THE ROLE OF LEK1 IN RECYCLING ENDOSOME TRAFFICKING AND ITS

FUNCTION IN HEART DEVELOPMENT

By

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To my wonderful daughter, Quinn McKenna, giving us joy everyday

and

To an amazing wife and mother, Audra, for always being there

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CHAPTER I

INTRODUCTION

Regulation of Proliferation and Differentiation

Cell proliferation and differentiation are two processes that require exquisite control during development to produce mature functional organs in the growing organism. Any misregulation of these processes can lead to altered cell division, which in turn, may result in formation of tumors or congenital malformations. Interestingly, each set of tissues has its own controls for these processes, since each tissue has its own unique niche and functions. The mechanisms controlling these functions are being elucidated, and we are just starting to understand the regulation of proliferation and differentiation during organogenesis and embryogenesis.

For the purposes of this document, the heart will be used as the model organ. Cells that comprise the functioning heart muscle, cardiomyocytes, do not retain the ability to proliferate after terminal differentiation. This is in contrast to other organs, for example epithelial cells of the digestive system. Enterocytes retain the ability to divide throughout their lifetime and have a continuous stem cell population to replenish lost and dead cells (Ross et al., 1995).

Cardiomyocytes have the ability to differentiate as they proliferate during cardiogenesis. A cascade of transcription factors has been identified, including Mesp1, 2, Nkx2.5, GATA 4, 5, 6, Mef2c, and the Tbx family of proteins, that

regulate the proliferation of the cardiac cell lineage (reviewed in Solloway and Harvey, 2003). Several studies have identified differential gene expression in mature cardiomyocytes, including genes that encode myosin heavy chain and light chain family of proteins, cardiac α-actin, tropomyosin, α-actinin, titan, and desmin (Franco et al., 1998; Lyons et al., 1995; Ross et al., 1996; Solloway and Harvey, 2003; Wang and Stockdale, 1998; Yutzey and Bader, 1995). The factors that control the balance of these processes are not yet well understood. For the purposes of our studies, the Retinoblastoma (Rb) family of genes is implicated in the regulation of proliferation and differentiation, at least in part, of the cardiomyocyte maturation pathway (Novitch et al., 1996; Papadimou et al., 2005).

In the developing mouse, cardiomyocytes continue to proliferate and differentiate until the first postnatal week. At this time, there are poorly defined signals that direct the exit of proliferating cells from the cell cycle and then initiate terminal differentiation. It is commonly speculated that this results in the inability of cells to reenter the cell cycle. Recently, what has become an area of great debate within the field (Barlucchi et al., 2003; Beltrami, 2003). It is unresolved whether or not a resident stem cell population exists within the heart, even though no clear stem cell population has yet been identified. Needless to say, the processes that regulate cardiomyocyte proliferation and terminal differentiation are poorly understood. Myocardial infarction is the leading cause of death in Americans (American Heart Association). If we were able to determine the underlying mechanisms of cardiomyocyte proliferation and differentiation, it may
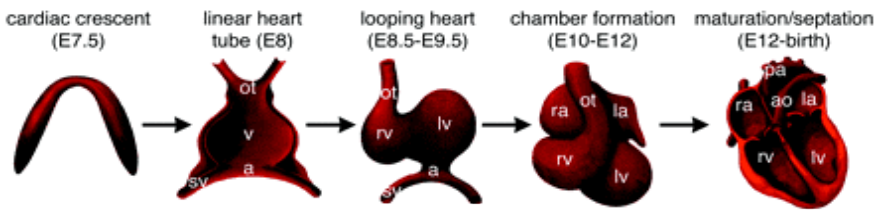
Figure 1: Summary of mouse heart development. 1) Cardiac crescent (E7.5). **2)** Linear heart tube (E8). **3)** Looping heart (E8.5-9.5). **4)** Chamber formation (E10-12). **5)** Maturation and septation (E12-birth) (Bruneau et al., 2002).

one day be possible to treat infarctions by reprogramming cells to proliferate and heal the damaged heart. Therefore, it is critical that we study and decipher the molecular regulators of cardiomyocyte proliferation and also for differentiation during development for basic scientific value and better treatment options.

