-kinase)/Akt and extracellular-signal-regulated kinase (ERK1/2) pathways (Barwe et al., 2012).

This study provides evidence that cells with Na,K-β knockdown (β-KD) show a reduction in the NCX1 protein expression and function leading to an increase in $[Ca^{2+}]_i$, with no significant increase in Na,K-β2 and Na,K-β3 subunit. In addition, these β-KD cells expressed an increase in the phosphorylation of downstream effectors of

PI3-K signaling pathway like Akt, p70S6K, GSK-3β and extracellular regulated kinase 1/2 (ERK). Previous studies in β-KD cells also showed an induction of mesenchymal phenotype associated with loss of polarity, contact inhibition and increased cell proliferation. The experimental results indicate that the enhanced cell migration and activation of ERK1/2 in β-KD cells was Ca^{2+}-dependent. I identified that this increase in ERK1/2 was due to calmodulin-associated activation of PI3-K (phosphatidyl inositol 3-kinase) protein kinase activity as determined by inhibition of calmodulin with antagonist W-13. Further these changes were reversed when NCX1 was overexpressed in β-KD cells, indicating that these changes observed in β-KD was a functional consequence of the loss of function.

Furthermore, functional inhibition of NCX1 with KB-R7943 also led to activation of ERK1/2, independent of Akt phosphorylation, and enhanced cell migration. This activation and enhanced migration were reiterated in porcine renal epithelial cells (LLCPK-1) and human renal epithelial cells (HREpiC) in addition to MDCK cells. Thus, the data reveal a novel role of forward mode NCX1 in regulation of cell migration in renal epithelial cells.

3.2 Materials and Methods

3.2.1 Cell Lines and Maintenance

DMEM supplemented with 10% fetal bovine serum, 2mM L-glutamine 25 U/mL penicillin, and 25 μg/mL streptomycin was used to grow MDCK and LLC-PK1

cells. Similarly MDCK-Na,K-β1 knockdown (β-KD) and rescue cells (β-KD/R) as described in (Barwe et al., 2012) were culture in supplemented DMEM. H9C2, rat cardiomyocytes were also culture in supplemented DMEM similar to MDCK cells.

β-KD cells were maintained in 10 μg/mL puromycin and β-KD/R cells were maintained in 10 μg/mL puromycin and 500 μg/mL neomycin. Full-length canine NCX1, a kind gift from Dr. Kenneth Philipson, UCLA (Ottolia et al., 2007), was transfected in β-KD cells using the calcium phosphate transfection method and NCX1 expressing β-KD cells were selected with 10 μg/mL puromycin and 100 μg/mL hygromycin post transfection. β-KD cells were also transfected with pWZL-neo delta-p85 and selected with 10 μg/mL puromycin and 500 μg/mL neomycin post transfection. The cells that survived the selection media were confirmed to express the transfected constructs and were utilized for the experiments.

Human Renal Epithelial Cells (HREpiC) purchased from ScienCell™ (Carlsbad, CA) were maintained as per manufacturer's recommendations and treated with inhibitors as indicated.

3.2.2 **Antibodies and Chemicals**

Monoclonal Na,K-β1 (M17-P5-F11) antibody from ThermoFisher Scientific Inc. (Waltham, MA) and monoclonal NCX1 antibody from Abcam® (Cambridge, MA) was used. Antibodies against phospho-p44/p42 MAPK (p-ERK1/2), total p44/p42 MAPK (T-ERK1/2), phospho Akt (Ser473), total Akt, phospho p70S6kinase (Thr389), phospho MLC2 and horseradish peroxidase conjugated secondary

antibodies against mouse and rabbit IgG were obtained from Cell Signaling Technology® (Lexington, KY). Monoclonal β-actin antibody was purchased from Sigma-Aldrich® (St. Louis, MO). KB-R7943, PD98059, U0126 and LY294002 were purchased from Tocris (Minneapolis, MN). MK-2206 and Y27632 were from Selleckchem (Houston, TX). W-13, KN-93, and ML-7 were from Cayman (Ann Harbor, MI). PMCA4 and SERCA2 were purchased from BIOSS antibodies (Woburn, MA).

3.2.3 **Immunoblot Analysis**

Cells were lysed in a buffer containing 20mM Tris (pH 7.4), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM sodium vanadate, 1mM phenylmethylsulfonyl fluoride (PMSF), 5mg/ml of antipapain, leupeptin, and pepstatin. For detection of NCX1 protein, cells were lysed in the lysis buffer supplemented with 2% sodium dodecyl sulfate (SDS). Based on protein estimation, 50 or 100 μg of cell lysates were boiled with loading buffer containing β-mercaptoethanol (β-ME) for 5 min, resolved by SDS-PAGE and transferred onto nitrocellulose membrane. The immunoblots were blocked in 5% non-fat dried milk in Tris buffered saline with 0.1% Tween 20 (TBST). Primary antibodies were diluted either in 5% bovine serum albumin (BSA) or non-fat dried milk in TBST and incubated overnight at 4°C. Secondary antibodies were diluted in 5% non-fat dried milk in TBST. Immunoblots were developed with chemiluminescent lightning system ECL or ECL Prime (GE Healthcare, Mickleton, NJ) according to the

manufacturer's recommendations. TINA 2.0 software (Open Source Image Analysis Environment) was utilized for immunoblot quantification and image analysis.

3.2.4 **Co-immunoprecipitation**

Cell and tissue lysates corresponding to 1 mg of protein were pre-cleared with Protein A Mag-agarose beads (GE Healthcare) and incubated overnight with control IgG, Na,K-β, or NCX1 antibodies pre-coupled to Mag beads for 4 h. The beads were washed and separated by SDS-PAGE, and the proteins bound to the beads were immunoblotted as described above. For resolution of Na,K-β protein (55 kDa) from heavy chain IgG band (50 kDa), immunoprecipitates were treated with peptide N-glycosidase F (New England Biolabs, Ipswitch, MA) as described previously (Barwe et al., 2005). The deglycosylated Na,K- β (32 kDa) appears as a single band. For co-immunoprecipitation, 1 mg of total protein was pre-cleared with Protein A Mag Agarose beads, GE® Biosciences (Pittsburg, PA), and incubated overnight with antibodies pre-coupled to the Mag beads with rabbit anti-mouse antibody for 4 h. The beads were washed, separated by SDS-PAGE and the proteins bound to the beads were subjected to immunoblotting as described above.

3.2.5 **GST Pull-down**

The cytoplasmic tail of Na,K-β, from amino acid 1-35 (β CD) was cloned in pGEX-5X vector (Invitrogen, Carlsbad, CA) as been described earlier (Barwe et al., 2005). GST and GST β-CD constructs were transformed in *Escherichia coli* (BL-21)

and induced with 0.25mM IPTG (isopropylthiogalactoside) for 4 h. The bacterial cells were centrifuged and reconstituted in the lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM MgCl2, 250 g/ml lysozyme, 1mMPMSF, and 10 g/ml each of antipain, leupeptin, and pepstatin) and then sonicated. The lysates were centrifuged at 13,000 rpm at 4°C for 15 min. The supernatant was rotated with glutathione-coupled agarose beads (Amersham Biosciences®) at 4°C for 1h. The glutathione beads coupled with protein were washed and the concentration of the bound fusion protein was assessed using coomassie-stained SDS gels. MDCK cells were lysed with lysis buffer used for immunoprecipitation and 1000µg of the lysate was incubated with 5 µg of GST or GST β-CD fusion proteins conjugated beads in separate tubes, overnight at 4°C. The beads were centrifuged, washed with the lysis buffer and immunoblotted for NCX1 protein as described above in immunoblotting.

3.2.6 **Cell Surface Biotinylation**

To quantitate the amount of protein expressed on cell surface, cell surface biotinylation was performed as described previously (Barwe et al., 2007). The cell surfaces of sub-confluent monolayers were labeled with 0.5µg/ml membrane-impermeable EZ-Link Sulfo-NHS-LC-Biotin, Pierce (Rockford, IL) in TEA buffer (150mM NaCl, 10mM triethanolamine pH 9, 1mM $CaCl_2$, 1mM $MgCl_2$) on ice and quenched with buffer containing (50mM NH_4Cl in PBS, 0.1mM $CaCl_2$, 1mM $MgCl_2$) and lysed in 1000 µl of lysis buffer (150mM NaCl, 20mM Tris pH 8, 5mM EDTA, 1%

Triton X-100, 0.1% BSA, 1mM PMSF, 5μg/ml antipain, leupeptin, and pepstatin). The lysate was rotated with 30 μl Ultralink streptavidin beads (Pierce) overnight at 4°C. The beads were washed with lysis buffer and the bound proteins were resolved on SDS-PAGE and immunoblotted for NCX1 and Na,K-β as described above.

3.2.7 **Endocytosis Biotinylation Assay**

One set of control plates were retained on ice with no treatment and three sets of culture plates were treated with NHS-acetate, 2 mg/ml in buffer A, pH 7.8 (Buffer A - 10mM NaCl, 250 mM NaOH, 10% FBS. buffer containing 150 mm NaCl, 50 mm HEPES, pH 7.5, 5 mm EDTA, 1% Triton X-100, 0.1% SDS, protease inhibitors) for 1 h at 4 °C, replacing NHS-acetate every 15 min. After the incubation periods two sets of culture plates were transferred back to 37 °C, one dish was incubated for 30 min, and the second dish was incubated for 60 min. All four culture plates were then biotinylated with 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL) at 4°C for 40 min, replacing biotin every 20 min. The free sulfhydryl groups of biotin were quenched in 50 mM ammonium chloride before lysis for 15 min. The cells were lyzed using buffer as mentioned above in the immunoblotting. The lysates were sonicated, centrifuged and biotinylated proteins were separated with magnetic streptavidin beads overnight at 4° C. The lysates were treated similar to the cell surface biotinylation experiment.

3.2.8 **qRT-PCR Analysis**

RNA was obtained by TRIzol® extraction method and cDNA was prepared prior to setting up the qPCR reaction by iScript™ cDNA Synthesis Kit (Bio-Rad) as per manufacturer's instructions. The cDNA was amplified via real-time polymerase chain reaction using the SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: (a) Na,K-β (forward: TTACCCTTACTACGGCAAGCTCCT, reverse: TTCAGTGTCCATGGTGAG GTTGGT), (b) NCX1 (forward: TTAGCCGTTGTGGCTCTCTT, reverse: TGTAG ACCATGGCCACAAAA), (C) GAPDH (forward: GCTGTCCAACCACA TCTCCTC, reverse: TGGGGCCGAAGATCCTGTT). Quantitative PCR was performed in a 384 well plate in a 7900HT Fast Real-Time PCR system (Applied Biosystems). Samples were assayed in triplicates and RNA levels were calculated by relative quantification (RQ) normalizing the samples to endogenous control GAPDH. The graphs represent the average of the relative mean expression level (RQ value) of three different experiments. The error bars represent the standard error of the RQ value (GraphPad Software).

3.2.9 **Immunofluorescence and Confocal Microscopy**

Cells were cultured on glass coverslips and fixed with either ice-cold methanol (−20°C) or 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature. The coverslips were incubated overnight at 4°C with primary antibodies diluted in 1% BSA in PBS with 100 μM calcium chloride and 1 mM magnesium

chloride, followed by Alexa-488™, Alexa-546™ or Alexa-633™ conjugated secondary antibodies and TO-PRO®-3-Iodide (Life Technologies, Grand Island, NY) for nuclear staining. The coverslips were mounted on glass slides with ProLong gold antifade reagent (Life Technologies). The images were captured using Leica TCS SP5 Confocal Microscope (Leica microsystems, Buffalo Grove IL).

3.2.10 **Wound-healing Migration Assay**

Cells (200,000) were seeded in a 6-well tissue culture plate 24 h before a wound was made by scratching across the bottom of the well with a pipet tip. Wounded cell monolayers were washed thrice with phosphate-buffered saline (PBS) to remove detached cells. Culture dishes were returned to the incubator for recovery of the wound in the presence or absence of indicated inhibitors in serum-free media for 16 h. The scratches were photographed using an inverted microscope under the same configuration at the start and end of the experiment. The images were used to calculate the distance migrated by the cell sheet. The rate at which the wound was closed was calculated using the formula, speed = distance/time.

3.2.11 **Calcium Measurements by Flow Cytometry**

Cells (5×10^5) were plated in a 6 well dish for 48 h. On the day of the experiment cells were versinized (detached without the use of trypsin) with 1X HBSS + 0.5 M EDTA at 37°C for 10 min, centrifuged at 3000 rpm for 3 min, and resuspended in complete medium. 1×10^5 cells were resuspended in Tyrodes buffer

without Ca^{2+} containing 0.5 μM Fluo4-AM in pluronic acid (both from Life Technologies) and incubated at 37°C for 20 min. Tyrodes buffer containing Ca^{2+} was added to the cell suspension to make the final Ca^{2+} concentration 0.5 mM before data acquisition using flow cytometer, BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). The fluorescence intensity was continuously monitored for 8-10 min. 100 μM Ouabain was added to the cells 2 min after the start of the experiment and changes in $[Ca^{2+}]_i$ were calculated as described previously (Khan et al., 2014).

3.2.12 **Calcium Imaging**

Cells (10,000) were plated on glass bottom dishes (MatTek Corp., Ashland, MA) and cultured until confluent. Cell cultures were then incubated with fluorescent Ca^{2+} indicator, FURA-2, AM (Life Technologies) at a concentration of 10 μM in a Pluronic acid/DMSO mixture in a 37ºC incubator for 30 min protected from light. The MatTek plates were washed twice with Hank's buffer saline solution (HBSS) containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM glucose, 10 mM N-(2-hydroxyethyl)piperazine-NP-(2-ethanesulfonic acid), pH 7.4. Cells were allowed to recover after loading in HBSS for 10 min before imaging. Xenon lamp equipped with quartz collector lenses was used to excite the cells and the cells were imaged with Nikon inverted microscope (Intracellular Imaging, Cincinnati, OH). The fluorescence intensity was measured continuously for 5 min to obtain baseline $[Ca^{2+}]_i$ values. The ratio of the fluorescence intensity emitted at 510 nm after excitation at 340 nm over excitation at 380 nm was used to calculate fold change in $[Ca^{2+}]_i$ concentrations as

described previously (Li et al., 1997). An average ratio of 30 cells was collected per trial experiment to calculate the mean $[Ca^{2+}]_i$ levels.

Alternatively, for quantitation of comparative intracellular response, cells were loaded with 10 μM Fluo-4, AM in a Pluronic acid/DMSO mixture for 20 min at 37 °C. The plates were then washed twice with HBSS solution with or without 2 mM $CaCl_2$. After recovery for 15 min, the fluorescence intensity corresponding to cytosolic Ca^{2+} was continuously measured by LSM 710 confocal microscope system (Zeiss, Thornwood, NY). The amplitude of the response was calculated by subtracting basal fluorescence obtained during initial 2 minutes from the maximum intensity of fluorescence after adding the 10 μM KB-R7943. Average of 15 cells was collected per field of view per trial experiment to calculate the mean response to KB-R7943.

3.2.13 **Statistical Analysis**

In order to determine the protein expression in the other cell lines when compared to the control, a densitometric analysis was carried out using TINA2.0 software. The fold changes were normalized to the loading control before relative comparison. The average $[Ca^{2+}]_i$ response to KB-R7943 was expressed as a percentage increase in intensity over baseline intensity levels. Paired T-Test was used to evaluate the differences between average of two groups using data from at least three independent experiments and P of < 0.05 was considered statistically significant.

3.3 Results

3.3.1 β-KD Cells Express Reduced Level of NCX1 Protein

MDCK cells with stable knockdown of Na,K-β using RNA-interference technique (β-KD) and with specific rescue of Na,K-β (β-KD/R) generated by introducing silent mutations within the shRNA recognition site in the Na,K-β cDNA have been described previously (Barwe et al., 2012). Immunoblot analysis confirmed 80% reduction in Na,K-β protein level in β-KD cells. These cells also showed 42% reduction in NCX1 protein level compared to MDCK cells when normalized to β-actin used as a loading control (Figure 3.1A).

This reduction in NCX1 protein in β-KD cells was consistent with the reduction of NCX1 in β-KO hearts as reported previously (Barwe et al., 2009). Changes in the protein levels of other Ca^{2+} transport proteins such as plasma membrane Ca^{2+}-ATPase (PMCA) and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) were not observed in β-KO hearts (Barwe et al., 2009).

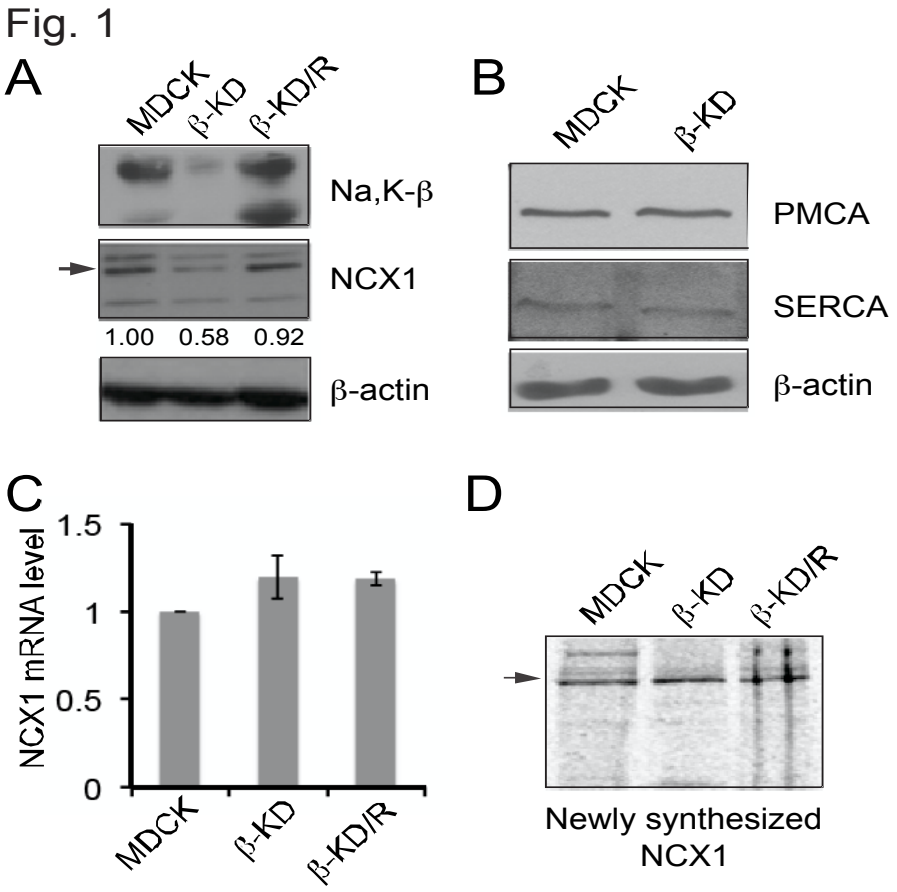


Figure 3.1. Knockdown of Na,K-β reduces NCX1 expression in MDCK cells: (A) Immunoblots showing Na,K-β, NCX1 and β-actin levels in the indicated cell lines. The arrow indicates 120 kDa NCX1 full length protein. The bands at 160 kDa and 70 kDa represent non-reduced exchanger and proteolytic fragment of NCX1 respectively. Quantification of NCX1 levels from three independent experiments expressed as a percentage of those in MDCK cells and normalized to β-actin loading control are indicated in the adjacent graph. The reduction in NCX1 protein level in β-KD cells is statistically significant (*P<0.05). (B) Immunoblots showing PMCA4, SERCA2 and β-actin levels in the indicated cell lines. (C) Graph showing NCX1 mRNA level in MDCK, β-KD and β-KD/R cells quantitated by qRT-PCR. Mean values from three independent experiments are plotted. Error bars denote s.e. of the Mean. (P = 0.247). (D) Cells were metabolically labeled with S^{35} methionine. Cell lysates were immunoprecipitated using anti-NCX1 antibody and resolved by SDS-PAGE. The newly synthesized NCX1 was detected by fluorography.

Similarly, PMCA and SERCA protein levels in β-KD cells were comparable to parental MDCK cells (Figure 3.1B), indicating that Na,K-β specifically regulates NCX1 expression. To determine whether this reduction in NCX1 expression in β-KD cells is posttranscriptional, the mRNA level of NCX1 was quantified. Compared to MDCK, β-KD and β-KD/R did not show a significant change in the transcript expression (Figure 3.1C). Further to identify if the reduction in the total protein level in β-KD is due to the reduced rate of synthesis of NCX1, i.e to determine if the reduction is due to a translational modification, S^{35} metabolic labeling was performed on these cells. The immunoprecipitation of S^{35} labeled and newly synthesized protein was comparable in MDCK, β-KD and β-KD/R cells (Figure 3.1D). This result suggests that the observed reduction in the NCX1 protein level is due to post-translational degradation.

3.3.2 **Cell Surface Expression of NCX1 is Diminished in β-KD Cells**

Since NCX1 localization to the cell surface is important for its exchanger activity, cell surface biotinylation assay was carried out to determine the level of this protein on the plasma membrane. As expected, the cell surface level of Na,K-β was reduced by 85% in β-KD cells. Unexpectedly, the NCX1 level on the cell membrane was almost diminished (by 89%), though the NCX1 total protein level was reduced only by 42% (Figure 3.2A).

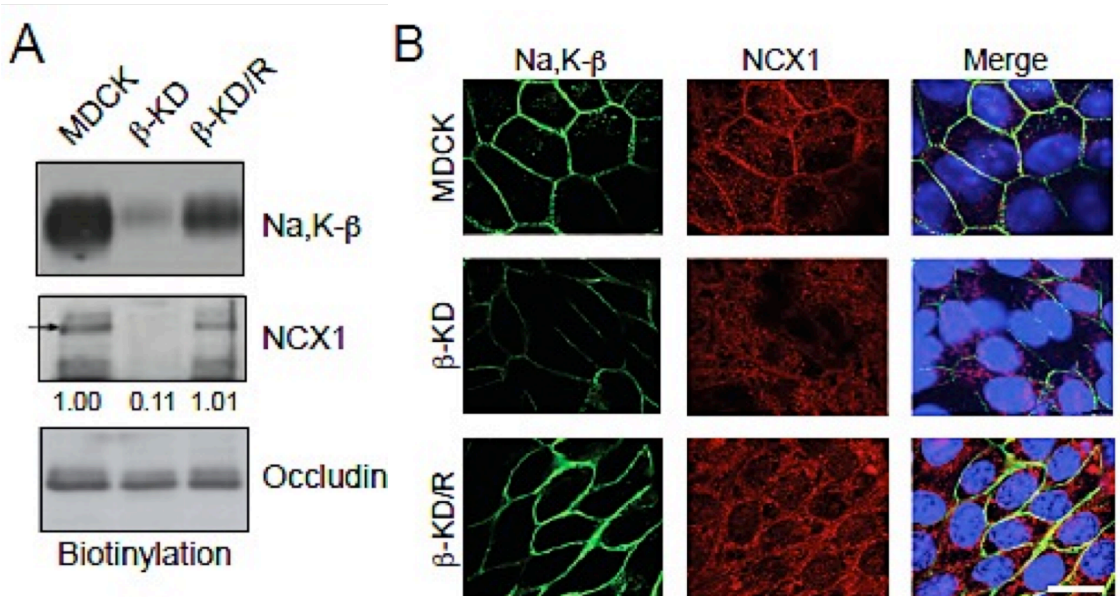


Figure 3.2. Na,K-β associates with NCX1 and its knockdown reduces NCX1 cell surface localization: (A) Cell surface proteins were pulled down with streptavidin beads and immunoblotted for indicated proteins. Immunoblots show cell surface levels of Na,K-β, NCX1 and occludin in MDCK, β-KD and β-KD/R cells. NCX1 cell surface levels expressed as a fold change with respect to MDCK cells are shown below the blot. The reduction in NCX1 cell surface levels in β-KD cells is statistically significant (*P = 0.003). (B) Representative images showing immunofluorescence staining of Na,K-β (green) and NCX1 (red) in MDCK, β-KD and β-KD/R cells. The TOPRO-3 stained nuclei are shown in blue. Scale bar = 25 mm.

Immunofluorescence analysis confirmed that NCX1 staining was diminished in β-KD cells. Moreover, while NCX1 was localized to the membrane in MDCK and β-KD/R cells, such localization was not prominent in β-KD cells (Figure 3.2B). β-KD/R cells with renewed Na,K-β expression had elevated NCX1 protein, highlighting the specificity of Na,K-β in the regulation of NCX1. Na,K-β associates with NCX1 in

MDCK cells Immunofluorescence analysis also showed that Na,K-β and NCX1 co-localize on the cell surface in MDCK and β-KD/R cells. To test the possibility that Na,K-β interacts with NCX1 and enhances its membrane stability, a co-immunoprecipitation analysis was performed. The immunoprecipitation of NCX1 by Na,K-β antibody indicates that Na,K-β associates with NCX1.