Heart Organogenesis

Although it has been one of the most extensively studied processes, regulation of cardiac muscle development is still not well understood. Fate mapping studies during gastrulation have shown that two sets of cells migrate from the primitive streak to two bilateral areas forming cardiac mesodermal sheets in the developing embryo (Garcia-Martinez and Schoenwolf, 1993). These cells commit to a cardiac fate (Gonzalez-Sanchez and Bader, 1990), and it is thought, at least in part, that signals from the surrounding endoderm are responsible for their committment (Jacobson and Duncan, 1968; Linask and Lash, 1986; Nascone and Mercola, 1995). The mesodermal layer, or the cardiac crescent, folds toward the ventral midline to form the linear heart tube (DeHann, 1967; Linask and Lash, 1986). Two cell types are specified at this time, the cells of the inner lining of the heart, or endocardium, and the cells that will develop into the muscle structure, or the myocardium (Garcia-Martinez and Schoenwolf, 1993; Inagaki et al., 1993). As seen in Figure 1, the linear heart tube consists of a single ventricle and a single atrium. The tube soon contorts into an S-shaped tube, and with extensive remodeling, eventually develops into a four-chambered heart (Carlson et al., 1981).
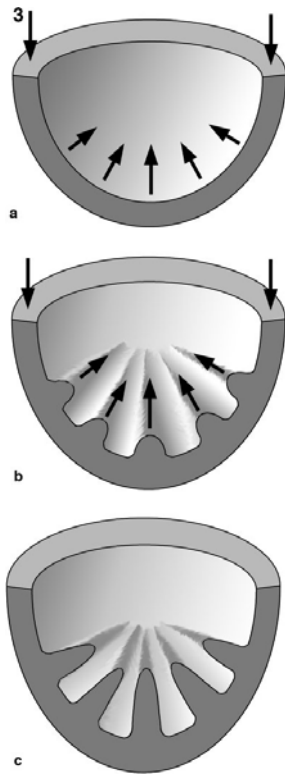
Figure 2. Trabeculation. Conceptualization of the development of ventricular trabeculae after E8 in the developing mouse heart. (Sedmura et al., 2000)

Maturation of the Heart Wall

The bilateral heart crescent fuses at murine E8. At this stage, the heart consists of three layers: an inner layer of endothelial cells, a middle layer of extracellular matrix, and an outer layer of myocardium that is 1-2 cells thick (Inagaki et al., 1993). Cardiac myocytes in the heart tube are actively dividing at a much higher rate in the single ventricle than those in the atrium. Finger-like projections, termed trabeculae, start to form in the ventricle at murine E10.5 and will form at a later time in the atrium (Figure 2; Challice and Viragh, 1974; Icardo, 1996; Icardo and Fernandez-Teran, 1987). Dividing cells exist in the base of the trabeculae and are "pushed" up to form the projections (Luo et al., 2001; Radice et al., 1997). As cardiomyoctes move up the trabeculae, they cease proliferation and differentiate, as defined by the expression of structural and contractile specific genes and cessation of mitosis (Challice and Viragh, 1974; Mikawa et al., 1992; Thompson et al., 2002). As trabeculae mature, they compact to form the thick myocardial wall of the adult heart (Mikawa et al., 1992). Data suggest that neuregulin signaling from the endocardium and the erbB2 and erbB4 receptors of the ventricular trabeculae are involved in myocardial compaction and maturation (Birchmeier and Zhao, 1995; Gassmann, 1995; Kasahara et al., 1998; Lee et al., 1993). Proliferation continues in the maturing myocardium, and contractile and structural gene expression occurs throughout heart development. All myocytes cease proliferation and permanently withdraw from the cell cycle, or terminally differentiate, a few days after birth (myocyte development reviewed in Sawyer et al., 1998). Rb family members are thought to have a regulatory role in

cardiomyocyte proliferation and terminal differentiation (Kablar et al., 1998; Pexieder et al., 2000; Zacksenhaus et al., 1997).

The LEK Family of Proteins

Our laboratory discovered Lek1, the murine member of the LEK family of proteins. The identified members of the family include Lek1, CMF1, only found in the chicken, and the human protein, CENP-F/Mitosin. The name of the family comes from the amino acid composition of the proteins comprising of approximately 40% leucine (L), glutamic acid (E), and lysine (K). All the proteins are relatively large in size (> 300 KD) and have similar domain structures. The N-terminus is comprised of numerous leucine zippers. The central region contains a spectrin repeat along with leucine zippers and is predicted to fold into a coiled coil structure (Goodwin et al., 1999). The C-terminus contains a functional nuclear localization sequence (NLS), leucine zippers, an atypical Rb binding site, a helix-loop-helix (HLH) dimerization domain, and a farnesylation domain (Figure 3; Goodwin et al., 1999; Liao et al., 1995; Redkar et al., 2002; Zhu et al., 1995a). Even though the family members all share structural similarities and are relatively large in size, their subcellular localizations and functions during cellular processes are diverse.

The human members of the LEK family are CENP-F/Mitosin, and initially, two laboratories used different methods to identify the same protein (Liao et al., 1995; Zhu et al., 1995b). Mitosin was identified first because of its ability to bind Rb, while CENP-F was identified as a human autoimmune antigen in patients with systemic autoimmune diseases. They are two proteins that are nearly

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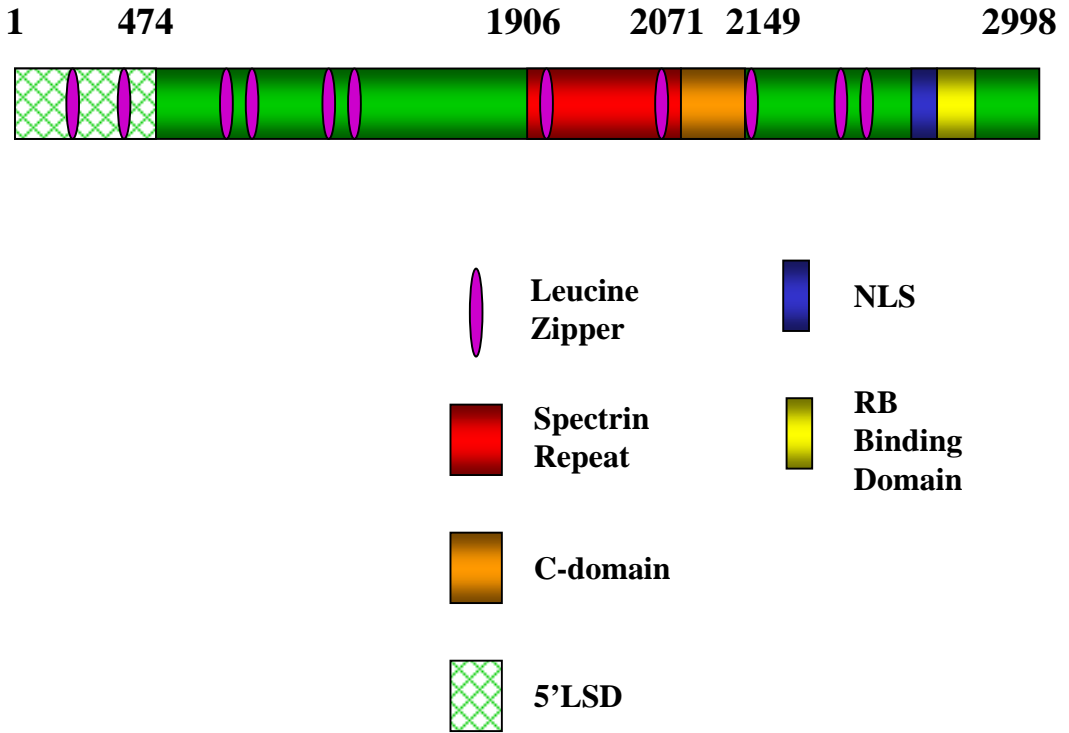
Figure 3. Structure of LEK family of proteins. The family includes Lek1 (mouse), CENP-F/Mitosin (human), and CMF1 (chicken). All are relatively large in size and share similar domain structures, yet they display divergent functions.

identical in sequence, but they have divergent C and N termini. They will be referred to as CENP-F/Mitosin for the purposes of this document. CENP-F/Mitosin expression is cell cycle dependent and one of the earliest proteins to assemble at the kinetochore. Protein accumulates in late S phase and peaks in early M phase (Zhu et al., 1995b) and is highly expressed in various malignancies (Landberg et al., 1996). Such an expression pattern makes it a useful cancer marker (de la Guardia et al., 2001; Erlanson et al., 1999; Rattner et al., 1997; Shigeishi et al., 2005). CENP-F/Mitosin demonstrates dynamic subcellular localization patterns during mitosis. It is a nuclear protein that associates at the kinetochore plate and spindle apparatus in early M phase, localizes to the spindle midzone in anaphase, the midbody in telophase, and then is completely absent at the end of cell division (Liao et al., 1995; Zhu et al., 1995a; Zhu et al., 1995b). The protein also contains a farnysalation site, a CAAX domain required for proper nuclear envelope and kinetochore localization. The domain is required for proper M phase progression and protein degradation (Ashar et al., 2000; Hussein and Taylor, 2002). Some oncogenes, for example the Ras protein, also undergo farnesylation, which is important for the protein's biological activity. Regulation at such sites is being investigated for possible therapeutic development (Russo et al., 2004).

CENP-F/Mitosin associates with the kinetochore through a core region that all LEK family members have, at least to some degree (Zhu, 1999). The kinetochore is required for microtubule (MT) attachment and proper M phase progression during the cell cycle (Biggins and Walczak, 2003; Cleveland et al.,

2003; Maiato et al., 2004). Kinetochores are macromolecule structures composed of many different proteins that assemble into a highly organized complex at the centromeres of chromosomes during mitosis. They are important for MT attachment and maintance during chromosome segregation. Proper cell division is dependent upon accurate kinetochore assembly. As a result, kintochores are critical at the G2/M checkpoint. Studies over the last few decades have begun to define and characterize the molecular composition of the kinetochore-centrosome complex during mitosis and describe the segregation event.