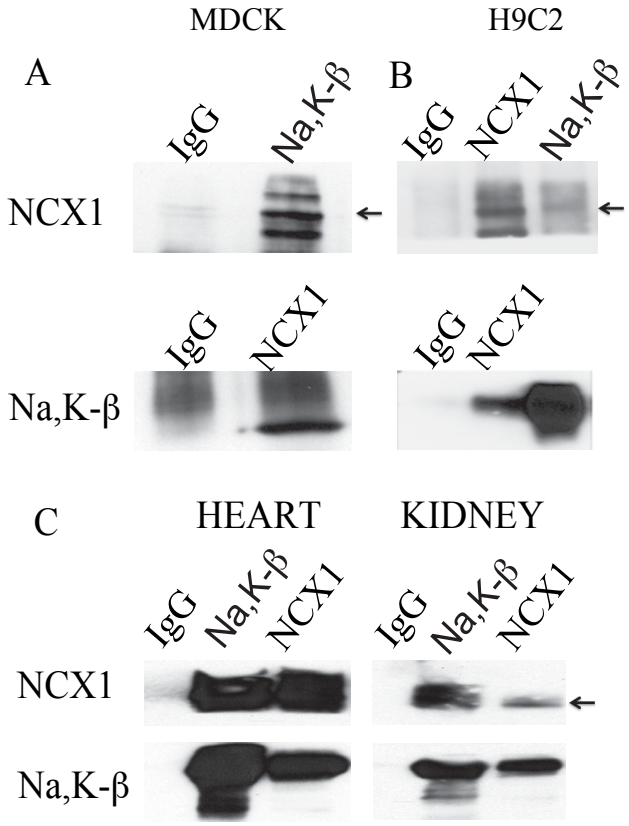


Figure 3.3. Association of Na,K-β with NCX1 in MDCK, H9C2 cells and tissue lysates from heart and kidney: A) Immunoblots demonstrating the association of NCX1 and Na,K-β by co-immunoprecipitation analysis in MDCK cells. Representative blots from three independent experiments are shown. B) Immunoblots representing association of NCX1 with Na,K-β and the reverse immunoprecipitation in H9C2, cardiomyocytes. C) Co-immunoprecipitation of NCX1 and Na,K-β in heart and kidney tissue lysates from mice.

This was further validated by reverse immunoprecipitation with NCX1 antibody and immunoblotting for Na,K-β (Figure 3..3A). Further the interactions of these two proteins were tested in different cell types such as H9C2, heart and kidney tissue (Figure 3.3B,C).

3.3.3 β-KD Cells Have Reduced NCX1 Activity and High Baseline Level of $[Ca^{2+}]_i$

NCX1 activity is required for ouabain-induced increase in $[Ca^{2+}]_i$ (Blaustein, 1993). Therefore, the effect of ouabain on $[Ca^{2+}]_i$ was tested to determine whether NCX1 function is altered in β-KD cells. Treatment of MDCK and β-KD/R cells with 100 μM ouabain elicited an immediate increase in $[Ca^{2+}]_i$ shown by the spike in fluorescence intensity above the threshold, i.e the percentage of response to ouabain treatment above the baseline Ca^{2+} level (Figure 3.4A). However, the percentage of response to ouabain treatment was 50% lower in β-KD cells compared to MDCK cells, indicating that β-KD cells have reduced NCX1 activity, consistent with reduced NCX1 protein. NCX1 is the major Ca^{2+} extrusion mechanism in kidney. To test the possibility that the reduced NCX1 in β-KD cells affects $[Ca^{2+}]_i$ concentration, the baseline intracellular free Ca^{2+} levels were measured by Fura-2AM ratiometric imaging.

The baseline $[Ca^{2+}]_i$ was 2.1-fold higher in β-KD cells compared to MDCK cells. The restoration of Na,K-β in β-KD/R cells reverted the baseline Ca^{2+} level similar to MDCK cells. Thus, the baseline $[Ca^{2+}]_i$ levels were inversely proportional to

NCX1 protein levels (Figure 3.4B). β-KD cells migrate faster and require $[Ca^{2+}]_i$ for increased migration rate.

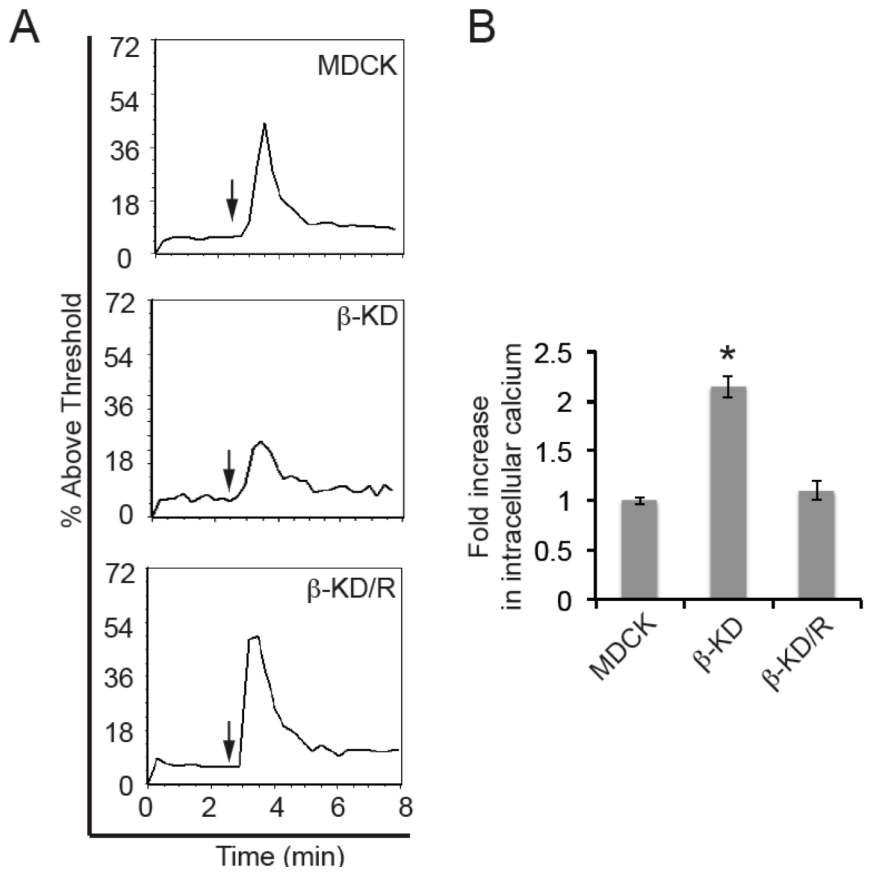


Figure 3.4: β-KD cells are ouabain insensitive and have elevated baseline $[Ca^{2+}]_i$ due to reduced NCX1 levels: (A) The plots show flow cytometric analysis of $[Ca^{2+}]_i$ in MDCK, β-KD and β-KD/R cells. The data is represented as percentage of fluorescence intensity above the threshold following the addition of 100 μM ouabain (arrow). Representative plots from three independent experiments in triplicates are shown. (B) The graph shows fold change in baseline $[Ca^{2+}]_i$ concentrations compared to MDCK cells. Average values from three independent experiments are plotted. Error bars denote s.e. of the mean. Asterisk indicates statistical significance (*$P < 0.0001$).

Because enhanced $[Ca^{2+}]_i$ can increase cell migration and Na,K-β is a motility suppressor (Barwe et al., 2005; Rajasekaran et al., 2001b), the rate of cell migration in β-KD cells was quantified by two different techniques. In a scratch wound healing assay, β-KD cells (13.6 ± 1.5 μm/h) showed 1.8-fold higher migration rate than MDCK (8.2 ± 0.5 μm/h) and β-KD/R (7.8 ± 0.7 μm/h) cells (Figure 3.5A,B). Wound-healing assay was performed in the presence of Ca^{2+} chelator, BAPTA-AM, to determine if the enhanced migration rate in β-KD cells was Ca^{2+} dependent.

Treatment of β-KD cells with BAPTA-AM reduced migration rate (5.6 ± 0.3 μm/h) (Figure 3.5A,B) indicating that migration is Ca^{2+} dependent in β-KD cells. In ECIS (electrical cell-substrate impedance sensing) wound-healing assay determine the resistance of cells grown on electrodes is continuously monitored. A wound is created by the application of high current to the cell-covered electrode resulting in cell death and causing subsequent drop in the resistance. The rate of migration is calculated by determining how quickly the resistance reverts to the levels of the cell-covered electrode due to the migration of cells on to the electrode (Keese et al., 2004). β-KD cells showed 2.4-fold higher migration rate when compared to parental MDCK cells (Figure 3.5C). The differences in fold increase in the rate of migration could be due to the variances in the sensitivity of the equipment employed for migration analysis and the changes in local cell density as described previously (Treloar and Simpson, 2013).

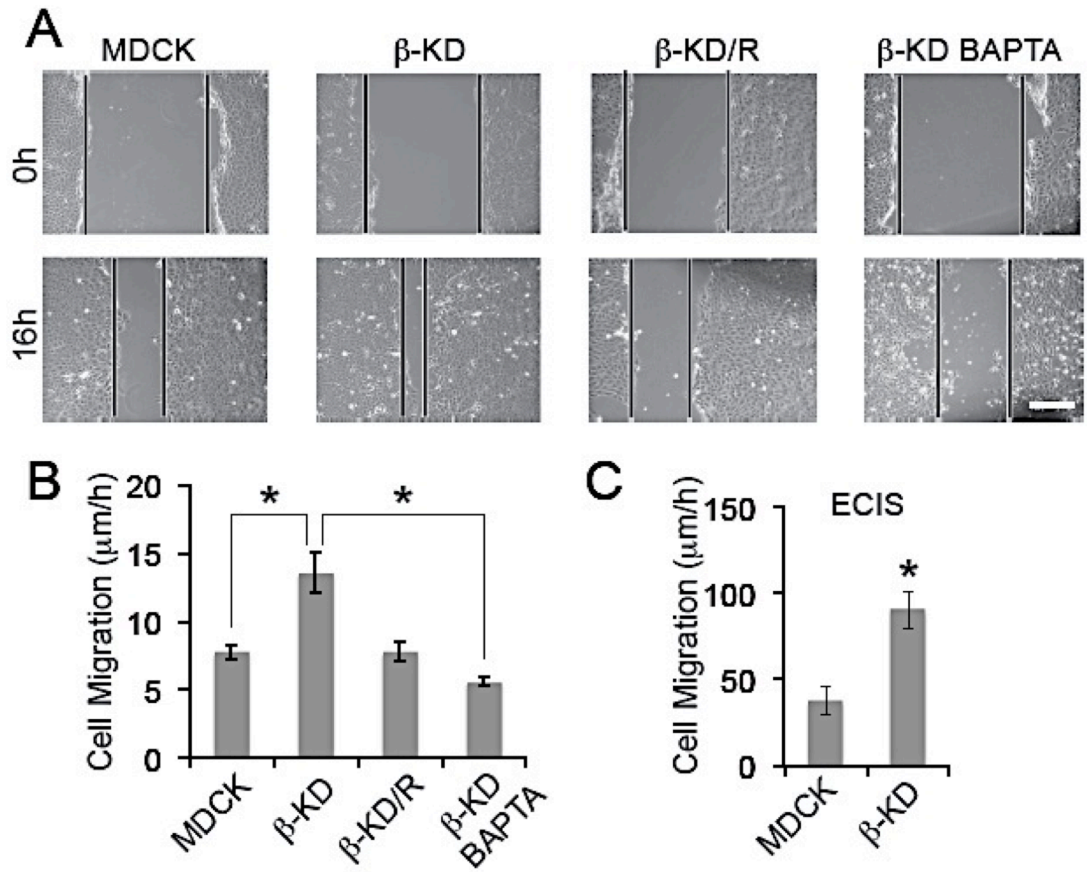


Figure 3.5. The role of Ca^{2+} in inducing cell migration in β-KD cells: (A) Representative images of the wound at 0 h and after 16 h in wound healing assay. Similar images were used to calculate the distance of migration over 16 h. Scale bar = 100 μm. (B) The graph respresents the average rate of migration calculated from three independent experiments in triplicates. Error bars denote s.e. of the mean and the asterisks indicate statistical significance (*P < 0.05). (C) The graph respresents the rate of migration obtained by ECIS wound healing assay from three independent experiments. Error bars denote s.e. of the mean and the asterisks indicate statistical significance (*P < 0.05).

3.3.4 **Cell Migration in β-KD Cells is Dependent on ERK1/2**

Previously it was demonstrated that β-KD cells have high ERK1/2 and Akt activation mediated by phosphoinositide-3 kinase (PI3-K) (Barwe et al., 2012). These signaling pathways are known to play significant roles in inducing cell migration (Huang et al., 2004; Qian et al., 2004; Zhou et al., 2003). Therefore, in order to determine which of these signaling molecules are regulated by $[Ca^{2+}]_i$, β-KD cells were treated with BAPTA-AM and the phosphorylation status of ERK1/2 and Akt was determined by immunoblot analysis.

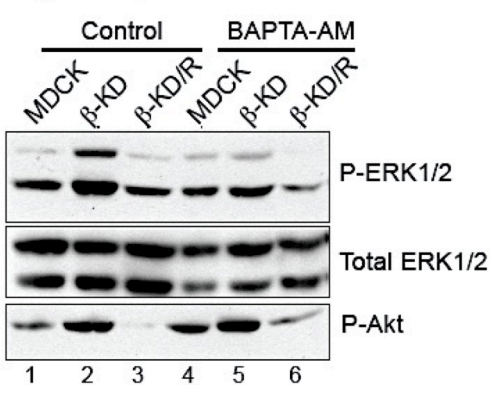


Figure 3.6. Ca^{2+} dependent phosphorylation of ERK in β-KD cells:Immunoblots showing the phosphorylation status of ERK1/2, Akt (Ser 473), and total ERK1/2 (loading control) in indicated cell lines in control conditions and treated with 10 μM BAPTA-AM. Representative blots from four independent experiments are shown.