Several reports demonstrate chromosome condensation or misalignment when Cenp-F/Mitosin is depleted (Bomont et al., 2005; Holt et al., 2005; Yang et al., 2005). Reports show that altering endogenous CENP-F/Mitosin function delays cell cycle progression, because microtubule (MT) dynamics and attachment at the kinetochore are affected (Bomont et al., 2005). Studies differ in what checkpoint is activated to cause the delay. In relation to our studies, CENP-F/Mitosin also interacts with Nudel/NudE, a protein that associates with dynein and the MT network through the Lis1 pathway (Soukoulis et al., 2005; Yan et al., 2003). Studies have identified this interaction as being critical for MT dynamics. Lastly, during interphase, mitosin remains nuclear and appears to sequester and/or activate certain transcription factors (Zhou et al., 2005). CENP-F/Mitosin is a multifunctional LEK protein that has a dynamic localization pattern throughout the cell cycle and is vital for cell cycle progression. It has critical functions in cell cycle progression, which is a MT-based process.

The chicken family member, CMF1, shares similar structural properties as the other LEK family members but demonstrates a divergent expression pattern from that of other LEK proteins. CMF1 was identified from a chicken expression library utilizing an antiserum to the basic helix-loop-helix domain of MyoD, an essential muscle transcription factor (Wei et al., 1996). Like other family proteins, it also contains a functional Rb binding domain (Redkar et al., 2002). Unlike other LEK roteins, the distribution of CMF1 appears to be restricted to skeletal and cardiac muscles during development, as it is highly expressed during early stages of embryogenesis and drops dramatically after embryonic day 7 (Pabon-Pena et al., 2000). It has not been detected in the adult, so it is postulated that the protein is critical for myocyte development, a shared function of mouse Lek1 (Papadimou et al., 2005).

CMF1 has been determined to have a functional nuclear localization sequence (NLS) by utilizing chimera reporter proteins (Redkar et al., 2002). Yet, it is observed predominantly in the cytoplasm during myocyte proliferation and differentiation. *In vitro* studies have shown nuclear CMF1 in primary myoblast and then a cytoplasmic distribution after differentiation (Dees et al., 2000; Pabon-Pena et al., 2000), however one group does report nuclear CMF1 in the developing embryo (Redkar et al., 2002). Analyses examining CMF1 expression patterns also show that it precedes the expression of sarcomeric myosin heavy chain, a marker for differentiated myocytes (Bader et al., 1982). Notably, inhibition of the endogenous protein results in a substantial decrease in sarcomeric heavy chain expression, indicating an inhibition of myocyte
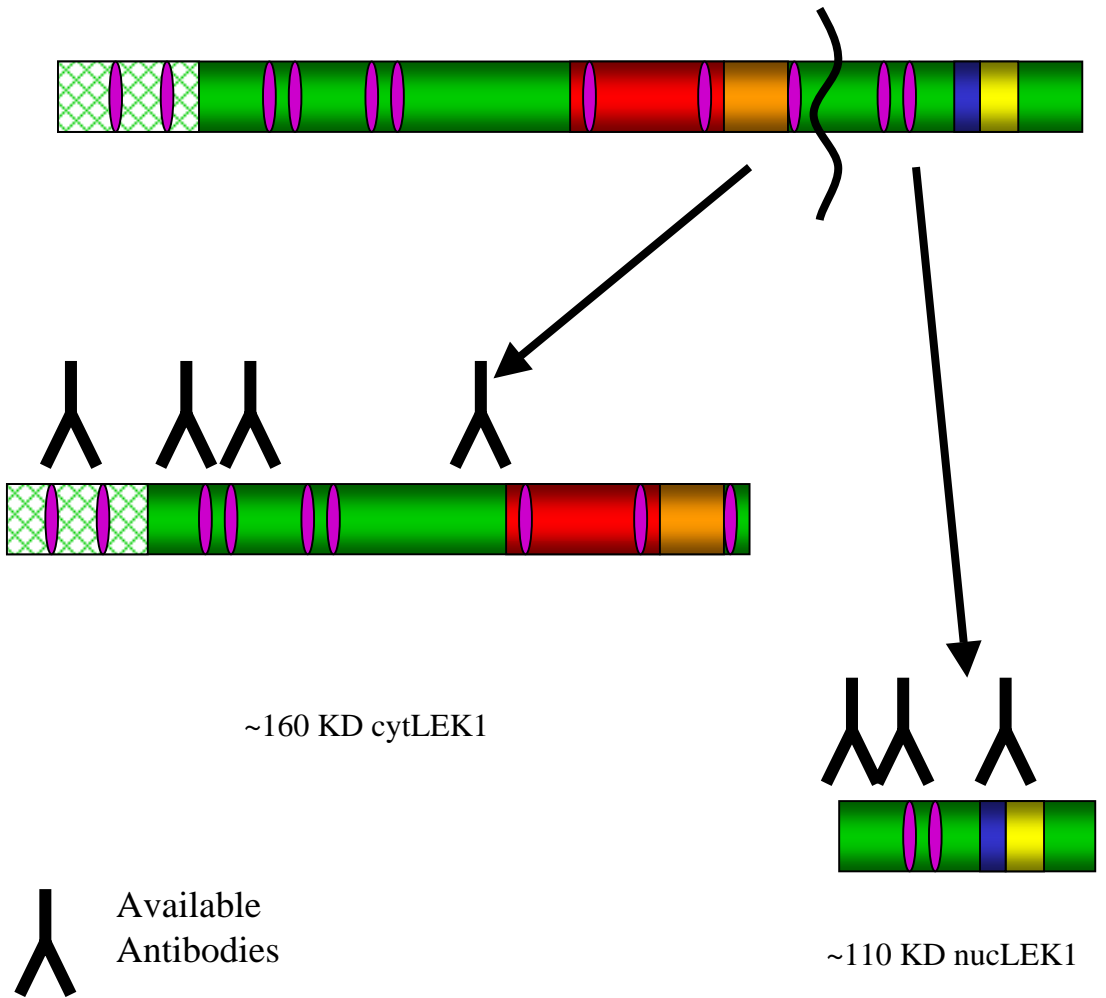
Figure 4. Lek1 undergoes a post-translational modification. The event produces an N-terminal cytLEK1 that localizes to the cytoplasm, while the C-terminal nucLEK1 immediately localizes to the nucleus. We have four available antibodies to cytLEK1, while we have three for nucLEK1. These antibodies are important for characterizing the Lek1 knock-out (Chapter V).

differentiation (Dees et al., 2000; Wei et al., 1996). Therefore, it is proposed that CMF1 plays an important role in the proliferation and differentiation of skeletal and cardiac myocytes, which is also a proposed function of the mouse family member Lek1 (Papadimou et al., 2005).

The mouse member of the LEK family of proteins is Lek1. It differs from other family members in its expression pattern and appears to have divergent cellular functions. *Lek1* was originally cloned utilizing reverse transcriptase polymerase chain reaction (RT-PCR). Degenerative primers based on conserved sequences of the LEK family were used against an E9.5 mouse heart library (Goodwin et al., 1999). The resulting clone fragments were then used to screen a E8.5 whole mouse cDNA library. Southern blot analyses demonstrated that there was a single copy of *Lek1* in the mouse genome, while northern blot analysis showed two bands approximately 10 kB, indicating that the mRNA may be alternatively spliced. Unique to the family, Lek1 undergoes post-translational modification. Previous studies in our laboratory have demonstrated that there is a post-translational cleavage event by an unknown enzyme at an unknown site that produces an N-terminal nucLEK1 and a C-terminal cytLEK1 (Figure 4). Early studies concentrated on nucLEK1 function in the nucleus (Ashe et al., 2004; Goodwin et al., 1999).

Northern blot and immunolabeling experiments demonstrated the expression pattern of Lek1 in the developing embryo (Figure 5). The highest levels of mRNA expression are at E8.5 in the whole embryo, and E9.5 in the head and caudal regions posterior to the heart. Interestingly, Lek1 message
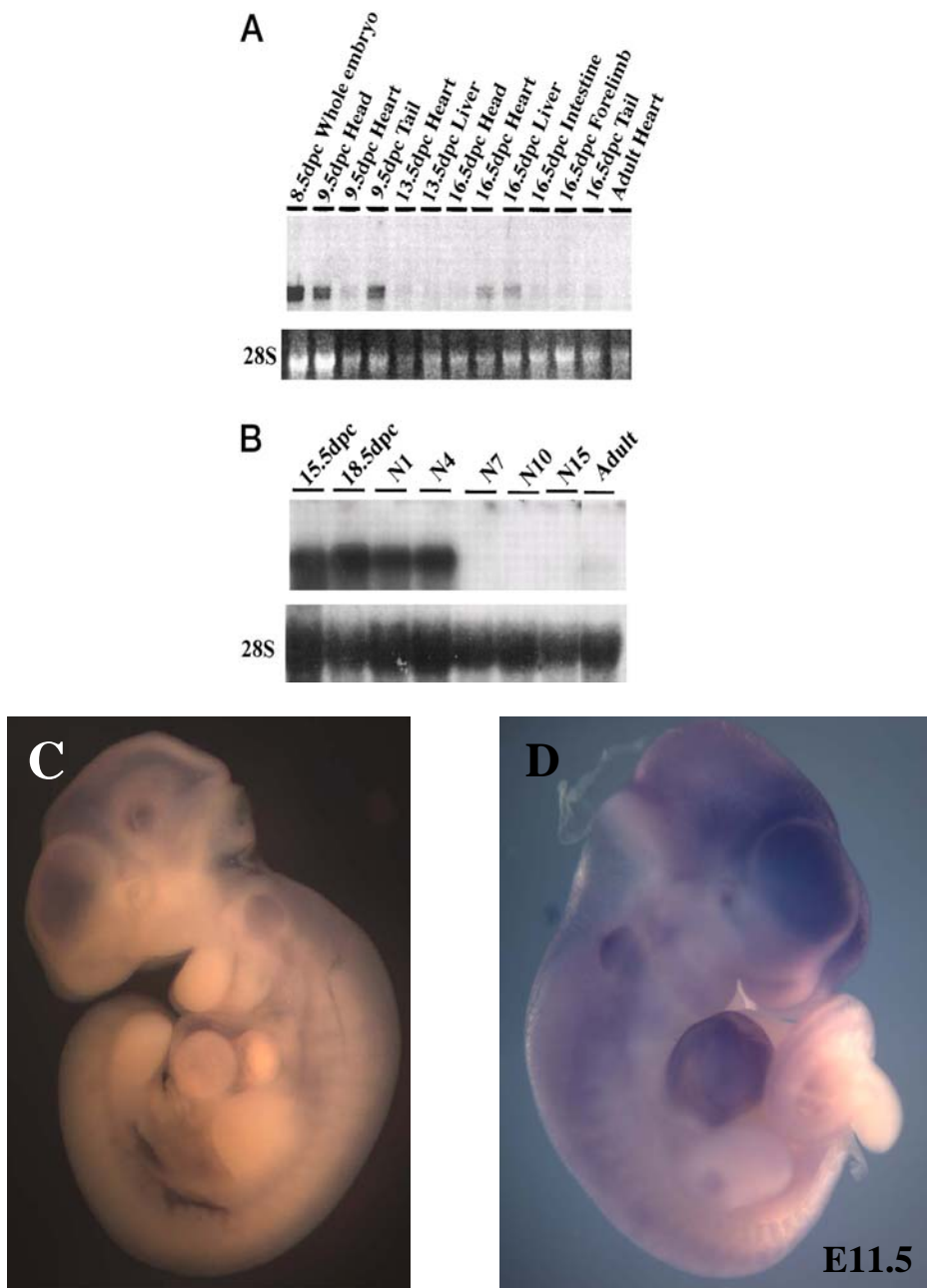
Figure 5. mRNA expression during development. A) Northern blot analysis demonstrates that Lek1 mRNA expression is highest at E9.5 in the developing mouse. It also shows that the heart and liver retain high levels of expression at E16.5. **B)** Transcript expression is high until N4 and is then severely down regulated and not found in the adult (A-B: Goodwin et al., 1999). **C-D)** *In situ* analyses at E11.5: The control sense-probe shows no significant background **(C)**, while the antisense-probe demonstrated high expression in the developing heart and in the brain.