BAPTA-AM treatment of β-KD cells reduced phosphorylated ERK1/2 to levels similar to MDCK cells, but the Akt phosphorylation remained high (Figure 3.6), indicating that ERK1/2 activation was Ca^{2+}-dependent and was likely involved in the regulation of cell migration. In order to determine the role of ERK1/2 and Akt activation in cell migration, β-KD cells were treated with specific inhibitors of these signaling pathways (PI3-K inhibitor – LY294002, MEK inhibitor – UO126, PD98059, Akt inhibitor – MK2206).

Treatment with inhibitors of MEK1/2 (upstream regulator of ERK1/2), PD98059 and UO126, reduced the migration rate of β-KD cells from 9.5 ± 0.9 μm/h to 4.7 ± 0.7 μm/h and 5.7 ± 1.1 μm/h respectively, indicating a significant role for ERK1/2 in migration (Figure 3.7A). As it was shown previously that ERK1/2 phosphorylation in β-KD cells is PI3-K-dependent at least in part (Barwe et al., 2012), I tested the role of PI3-K in β-KD cell migration. PI3-K inhibition by LY294002 reduced phosphorylated ERK1/2 (47.9 ± 1.3% of control) and also reduced the rate of migration (4.5 ± 1.5 μm/h) indicating that PI3-K is involved in inducing cell migration via its effect on ERK1/2.

Because Akt is a downstream target of PI3-K and is activated in β-KD cells, I tested whether Akt was also involved in the regulation of cell migration. Treatment with MK2206, a specific Akt inhibitor (10.9 ± 1.2 μm/h), did not alter the migration rate of β-KD cells (9.5 ± 0.9 μm/h) ($P = 0.355$) indicating that migration in β-KD cells is Akt-independent. Immunoblot analysis of inhibitor-treated cells confirmed the reduction in phosphorylated ERK1/2 by PD98059, UO126 and LY294002 (Figure. 7B). MK2206 did not change phosphorylated ERK1/2 levels significantly (83.9 ± 1.9%). However, phosphorylation of Akt and p70S6 kinase, a downstream target of Akt, was reduced following LY294002 and MK2206 treatments (Figure 3.7B).

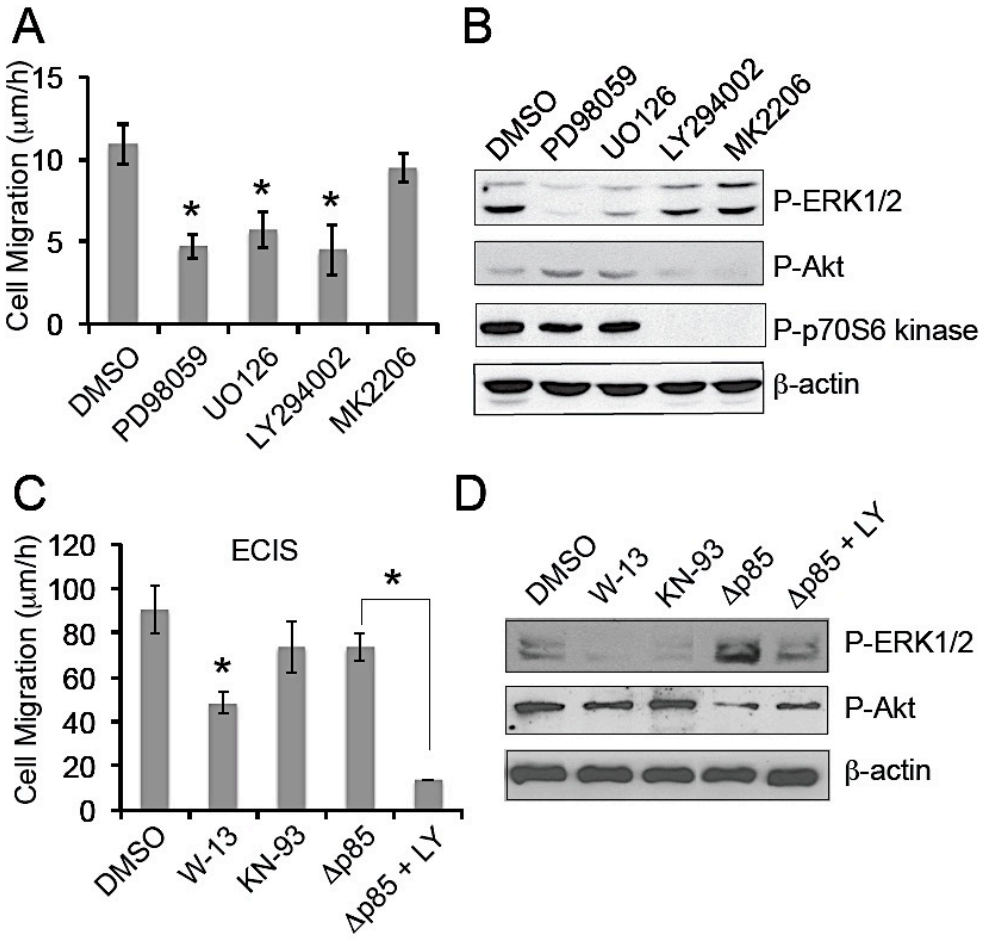


Figure 3.7. Increased migration in β-KD cells is dependent on phosphorylation of ERK1/2: (A) The graph shows the average rate of migration of β-KD cells following treatment with different inhibitors from three independent experiments performed in triplicates. Error bars denote s.e. of the mean. Treatment with all inhibitors except MK2206 attained statistical significance (*P < 0.05). (B) The immunoblots show the levels of phospho-ERK1/2, phospho-Akt, phospho p70S6 Kinase and β-actin following inhibitor treatment in β-KD cells. Representative blots from three independent experiments are shown. (C) The graph shows the average rate of migration by ECIS wound healing assay in β-KD and β-KD/ΔP85 cells after treatment with different inhibitors from three independent experiments. Error bars denote s.e. of the mean. β-KD cells treated with W-13 and β-KD/ΔP85 treated with LY294002 attained statistical significance (*P < 0.05). (D) The immunoblots show the levels of phospho-ERK1/2, phospho-Akt and β-actin following inhibitor treatments. Representative blots from three independent experiments are shown.

3.3.5 **Calmodulin-Activated PI3-Kinase is Involved in ERK1/2 Activation and Cell Migration in β-KD Cells**

To confirm the involvement of PI3-K in cell migration, β-KD cells were transfected with a dominant negative mutant of the PI3-K regulatory subunit p85, lacking the binding site for the catalytic subunit p110 (β-KD/Δp85) (Zhao et al., 2003). Surprisingly, the Δp85 cells migrated at rates similar to β-KD cells (Figure 3.7C). Although these cells had reduced Akt activation consistent with suppression of PI3-K signaling, there was no reduction in ERK1/2 phosphorylation (Figure 3.7D). PI3-K is a dual specificity kinase (Carpenter et al., 1993; Dhand et al., 1994), which can directly phosphorylate MEK resulting in ERK1/2 activation apart from its well-known lipid kinase activity (Bondeva et al., 1998), LY294002 inhibits both activities of PI3-K (Rondinone et al., 2000). β-KD/Δp85 cells treated with LY294002 showed reduced migration and ERK1/2 phosphorylation (Figure 3.7C,D) indicating that the protein kinase activity of PI3-K rather than the lipid kinase activity is involved in ERK1/2 phosphorylation and migration in β-KD cells. Enhanced $[Ca^{2+}]_i$ can activate PI3-kinase via Ca^{2+} sensor calmodulin (CaM) or calmodulin dependent kinase II (CaMKII), which are key players in Ca^{2+}-mediated cell migration (Easley et al., 2008; Joyal et al., 1997; Rotfeld et al., 2014).

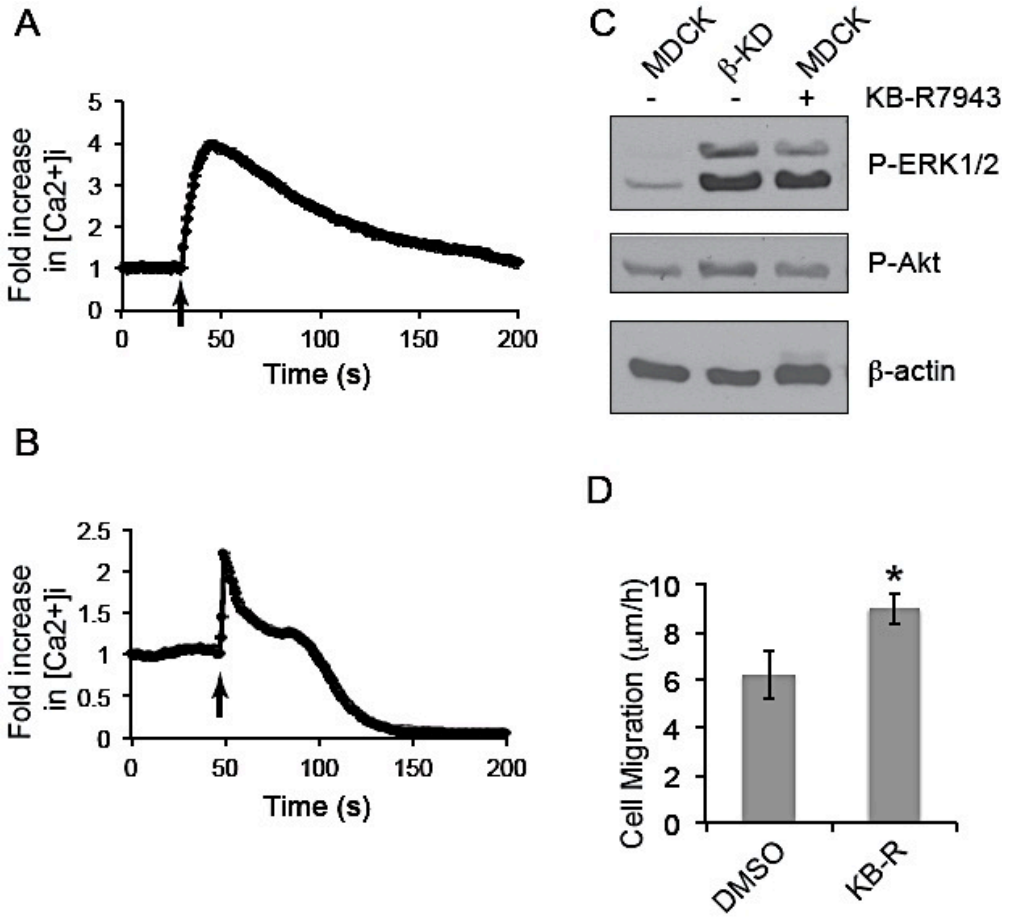


Figure 3.8. Inhibition of NCX1 activity increases $[Ca^{2+}]_i$, activates ERK1/2 and increases rate of migration in MDCK cells: Plots depicting the $[Ca^{2+}]_i$ response of MDCK cells loaded with 10 μM Fluo-4, AM to KB-R7943 with (A) or without (B) Ca^{2+} in the medium. Arrow indicates the time of KB-R7943 addition. Note the increase in $[Ca^{2+}]_i$ concentration upon addition of 10 μM KB-R7943 (arrow). (C) Immunoblots show the phosphorylation status of ERK1/2 and Akt with β-actin as loading control in MDCK, β-KD and MDCK cells treated with 10 μM KB-R7943. Representative blots from three independent experiments are shown. (D) The graph denotes the average rate of migration of MDCK cells with or without 10 μM KB-R7943 treatment as measured by wound healing assay from three independent experiments performed in triplicates. Error bars denote s.e. of the mean and the asterisk indicates statistical significance (*$P < 0.05$).

β-KD cells were treated with W-13 or KN-93, which inhibit CaM and CaMKII respectively. W-13 reduced β-KD cell migration by 47% (P = 0.001), while KN-93 did not significantly alter cell migration rate (P = 0.173) (Figure 3.7C). W-13 also reduced ERK1/2 phosphorylation by 90% (Figure 3.7D). Taken together, these results indicate that CaM is involved in inducing ERK1/2 dependent cell migration in β-KD cells.

3.3.6 Pharmacological Inhibition of NCX1 Induces PI3-K Dependent ERK1/2 Activation and Promotes Cell Migration in Renal Epithelial Cells

To determine if the functional inhibition of NCX1 is sufficient to activate ERK1/2 and induce migration, I blocked the Na^+/Ca^{2+} exchange current with KB-R7943 that has been shown to inhibit both inward and outward Ca^{2+} flux (Amran et al., 2003; Billman, 2001). Treatment of MDCK cells with 10 μM KB-R7943 increased $[Ca^{2+}]_i$ in the presence or absence of Ca^{2+} in the culture medium (Figure 3.8A,B). KB-R7943 treated MDCK cells also showed 2.8-fold increase in ERK1/2 phosphorylation but phosphorylated Akt level remained comparable to MDCK cells (Figure 3.8C). Furthermore, KB-R7943 treatment in MDCK cells increased the migration rate by 1.5 fold (Figure 3.8D), highlighting the role of NCX1 in the regulation of cell migration.