increases in the heart and liver through development, while in other organs it decreases as the embryo ages. In the heart specifically, Lek1 transcript is detected at high levels until P4 and then disappears by P7. nucLEK1 protein expression demonstrates a similar pattern and is not found in the heart post N5 (Goodwin et al., 1999). Coincidently, this is the same time in which cardiomyocytes permanently exit the cell cycle and terminally differentiate (Soonpaa and Field, 1998; Soonpaa et al., 1996). From these initial data, we postulated that Lek1 has a role in the regulation of proliferation and differentiation during cardiogenesis.

In order to test our hypothesis for a Lek1 function in cardiomyocyte proliferation and/or differentiation, the Rb binding domain of nucLEK1 was examined because of Rb's role in these processes (Ashe et al., 2004). NucLEK1, like other LEK family members (Redkar et al., 2002; Zhu et al., 1995b), binds all Rb family members, Rb, p107, and p130. NucLEK1 was found to associate with both transiently expressed and endogenous Rb proteins, through the "pocket domain" of nucLEK1 (Ashe et al., 2004). Other proteins, such as the viral oncogenes E1A, E7, and T-antigen, and E2Fs, have been found to interact with Rb proteins through their pocket domain (Knudsen and Wang, 1997). Rb regulators bind the pocket region of Rb to interfere with protein interaction (Resnitzky et al., 1994). Rb family proteins have important functions in cell cycle regulation, and its roles in the control of differentiation and apoptosis are being elucidated (Yee et al., 1998). We propose that nucLEK1 binds Rb family proteins, therefore inhibiting their function. The interaction maintains cells

in a proliferative, non-differentiative state during embryogenesis. nucLEK1 is then downregulated, Rb can bind proliferative factors, and differentiation proceeds.