Fig. 7

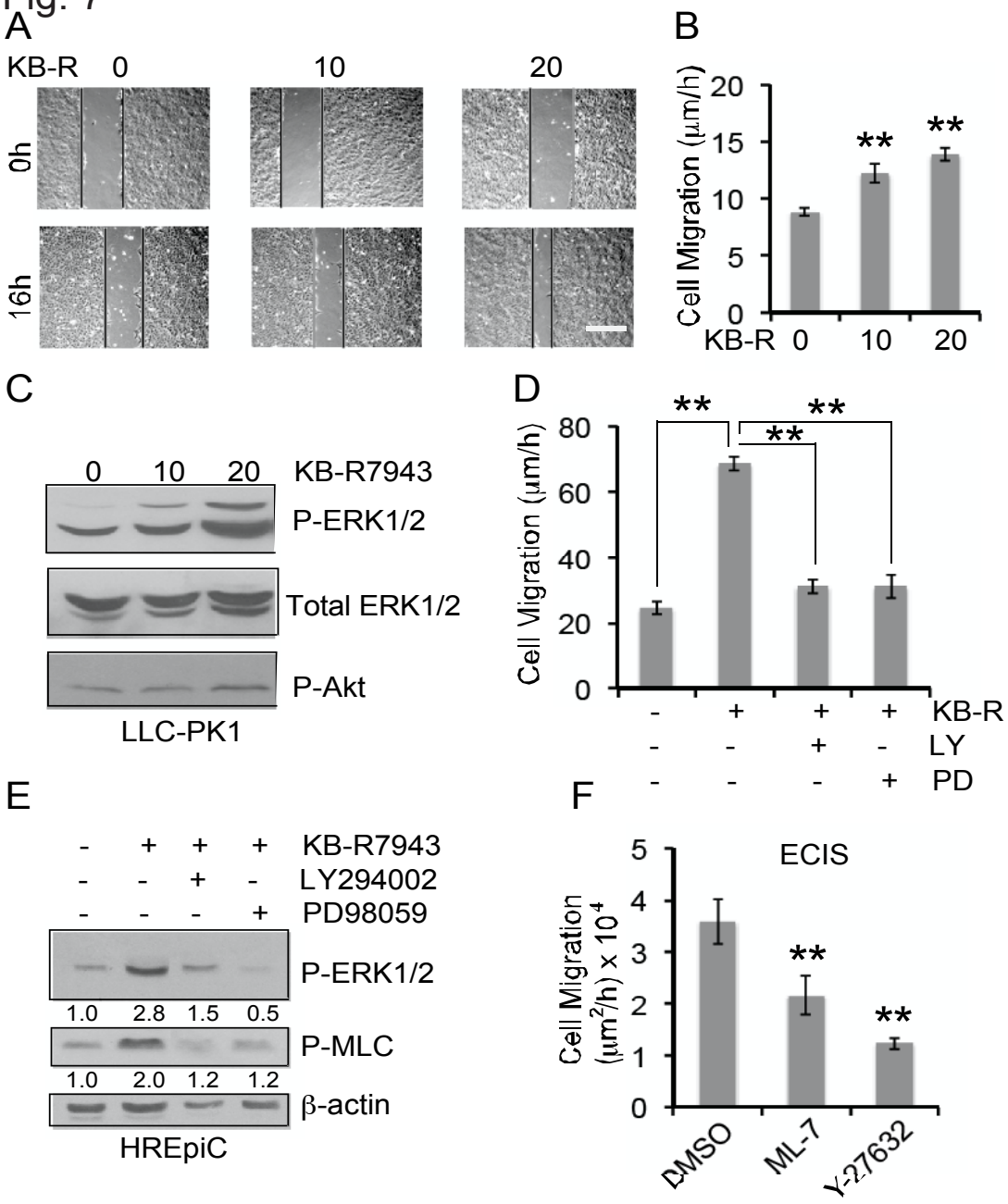

A
KB-R 0 10 20
0h
16h
B
Cell Migration (μm/h)
20
15
10
5
0
**
**
KB-R 0 10 20
C
0 10 20 KB-R7943
P-ERK1/2
Total ERK1/2
P-Akt
LLC-PK1
D
Cell Migration (μm/h)
80
60
40
20
0
**
**
**
- + + + KB-R
- - + - LY
- - - + PD
E
- + + + KB-R7943
- - + - LY294002
- - - + PD98059
P-ERK1/2
1.0 2.8 1.5 0.5
P-MLC
1.0 2.0 1.2 1.2
β-actin
HREpiC
F
ECIS
Cell Migration (μm²/h) x 10⁴
5
4
3
2
1
0
**
**
DMSO
ML-7
Y-27632

Figure 3.9. NCX1 inhibition by KB-R7943 increases ERK1/2 and MLC phosphorylation, and rate of migration in renal epithelial cells in a PI3-K dependent manner: (A) Representative images of the wound in LLC-PK1 cells treated with different concentrations of KB-R7943 at 0 h and after 16 h. Scale bar = 100 μm. (B) The graph represents the mean rate of migration calculated from three independent experiments in triplicates. Error bars denote s.e. of the mean and the asterisks indicate statistical significance (**P < 0.005). (C) Corresponding immunoblots show the phosphorylation status of ERK1/2 and Akt with total ERK1/2 as loading control in LLC-PK1 cells treated with indicated concentrations of KB-R7943 for 16 h. (D) The graph represents the average rate of migration of HREpiC cells treated with 10 μM KB-R7943 in the presence or absence of 10 μM LY294002 or 10 μM PD98059 for 10 h. Error bars denote s.e. of the mean and the asterisks indicates statistical significance (**P < 0.005). (E) Corresponding immunoblots show the phosphorylation status of ERK1/2 and MLC with β-actin as loading control in HREpiC cells. Quantification from three independent experiments expressed as a fold change normalized to β-actin loading control are indicated below the blot. (F) The graph shows the average rate of migration in β-KD cells treated with DMSO, 5 μM ML-7 or 5 μM Y-27632 for 16 h by ECIS wound healing assay from three independent experiments. Error bars denote s.e. of the mean (**P < 0.005).

KB-R7943 also showed progressively increasing rate of cell migration in LLC-PK1 cells. (Figure 3.9A,B) accompanied by a dose-dependent increase in ERK1/2 phosphorylation (Figure 3.9C). Likewise, primary human renal epithelial cells, HREpiC, also showed 2.8-fold increase in migration rate with 10 μM KB-R7943 treatment (Figure 3.9D). Similar to MDCK and LLC-PK1 cells, NCX1 inhibition in HREpiC cells induced 2.8-fold increase in ERK1/2 phosphorylation (Figure 3.9E). Furthermore, MEK inhibitor PD98059 prevented cell migration induced by KB-R7943 (Figure 3.9D) indicating that increased migration by NCX1 inhibition is ERK1/2 dependent. PI3-K inhibitor LY294002 suppressed KB-R7943 induced cell migration rate (Figure 3.9D) and ERK1/2 phosphorylation (Figure 3.9E) indicating that PI3-K

dependent ERK1/2 activation is required for increased cell migration following NCX1 inhibition.

3.3.7 NCX1 Inhibition Mediates Migration by PI3-K/ERK Dependent Increase in MLC Phosphorylation

Myosin light chain kinase (MLCK) and Rho-associated protein kinase (ROCK) are known substrates of ERK1/2 involved in increased cell migration (Huang et al., 2004). β-KD cells treated with MLCK or ROCK inhibitors showed reduced migration supporting the involvement of MLCK and ROCK in mediating cell migration (Figure. 9F). Both MLCK and ROCK phosphorylate myosin light chain (MLC) and enhance migration (Ikebe and Hartshorne, 1985; Totsukawa et al., 2000). KB-R7943 increased MLC phosphorylation by 2.0-fold in HREpiC cells (Figure 3.9E). This increase was abrogated by inhibition of PI3-K and ERK1/2 activation is required for increased cell migration following NCX1 inhibition.

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migration (Ikebe and Hartshorne, 1985; Totsukawa et al., 2000). KB-R7943 increased MLC phosphorylation by 2.0-fold in HREpiC cells (Figure 3.9E). This increase was abrogated by inhibition of PI3-K and ERK1/2 confirming that PI3-K/ERK regulates KB-R9743 induced migration via MLC phosphorylation.

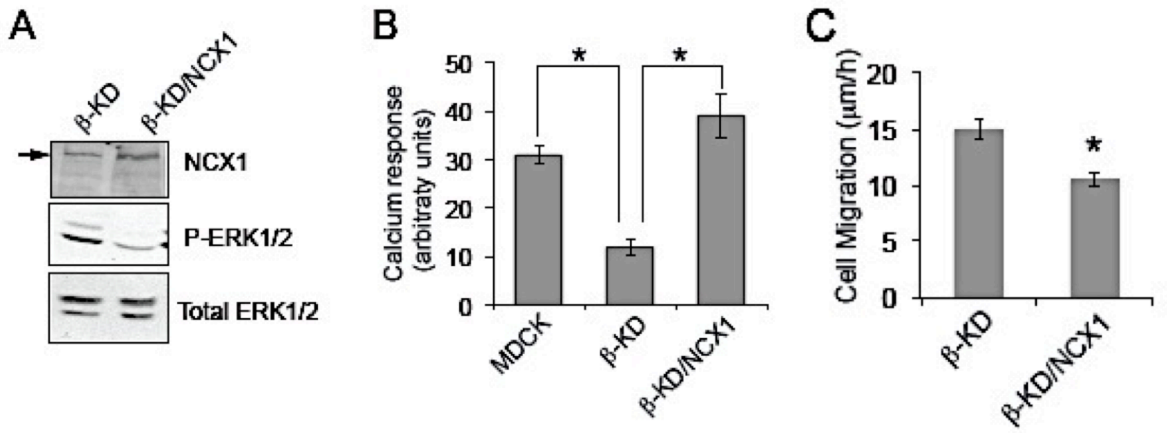

Figure 3.10. Over-expression of NCX1 in β-KD cells reduces ERK1/2 phosphorylation, exhibits response to NCX1 inhibitor and decreases migration rate: (A) Representative immunoblots showing NCX1, phosphorylated ERK1/2, and total ERK1/2 levels in indicated cell lines. (B) The graph represents the mean amplitude of calcium response following treatment with 10 μM KB-R7943 in MDCK, β-KD and β-KD/NCX1 from at least 15 individual cells measured by confocal microscopy. Similar data was obtained in three independent experiments. Error bars denote s.e. of the mean and the asterisks indicate statistical significance (*P< 0.05). (C) The graph depicts the average rate of migration as determined by wound healing assay from three independent experiments in triplicates. Error bars denote s.e. of the mean and the asterisk indicates statistical significance (*P< 0.05).

3.3.8 **Overexpression of NCX1 in β-KD Cells Suppresses ERK1/2 Activation and Cell Migration**

To confirm the role of NCX1 in the regulation of ERK1/2 and cell migration, I overexpressed NCX1 in β-KD cells (β-KD/NCX1). Immunoblot analysis showed that the levels of NCX1 were 2.0 ± 0.1 fold higher in β-KD/NCX1 cells (Figure 3.10A). Higher NCX1 levels were accompanied by 75% reduction in ERK1/2 phosphorylation compared to β-KD cells. Unlike MDCK cells, β-KD cells do not show an increase in $[Ca^{2+}]$ following KB-R7943 treatment. However, β-KD/NCX1 cells treated with KB-R7943 showed enhanced Ca^{2+} similar to MDCK indicating that NCX1 activity was restored by overexpression of NCX1 (Figure 3.10B). Finally, overexpression of NCX1 in β-KD cells suppressed migration rate in a wound-healing assay (Figure 3.10C). Taken together, these data indicate that NCX1 expression in β-KD cells was sufficient to reduce Ca^{2+}-induced ERK/12 activation and cell migration.

3.4 Discussion

These data show that reduced NCX1 protein in β-KD cells was due to posttranslational alteration and was diminished at the cell surface. In addition the data suggests that Na,K-β associates with NCX1 and its membrane stability is affected in the absence of Na,K-β in β-KD cells. Further the reduction in NCX1 expression of Na,K-β knockdown cells was associated with increased $[Ca^{2+}]_i$ and enhanced rate of migration. Interestingly, the increased motility in β-KD cells required

Ca^{2+}/calmodulin/PI3-K mediated ERK1/2 activation for motility. Treatment of MDCK, LLC-PK1 and HREpiC cells with NCX1 inhibitor increased $[Ca^{2+}]_i$, ERK1/2 phosphorylation, and enhanced migration similar to β-KD cells, suggesting that the increased migration in β-KD cells is via suppression of NCX1 activity. This KB-R7943 induced migration was associated with PI3-K and ERK1/2 dependent phosphorylation of MLC. Furthermore, β-KD cells with restored expression of NCX1 activity showed reduced ERK1/2 phosphorylation and migration. A model summarizing the mechanism of migration induced by reduced NCX1 activity is outlined (Figure 3.11).

NCX1 is a bidirectional ion counter transporter, which can function either in the Ca^{2+} efflux or Ca^{2+} influx mode. NCX1 activity and the directionality of ion transport is driven by the membrane potential and the transmembrane electrochemical gradients of Na^+ and Ca^{2+} (Blaustein and Lederer, 1999; Liao et al., 2012; Philipson and Nicoll, 2000). The major function of NCX1 in the kidney is Ca^{2+} extrusion where it plays an important role in net transepithelial Ca^{2+} absorption and is responsible for the majority of Ca^{2+} extrusion (Kip and Strehler, 2003). The importance of NCX1 in Ca^{2+} efflux is evident by the rise in $[Ca^{2+}]_i$ in β-KD cells, which express reduced levels of NCX1. Although bidirectional NCX1 inhibitor KB-R7943 is more potent in inhibiting the reverse than the forward mode (Iwamoto et al., 1996), its sensitivity is dictated by the splice variant of NCX1 expressed by the cells. The splice variant expressed in the kidney, NCX1.3 (Dunn et al., 2002), is inhibited in its forward mode

by KB-R7943 with IC50 values of 2-3 μM (Hamming et al., 2010). Treatment with KB-R7943 in Ca^{2+} free medium also caused an increase in $[Ca^{2+}]_i$ (Figure 3.5B). Taken together, the Ca^{2+} efflux activity of NCX1 is involved in the regulation of cell migration in my study.

Many ion transporters regulate cell migration by maintaining housekeeping functions inside the cell such as membrane potential, cell volume, $[Ca^{2+}]_i$ concentration and pH (Schwab et al., 2012). NCX1 has been reported to promote the migration of a diverse group of non-epithelial cells such as endothelial cells, oligodendrocytes, myofibroblasts, microglia, and pancreatic cancer cells (Andrikopoulos et al., 2011; Chifflet et al., 2012; Dong et al., 2010; Ifuku et al., 2007; Kemeny et al., 2013; Raizman et al., 2007; Sakamoto et al., 2009; Tong et al., 2009). Inhibition or knockdown of NCX1 reduced $[Ca^{2+}]_i$ and prevented migration. This apparent contrast to the findings could be explained by the directionality of Ca^{2+} flux by NCX1 in these cell types. This would suggest that NCX1 regulation of cell migration might be dependent on the directionality of ion transport. However, the underlying commonality in these reports and ours is that a rise in $[Ca^{2+}]_i$ was required for increase in cell migration. Migration is a Ca^{2+} dependent process as most of the regulators of motility that coordinate focal adhesion dynamics and interaction with cytoskeletal proteins are Ca^{2+} sensitive (Prevarskaya et al., 2013; Wiegert and Bading, 2011).

Ouabain, a specific inhibitor of Na,K-ATPase activity leads to an increase in $[Na^{+}]_i$. This increase reduces the driving force for the Na^{+} influx through NCX1,

subsequently resulting in an increase in $[Ca^{2+}]_i$ (Blaustein, 1993). NCX1 knockout mice conclusively demonstrated that ouabain-induced increase in $[Ca^{2+}]_i$ and enhanced cardiac contractility requires NCX1 activity (Reuter et al., 2002). Therefore, ouabain sensitivity is dependent on NCX1 as well as Na,K-ATPase. Previous studies showed that the Na,K-β KO cardiomyocytes and hearts with greatly reduced NCX1 were insensitive to ouabain-induced Ca^{2+} transients and cardiac contractility (Barwe et al., 2009). Consistent with these observations, β-KD with reduced NCX1 protein did not respond to ouabain, further confirming that NCX1 functions in the Ca^{2+} efflux mode. Migrating cells have a Ca^{2+} gradient inside the cell with high Ca^{2+} levels towards the retracting end and low Ca^{2+} at the leading end (Schwab et al., 1997), thus making it amenable to respond to Ca^{2+} sparks that trigger forward movement. Other ion transporters involved in migration have a polarized distribution. The localization of NCX1 in a migrating cell has not been determined.

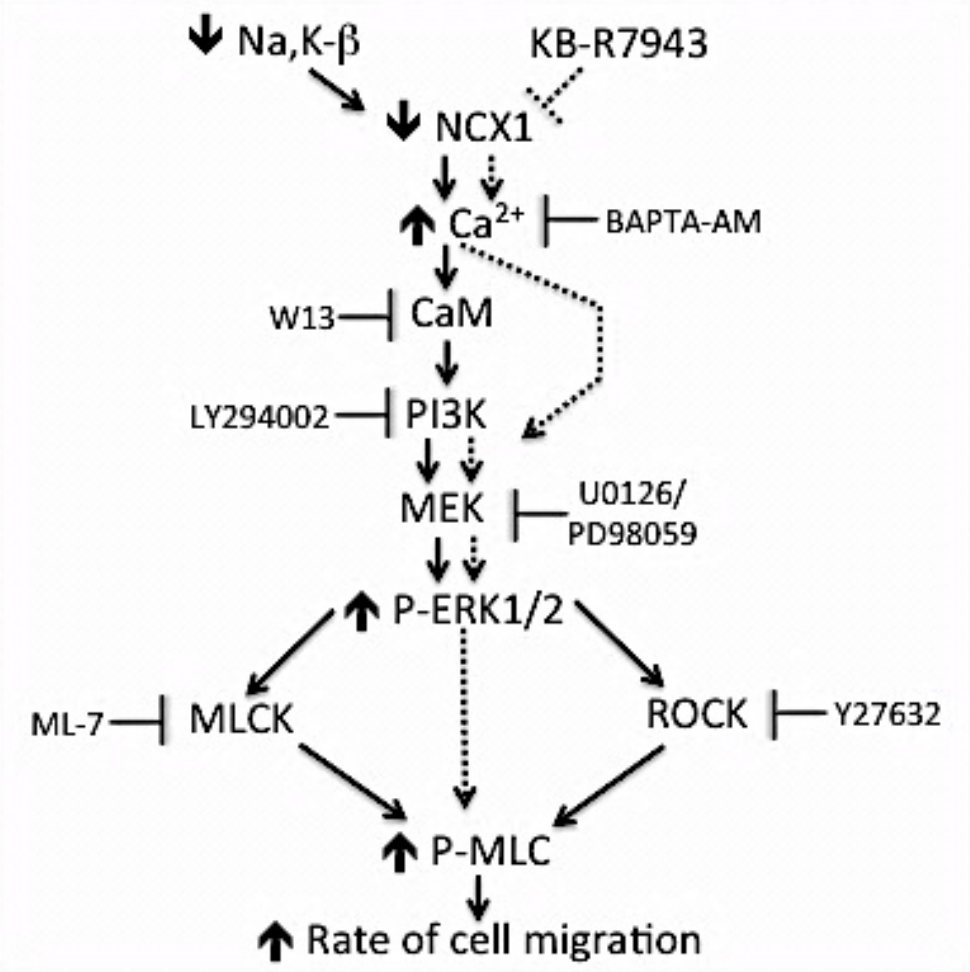

Figure 3.11. Schematic model of regulation of migration by reduced protein expression or functional inhibition of NCX1 in renal epithelial cells: The solid arrows indicate the consequences of the reduction in the NCX1 protein level whereas the dotted lines indicate the consequences of functional inhibition of NCX1.

The catalytic subunit of PI3-K, p110 subunit, is also a dual specificity kinase, which possesses a serine/threonine protein kinase activity in addition to its well-known lipid kinase activity (Carpenter et al., 1993; Dhand et al., 1994). The lipid kinase activity is responsible for Akt phosphorylation and the protein kinase activity

mediates ERK1/2 activation by direct phosphorylation of MEK (Bondeva et al., 1998). Increased lipid kinase activity in gain-of-function oncogenic mutants of PI3-K p110 was accompanied by enhanced protein kinase activity (Buchanan et al., 2013). Previous study has shown that direct binding of CaM to the SH2 domain of p85 subunit of PI3-K results in increase of its lipid kinase activity (Joyal et al., 1997), however, the effect on protein kinase activity was not tested. Therefore, it is tempting to speculate that CaM could also induce protein kinase activity of PI3-K in renal epithelial cells. However, the exact mechanism by which CaM activates PI3-K protein kinase activity remains to be determined.

Previous studies have demonstrated that replenishing Na,K-β in highly motile MSV-MDCK cells reduced cell motility (Barwe et al., 2005; Rajasekaran et al., 2001b) and that knockdown of Na,K-β prevented epithelial lumen formation via PI3-K dependent ERK1/2 activation (Barwe et al., 2012). This is the first report showing that knockdown of Na,K-β in MDCK cells increased rate of cell migration, and that increased $[Ca^{2+}]_i$ following NCX1 reduction was required for ERK1/2 activation and enhanced migration in β-KD cells. Thus, it is likely that reduced NCX1 is a downstream mediator of the β-KD phenotype. The mechanism by which Na,K-β regulates NCX1 expression remained to be determined. Since Na,K-β acts as a chaperone for the membrane trafficking of Na,K-α, it is likely that it plays a similar role in aiding the trafficking of NCX1 to the membrane. The immunofluorescence data

showing reduced NCX1 membrane localization in β-KD cells and the ouabain insensitivity supports this possibility.

The role of Na,K-β as a motility and tumor suppressor in conjunction with reduced expression of Na,K-β in several carcinomas has been established (Barwe et al., 2005; Inge et al., 2008b; Rajasekaran et al., 1999; Rajasekaran et al., 2005; Vagin et al., 2006). Similar to Na,K-β levels, NCX1 transcript level is also reduced in various subtypes of renal carcinoma (Figure 3.12). Therefore, given the role of NCX1 in cell migration in epithelial cells, it is likely that reduced expression of NCX1 is also a prognostic indicator of carcinoma progression. Experiments are in progress to address the alternate functions of NCX1 in renal cancers.

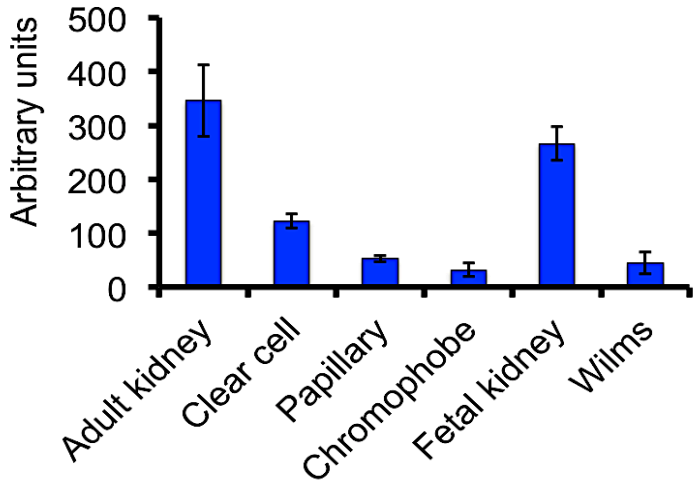

Figure 3.12. Analysis of microarray data: A comparison of NCX1 transcript levels in different types of renal cancers. $P < 0.001$ for all RCC types and $P<0.005$ for Wilms. All these tumors show a significant reduction in NCX1 transcript levels.

Chapter 4

DISCUSSION AND CONCLUSIONS

4.1 What is Known and What is New About the Functions of Na,K-ATPase

Apart from its well-known function in the regulation of ion homeostasis, Na,K-ATPase activity has been shown to be involved in a number of cellular processes. Contreras et al., in 1999 reported that inhibition of the pump activity reduces transmembrane epithelial resistance (TER) due to loss of cell attachment and cell-cell adhesion. This was followed by a decade of research in identifying various novel and moonlighting functions of Na,K-ATPase in 1) formation of tight junctions, 2) epithelial cell polarity, 3) cell-cell adhesion, 4) migration 5) protein-protein interactions (scaffolding platform for signal transduction) and 6) molecular chaperoning. These functions are mediated either by Na,K-α/β heterodimer or Na,K-β solely. Identification of various moonlighting functions of Na,K-ATPase has been chronicled in Table 1.

4.2 Characteristics Exhibited by Na,K-β Independent of Na,K-ATPase Ion Transport Activity

As additional functions of Na,K-ATPase were discovered, the necessity to clarify whether these functions are dependent on the pump activity of Na,K-α or on

the protein-protein interactions mediated by Na,K-β occurred. In MSV-MDCK cells, Na,K-β expression, and not Na,K-α restored epithelial cell polarity and phenotype along with E-cadherin. In addition Na,K-β expressing cells reduced the rate of cell motility and invasiveness independent of Na,K-α expression (Barwe et al., 2005; Espineda et al., 2004; Inge et al., 2008b; Rajasekaran et al., 2003c; Rajasekaran et al., 2001b). These data indicated that Na,K-β has an important part in mediating cellular processes by itself, independent of its cognate partner, Na,K-α.