To test our hypothesis, experiments were conducted that disrupted Lek1 function. Morpholino (MO) antisense oligomer technology was utilized to test knock-down of Lek1. NIH 3T3 fibroblasts were depleted of Lek1, presumably activating Rb family member function, and the cell population arrested in G1/S (Ashe et al., 2004). Therefore, we conclude that Lek1 is required for NIH 3T3 cell proliferation. Evidence also suggests that Lek1 is required for C2C12 differentiation. C2C12 cells express Lek1 in their proliferative, non-differentiated state. In C2C12 cells that are depleted of Lek1, cells form myotubes and accumulate sarcomeric myosin heavy chain expression, indicating that the cells have differentiated (Ashe and Bader, unpublished data). These data on Lek1 support a role for its interaction with Rb family proteins resulting in regulation of proliferation and differentiation during embryogenesis.

cytLek1, Nde1, and the Microtubule Network

cytLEK1 is the N-terminal portion of Lek1 that immediately localizes to the cytoplasm (Pooley et al., 2006; Soukoulis et al., 2005). It contains a spectrin repeat that has been shown to be important for cytoskeletal interaction (Djinovic-Carugo et al., 2002). Since cytLEK1 contains this repeat and the function(s) of this cleavage product were unknown, it was initially hypothesized that cytLEK1 may have a role with the cytoskeletal network. A yeast-two hybrid (Y2H) screen was performed utilizing the spectrin repeat region and 5' and 3' flanking

sequences of cytLEK1 as bait to screen an E17.5 embryonic mouse heart library. Out of several sequenced clones, Nde1 (formally NudE), a member of the Lis1 pathway, was identified most often (Soukoulis et al., 2005).

Nde1 and Nudel are protein isoforms and mammalian homologs of the *nude* gene in *A. nidulans* and are members of the Lis1 pathway (Efimov and Morris, 2003). They are highly conserved among mammals, demonstrate similar expression patterns, and have overlapping functions. Yet it is unclear what their separate roles may be. Only Nde1 will be referred to for the purposes of this document. Studies examining Nde1 mRNA and protein expression patterns show that it is found at high levels in the developing brain, heart, skeletal muscle, and testes (Feng et al., 2000; Niethammer et al., 2000; Sasaki et al., 2000; Yan et al., 2003). Expression is first detected during embryogenesis at E11 and peaks five days later when it slowly decreases and is found at low levels in the adult (Feng et al., 2000). Data suggest that Nde1 function is important during embryogenesis, and its function in the adult remains elusive. Lek1 has a similar expression pattern, and since we identified it as a cytLEK1 interacting protein in our Y2H screen, we initially speculated that cytLEK1 and Nde1 may function together in a common pathway.

Nde1 was originally discovered by a Y2H screen utilizing Lis1 as bait (Feng et al., 2000). It is also interesting to note that Y2H screens conducted by other groups utilizing the LEK family protein CENP-F/Mitosin as bait also identified Nde1 as an interacting protein (Feng et al., 2000; Yan et al., 2003). Subsequent studies on the Nde1-Lis1 interaction revealed additional interaction

with the microtubule associated motor dynein (Feng et al., 2000; Niethammer et al., 2000; Sasaki et al., 2000).

In most cells, Nde1 has a cytoplasmic distribution with high perinuclear expression pattern and significant localization at the centrosome (Feng et al., 2000). It must be pointed out that Nde1 localization is not exclusive to the centrosome, as it does localize throughout the perinuclear region and in the cell periphery, similar to cytLEK1. Nde1 has also been co-immunoprecipitated with γ-tubulin, a key component of the centrosome (Feng et al., 2000). cytLEK1 colocalizes to a high degree with the Lis1 pathway members Nde1, Lis1, and dynein (Figure 6; Soukoulis et al., 2005). Colocalization between the proteins is not absolute, and this result is expected given that all these proteins have been shown to have multiple functions. Further subcellular localization experiments showed that Nde1 colocalized with dynein on mitotic spindles and on the kinetochore (Feng et al., 2000; Yan et al., 2003). Overexpression of Nde1 in mammalian cells significantly disorganizes the microtubule network, while the centrosome is altered and dispersed (Feng et al., 2000). From these data, it is speculated that Nde1 association with Lis1 and dynein functions in microtubule processes and centrosome formation. Even though the microtubule network, dynein, and associated Lis1 pathway proteins are well-studied, precise regulation of the network remains poorly understood.