Na,K-β regulating cell migration independent of pump function was shown by Na,K-ATPase inhibition. Although Na,K-β overexpressing cells migrated slower, migration of Na,K-ATPase inhibited cells were unaltered (Barwe et al., 2005). Identification of specific amino acid residues within the transmembrane domain of Na,K-β that mediate homo-oligomerization (glycine zipper) and those that regulate pump activity (heptad repeat) (Barwe et al., 2007) enabled the determination of minimal contribution of Na,K-ATPase pump activity in cell adhesion and lumen formation, a measure of epithelial polarization (Barwe et al., 2012).

Furthermore, studies have proven the role of Na,K-β in membrane trafficking of other ion transporters (BKCa, NKCC2) apart from Na,K-α. So my study focused on establishing the role of Na,K-β as a chaperone for membrane proteins that ascertain epithelial polarity and regulate ion homeostasis. The figure 4.1 summarizes what was known earlier and what I have shown in my dissertation study (highlighted in yellow).

Table 1: Chronological Order of Functions of Na,K-ATPase Identified

Characteristics	Function	Reference	Year
Ion homeostasis	**Pump activity**	(Heinz and Hoffman, 1965)	1957-1992
		(Gibson and Harris, 1968)	
		(Baker et al., 1969)	
		(Jorgensen and Skou, 1971)	
		(Hayslett et al., 1973)	
		(Skou and Esmann, 1992)	
Pump dependent	**TJ formation and maintenance**	(Contreras et al., 1999)	1999-2007
		(Rajasekaran et al., 2001a)	
		(Genova and Fehon, 2003)	
		(Paul et al., 2003)	
		(Rajasekaran et al., 2003a)	
		(Violette et al., 2006)	
		(Rajasekaran et al., 2007)	
	Epithelial polarity	(Contreras et al., 1999)	1999-2010
		(Rajasekaran et al., 2001a; Rajasekaran et al., 2001b)	
		(Vogelmann and Nelson, 2005)	
		(Rajasekaran et al., 2007)	
		(Bagnat et al., 2007)	
		(Liu et al., 2008)	
		(Zhang et al., 2010)	
	Cell-cell	(Gloor et al., 1990)	1990-2011
		(Chow and Forte, 1995)	
		(Rajasekaran et al., 2001b)	
		(Shoshani et al., 2005)	
		(Kitamura et al., 2005)	

Pump independent	**adhesion**	(Vagin et al., 2006)	
		(Barwe et al., 2007)	
		(Tokhtaeva et al., 2011)	
	Motility suppressor	(Rajasekaran et al., 2005)	2005-2015
		(Inge et al., 2008b)	
		(Balasubramaniam et al., 2015b)	
	Proliferation	(Haas et al., 2002)	2002-2013
		(Pedemonte et al., 2005)	
		(Yuan et al., 2005)	
		(Barwe et al., 2012)	
		(Xie et al., 2013)	
	Chaperone	(Ackermann and Geering, 1990)	1990-2015
		(Jha and Dryer, 2009)	
		(Carmosino et al., 2014)	
		(Balasubramaniam et al., 2015b)	

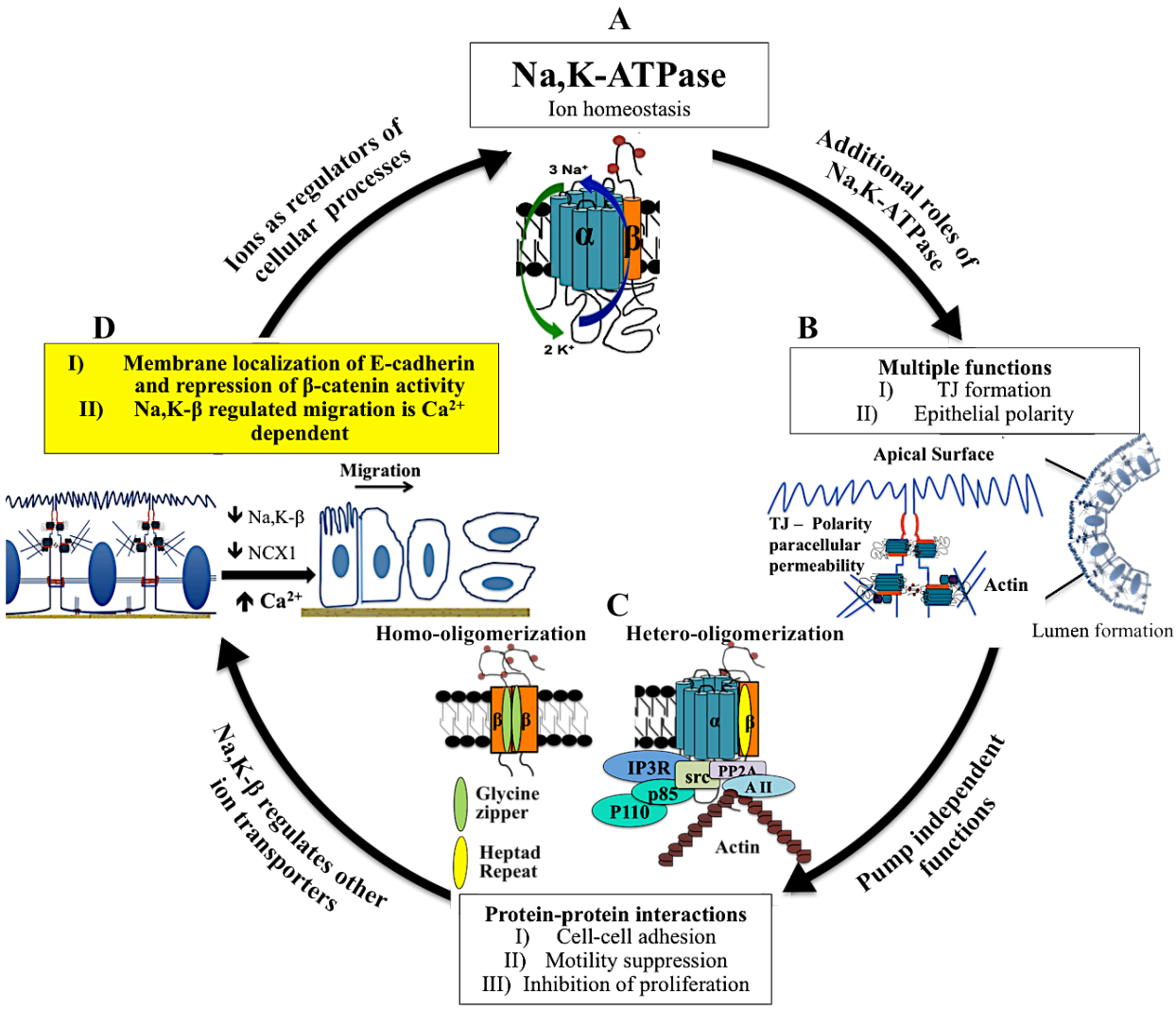
A
Na,K-ATPase
Ion homeostasis
3 Na+
2 K+
α
β
Additional roles of Na,K-ATPase
B
Multiple functions
I) TJ formation
II) Epithelial polarity
Apical Surface
TJ – Polarity
paracellular
permeability
Actin
Lumen formation
C
Homo-oligomerization
Hetero-oligomerization
Glycine zipper
Heptad Repeat
IP3R
src
PP2A
p85
A II
P110
Actin
Pump independent functions
Protein-protein interactions
I) Cell-cell adhesion
II) Motility suppression
III) Inhibition of proliferation
Na,K-β regulates other ion transporters
D
I) Membrane localization of E-cadherin and repression of β-catenin activity
II) Na,K-β regulated migration is Ca2+ dependent
Migration
Na,K-β
NCX1
Ca2+
Ions as regulators of cellular processes

Figure 4.1. Role of Na,K-ATPase in ion dependent regulation of epithelial cell functions: A) Since its discovery by Jens Skou in 1957, the role of Na,K-ATPase (composed of Na,K-α and Na,K-β subunits) in regulating ion homeostasis has been studied extensively. B) Studies in early 2000's elucidated other functions of Na,K-ATPase in the formation and maintenance of TJ, and preservation of epithelial polarity. A pictorial representation showing Na,K-ATPase localized to TJ regulating paracellular permeability and the formation of fluid-filled lumen is shown. C) Various groups have studied the pump independent roles of Na,K-ATPase in mid-late 2000's, specifically the role of protein-protein interactions in the regulation of cell-cell adhesion, and in suppression of motility and proliferation. The cartoon depicts protein-protein interactions mediated by Na,K-ATPase subunits. Na,K-β homo-oligomerization mediates cell adhesion, while hetero-oligomerization is involved in signaling. Na,K-α interaction with Src, p85 subunit of PI3-K and ankyrin is shown along with Na,K-β association with annexin II and PP2A. D) Additional functions of Na,K-β in the regulation of membrane localization of other ion transporters (such as NCX1, BKCa, NKCC2), apart from its cognate partner Na,K-α, have come to light in recent years. My work has shown that Na,K-β regulates membrane expression and localization of E-cadherin thereby suppressing β–catenin which is an important signaling mechanism involved in a number of cancer types. Further I have also shown that Na,K-β mediated migration is Ca^{2+} dependent, the loss of Na,K-β mediates reduction in NCX1 thereby increasing $[Ca^{2+}]i$ and activating PI3-K/ERK, another mechanism involved in cancer progression. In conclusion, Na,K-ATPase dependent cellular processes such as maintenance of the epithelial phenotype, inhibition of β-catenin transcriptional activity and suppression of migration are important characteristics, if not regulated stringently these steps will lead to EMT and cancer progression.

4.2.1 Na,K-β Complexes With E-cadherin by Associating With β-catenin, Regulating Cell-Cell Adhesion and Epithelial Polarity

E-cadherin is mediates cell-cell adhesion via Ca^{2+} dependent homophilic interactions between neighboring epithelial cells. Its role has been associated in development of epithelial polarity and maintenance of tissue architecture (van Roy and Berx, 2008). Similarly, Na,K-β also acts as a cell adhesion molecule, essential for maintaining the epithelial integrity and formation of tight junctions. Both these

proteins are found in adherens junctions and are important in the formation of polarized epithelial cells. In MSV-MDCK cells, restoring the expression of E-cadherin along with Na,K-β, not E-cadherin or Na,K-β alone resuscitated epithelial phenotype by inducing the formation of tight junctions and apico-basal polarity. This was associated with a reduction in $[Na^+]_i$, since previous studies had shown that localized increase $[Na^+]_i$ disrupted the TJs, it can be inferred that E-cadherin might also play an important role in ion homeostasis. In addition, detergent extraction assay indicated that the membrane anchorage of E-cadherin is stronger in the presence of Na,K-β (Rajasekaran et al., 2001b). Taken together, these results suggest that Na,K-β functions synergistically with E-cadherin.

The data from my study identified the molecular mechanism behind this synergism and indicated that Na,K-β acts as a chaperone for E-cadherin membrane expression and localization. Na,K-β, β-catenin and E-cadherin complexes together and Na,K-β cytoplasmic tail directly interacts with β-catenin. Reduction in Na,K-β expression, reduces cell surface expression of E-cadherin in β-KD cells, associated with an increase in β-catenin nuclear localization and transcriptional activity. Therefore I speculate that this is enabled by association of E-cadherin with Na,K-β, directly or by its association with β-catenin. The regulation of E-cadherin expression by Na,K-β emphasizes the significance of the chaperoning activity of Na,K-β in maintaining the structure and function of polarized epithelial phenotype, in addition to its direct role in regulation of some cell-cell adhesion and epithelial architecture.

4.2.2 Na,K-β Regulates NCX1 Expression and $[Ca^{2+}]_i$ That Governs Adhesion, Migration and Polarity

In addition to its role in regulating epithelial polarity, Na,K-β has been reported to regulate the expression of NCX1, governing $[Ca^{2+}]_i$ (Barwe et al., 2009). Na,K-β re-expression restored the $[Ca^{2+}]_i$ concentration suppressing the cell motility. Localized increase in intracellular ion concentration i.e increase in $[Na^+]_i$ during reduced Na,K-β has been shown to affect the formation of junctions (Rajasekaran et al., 2001a). In addition studies have also shown that increase in $[Na^+]_i$ increases $[Ca^{2+}]_i$ (Baker et al., 1969) and any increase in $[Ca^{2+}]_i$ is also known to disrupt epithelial junctional formation (Brown and Davis, 2002). This indicates that governance of intracellular Na^+ and Ca^{2+} by Na,K-β is critical for normal formation and function of TJ thereby maintaining the epithelial phenotype (Balasubramaniam et al., 2015b; Rajasekaran et al., 2001b).

Although Na,K-ATPase pump activity was not involved, restoring the expression and function of NCX1, in Na,K-β knockdown cells reverted $[Ca^{2+}]_i$, PI3-K/ERK signaling and enhanced migration (Balasubramaniam et al., 2015b). Moreover Na,K-β has been shown to regulate other ion transporters such as BKCa and NKCC2, besides Na,K-α. Based on these studies we can infer that Na,K-β facilitates its moonlighting functions by regulation of cell surface expression of ion transporters and thereby controlling ion homeostasis.

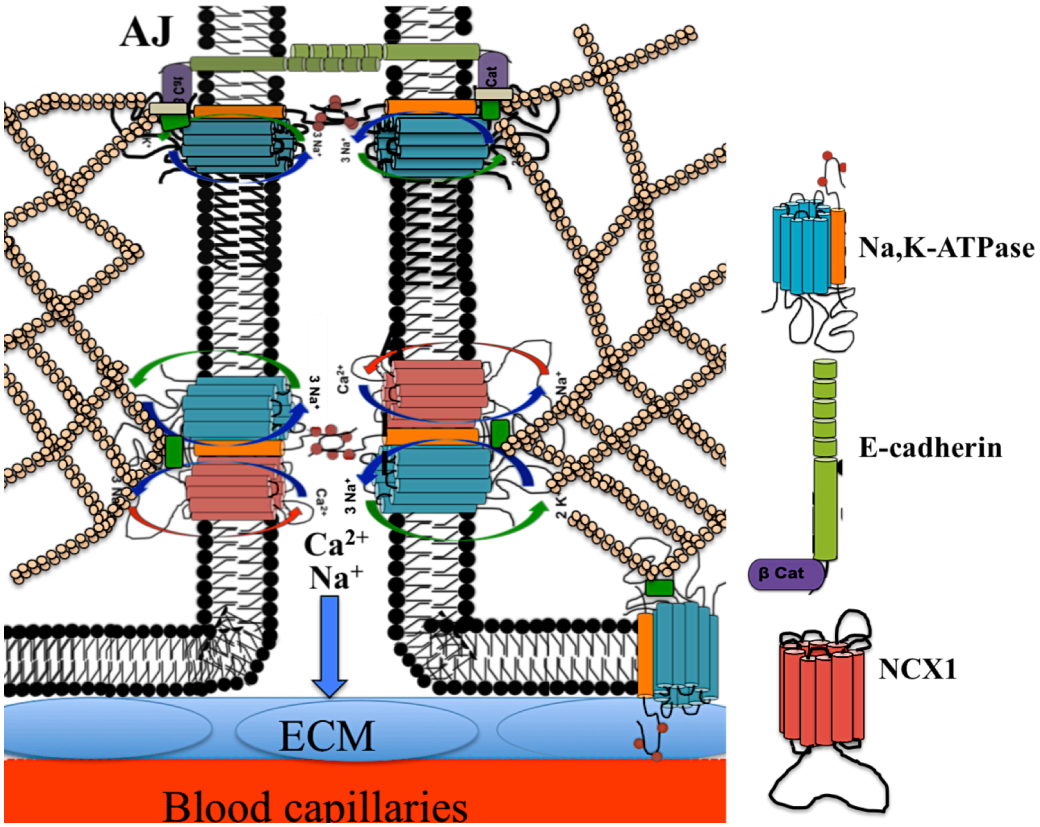

Figure 4.2. Functional coupling of Na,K-β with cell adhesion molecule E-cadherin and NCX1: Na,K-β (shown in orange) is known to associate with Na,K-α (shown in blue), its cognate partner. Na,K-β associates with E-cadherin (shown in green) via β-catenin and localizes to the adherent junction, stabilizing of E-cadherin on the membrane. In addition it also associates with NCX1 in the baso-lateral region and mediates the vectoral transport.

4.3 Conclusion

4.3.1 Na,K-β is a Chaperone for E-cadherin and NCX1

Na,K-α expression is dependent on the expression of Na,K-β, this is important in maintenance of ion homeostasis and regulation of paracellular diffusion in epithelial cells. Studies over the years have highlighted the importance of Na,K-ATPase in formation and maintenance of TJ that regulate polarized phenotype characteristic of epithelial cells (Rajasekaran et al., 2001a; Rajasekaran et al., 2001b). We provide evidence that Na,K-β complexes with E-cadherin via β-catenin and this association is essential for the E-cadherin surface expression and suppression of β-catenin transcriptional activity. Further, we elucidated that Na,K-β interacts with NCX1 and regulates its membrane expression and localization; this further ascertains the chaperoning role of Na,K-β. In addition, I found that Na,K-β indirectly governs $[Ca^{2+}]_i$ concentration and is essential in maintaining the signaling and migratory characteristics of the epithelial cell. Similarly Na,K-β has been shown to govern the membrane expression of ion transporters, BKCa that regulates membrane potential, and NKCC that is an important player in the regulation of Na^+ and Cl^- reabsorption. All these studies suggest that Na,K-β acts as a molecular chaperone for multiple membrane proteins, adhesion molecules and ion transporters, thereby regulating of ion homeostasis and epithelial phenotype.

4.3.2 **Na,K-β as a Conciliator of Consequences Occurring During EMT**

Na,K-β is a versatile protein involved in initiation of cell-cell adhesion, suppressing cell motility and preserving the epithelial architecture. Both Na,K-β and E-cadherin are essential for maintenance of the structure and function of polarized epithelia. During EMT, transcriptional factor snail, has been shown to repress the transcription of both Na,K-β and E-cadherin in cells in culture and in a number of cancer types (Cano et al., 2000; Rajasekaran et al., 1999). Further previous study from the lab has reported that during exposure to TGF-β1, the surface expression of Na,K-β is reduced, before the initiation of the downregulation of E-cadherin and upregulation of well-characterized EMT markers, associated with the induction of mesenchymal phenotype (Rajasekaran et al., 2010).

My study provides an explanation for this observation, that is the loss of Na,K-β, destabilizes E-cadherin, reducing its surface expression, destabilizing AJ. Any changes in AJ composition, weakens the epithelial cell architecture and affects the vectorial transport of ions mediated by epithelial cells. Further studies have shown that increased β-catenin transcriptional activity has been associated with increased proliferation and cell survival (Mann et al., 1999). Various groups have shown that nuclear accumulation of β-catenin contributes to the development and progression of cancer. This suggests that one of the consequence of reduction of Na,K-β is loss of epithelial phenotype and increased proliferation, trademarks of EMT.

Na,K-β has been reported to regulate ion transporters including Na,K-α, BKCa, NKCC2; in this study I have shown that Na,K-β chaperones and regulates

NCX1 expression also. In addition this regulation of NCX1 by Na,K-β plays an important role in Ca^{2+} dependent cell migration in renal epithelial cells. This suggests that during EMT, when there is a reduction in the expression of Na,K-β, this could affect the expression of NCX1, thereby causing an increase in the rate of migration, another trademark of EMT. Based on these studies we can infer that Na,K-β facilitates its moonlighting functions, at least in part, by 1) regulation of cell surface expression of E-cadherin and controlling β-catenin transcriptional activity involved in proliferation and cell survival, and 2) regulation of NCX1 expression involved in Ca^{2+} dependent cell migration, thereby governing ion homeostasis and epithelial phenotype (Balasubramaniam et al., 2015a) (Figure 4.2).

In diseased conditions such as EMT, fibrosis and cancer, associated with a loss of Na,K-β, the membrane localization and function of Na,K-α as well as other ion transporters and adhesion molecules is likely to be affected. This indicates that, the consequences of loss of Na,K-β is associated with changes in intracellular ionic milieu. These changes might lead to synchronous deterioration of ion homeostasis, epithelial polarity, increase in rate of proliferation and migration. The loss of Na,K-β could be the initial trigger before a plethora of events are unleashed, culminating in neoplastic lesions.

Chapter 5

FUTURE DIRECTIONS

Based on literature, we hypothesized that Na,K-β functionally synergizes with other membrane proteins to regulate cell-cell adhesion, migration and signaling. My studies have elucidated that Na,K-β chaperones E-cadherin and NCX1, thereby mediating the cellular processes mentioned above. My study has also shown that functional inhibition of NCX1 increases rate of migration and ERK signaling pathway. It would be of importance to determine if this signaling cascade has any role in the regulation of epithelial phenotype, as Ca^{2+} signaling is known to regulate a number of functions in the cell. Further, I have shown the role of NCX1 in regulation of migration in epithelial cells. An extension of this study with clinical relevance would be to determine if NCX1 regulates cell-cell adhesion or affects the expression of E-cadherin, formation of TJs mediating some of the characteristics exhibited by Na,K-β. Other characteristics may be independent of NCX1 activity. For example, activation of Akt in β-KD cells is independent of reduction in NCX1 mediated increase in $[Ca^{2+}]_i$, indicating that some functions of Na,K-β are independent of NCX1 activity. Such studies will aid in our understanding of the role of NCX1 in epithelial cell morphogenesis other than regulation of $[Ca^{2+}]_i$ concentration.

Na,K-β expression is reduced during EMT and a number of cancer types. Since my research has shown that E-cadherin and NCX1 expression is regulated by Na,K-β,,it might be of importance in categorization and prognosis of renal cancer subtypes exhibiting a reduction in Na,K-β associated with reduction in membrane expression of E-cadherin and NCX1. These results raise series of questions including: i) To determine if the loss of Na,K-β a cause or consequence of EMT? ii) Does loss of NCX1 promote EMT, i.e., contribute in the transformation of epithelial cells to fibroblasts by activating the Ca^{2+} dependent CaM/PI3-K/ERK signaling pathway? iii) Snail has been shown to down regulate both E-cadherin and Na,K-β during EMT. Does snail affect the expression of NCX1 too?

Earlier studies have shown that with TGF-β treatment, Na,K-β expression is reduced, followed by the reduction in the expression of E-cadherin with an associated increase in the fibroblastic markers and increase in fibroblastic characteristics (Rajasekaran et al., 2010). In order to determine whether Na,K-β is a cause or consequence of EMT, β-KD cells can be utilized and profiled for EMT characteristics and markers.

NCX1 is an important regulator of $[Ca^{2+}]_i$ concentration and regulator of Ca^{2+} dependent signaling, and given the significance of Ca^{2+} in EMT, it will be interesting to determine the role of NCX1 in induction of EMT. In addition, it will be of interest to determine if functional inhibition of NCX1 or knockdown will activate signaling

pathways contributing to EMT and upregulate fibroblastic proteins fibronectin, α-SMA.

TGF-β is known to mediate fibrotic EMT in a number of tissues through activation of smad and non-smad signaling pathways. Transcription factors, like Snail, are stimulated by TGF-β1-Smad signaling and play crucial roles in the induction of EMT. Snail binds to E-boxes and suppresses the transcription of genes involved in maintaining the epithelial phenotype. Previous studies have shown that both E-cadherin and Na,K-β have Snail binding E-boxes in their promoter region. Interestingly NCX1 also has 2 CANNTG motifs (E-boxes). It would be of significance to determine if Snail downregulates NCX1 during EMT or down regulation of NCX1 during EMT is Na,K-β dependent or Snail-independent. If NCX1 is a direct target of TGF-β1 mediated EMT, then MDCK cells treated with TGF-β will show reduced NCX1 transcript and increased Snail transcripts.

Previous studies have shown that Na,K-β regulates other ion transporters such as BKCa (8 transmembrane domain protein), NKCC2 (12 transmembrane domain) apart from Na,K-α (10 transmembrane domain protein) and NCX1 (10 transmembrane domain protein) , so a logical extension of the study would be to determine if Na,K-β chaperones other polytopic ion transporters similar to the ones mentioned above. Furthermore, it would be interesting to test if Na,K-β chaperones other TJ or AJ proteins such as claudins, nectins and JAM similar to E-cadherin regulation.

Aldosterone, a mineralocorticoid, has been shown to increase the transcription, subsequently upregulating the membrane expression of Na,K-ATPase and increase the sodium pumping activity in the cell membrane (Dooley et al., 2013). Based on literature we also know that Na,K-α expression will be upregulated only in the presence of Na,K-β. It will be of importance to understand the effect of aldosterone on Na,K-β expression, also to test if aldosterone can be used as an upregulator of Na,K-β expression in renal carcinoma cells and cause MET. Further studies have also shown that compounds related to glucocorticoids such as triamcinolone, dexamethasone, and fluorometholone can upregulate cell surface expression of Na,K-β, enhance cell-cell adhesion, reduce the rate of migration and invasion, in addition to inducing MET of renal cell carcinoma (RCC) cells (Huynh et al., 2015). It will be interesting to determine if the induction of MET observed in these cells is due to the upregulation of E-cadherin and NCX1 chaperoned by Na,K-β or by Na,K-β in itself.

All these findings will be of importance as the availability Na,K-β, NCX1, other ion transporters and junctional molecules on the membrane in the epithelial cells present them as potential therapeutic targets for prognosis and treatment during diseased state such as fibrosis and cancer. Selective inhibition or upregulation of these membrane proteins might have significance in clinical research, as ion transporters are increasingly used as pharmacological targets for development of novel drugs. Thus, NCX1 upregulator could potentially be used for treatment of renal cancers.

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