Since it is postulated that Nde1 functions in MT based processes through the Lis1 pathway, such activities were further characterized. Dynein is MT motor that is essential for the positioning of many organelles, vesicles, proteins, and
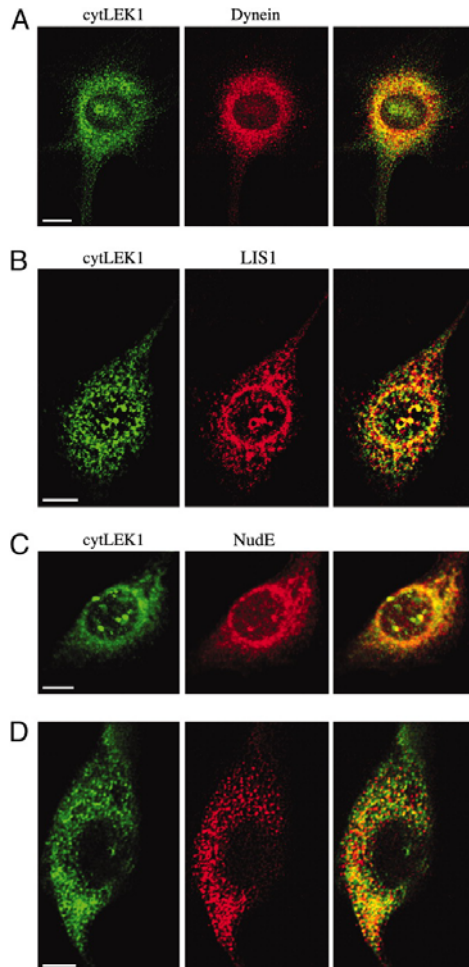
Figure 6. cytLEK1 colocalizes with Lis1 pathway proteins. Confocal microscopy was utilized to examine endogenous proteins in 3T3 fibroblasts. CytLEK1 is in green, while colocalizing proteins are visualized in red. **A)** CytLEK1 and dynein have a cytoplasmic distribution along with a high perinuclear distribution. The merged image demonstrates a high degree of colocalization between cytLEK1 and dynein. **B)** CytLEK1 is more diffuse than that of Lis1, but they still share a high degree of overlap, especially in the perinuclear region. **C)** Both cytLEK1 and Nde1 (formally named NudE) demonstrate significant colocalization as seen in the merged image. **D)** Deconvolution analysis of confocal data shows a more detailed localization of cytLEK1 and Nde1 (NudE1). Figure adapted from Soukoulis et al., 2005.

RNAs near the nucleus. Mitosis is a key process that is dependent on dynein. During the cell cycle, it has a wide set of functions including chromosome alignment, spindle orientation, and the movement of chromosomes toward opposite poles (Banks and Heald, 2001; Gibbons, 1996; Hirokawa et al., 1998). Important for our studies in characterizing Lek1 function in the following chapters, dominant-negative experiments have shown that altering proteins in the Lis1 pathway fragment and disperse lysosomes, endosomes, and Golgi. These effects were specific to dynein-mediated processes, because vesicles relying on kinesin motors were unaffected. Knock-down of Nde1 by RNAi supported these results (Liang et al., 2004b). Further experiments utilizing RNAi also show accumulation of MT near the nucleus, implying that the Lis1 pathway is required for MT transport to the periphery. The MT network also forms a connection from the cell cortex to the centrosome and nucleus. During cell migration, cells depleted of Nde1 have an increased distance between the nucleus and centrosomes (Shu et al., 2004; Smith, 2000). It is now apparent that Nde and the Lis1 pathway are required for such MT-based processes. It must also be noted that extensive studies have characterized the pathway in neuronal development and have shown that it is critical for proper neuronal development and function. In fact, mutations to this pathway are responsible for "smooth brain", or lissencephaly (Reiner et al., 1993). Studies describing Lis1 function in other tissues are more obscure.

The cytLEK1-Nde1 binding domains have been defined in each protein utilizing Y2H and coimmunoprecipitation studies. The necessary and sufficient

region in cytLEK1 to bind Nde1 is amino acids (aa) 2071-2149 and is referred to as myc-C (refer to Figure 3; Soukoulis et al., 2005). Interestingly, myc-C colocalized with Nde1 and γ-tubulin at the centrosome. This is of great interest, since Nde1 appears to have a function in establishing and maintaining centrosome integrity, a known function of the LEK family of proteins. When transfected into mammalian cells, myc-C acted as a dominant-negative protein. Experiments demonstrated that cells had a collapsed MT network, therefore also having a rounded-up phenotype. Interestingly, these results phenocopy what is observed in knock-down studies of Lis1 pathway members (Shu et al., 2004; Smith, 2000). As one might expect, myc-C transfected cells showed altered distribution of endogenous dynein, Lis1, and Nde1, confirming a cytLEK1 function in the pathway. Myc-C conferred detergent resistance and remained attached to the cytoskeleton, further demonstrating association with the MT network. Overexpression of myc-C also inhibited microtubule repolymerization after addition of nocodazole, while knock-down of cytLEK1 resulted in tight perinuclear focusing of microtubules around the nucleus. Both phenotypes are seen when altering proteins of the Lis1 pathway. From these data, we postulate that the myc-C region of cytLEK1 is responsible for its association and functions with the MT network through Nde1 and the Lis1 pathway.

SNAREs

Studies over the last 30 years have described the molecular mechanisms responsible for intracellular protein transport. Proteins traffic between

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membranous organelles which include the endoplamic reticulum, Golgi apparatus, endosomes, lysosomes, and the plasma membrane. In order for proteins to move between these membrane bound compartments, a series of budding and fusion events must occur between donor and acceptor membranes.

The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNAREs) family of proteins is responsible, at least in part, for membrane targeting and fusion (Figure 7; Sollner et al., 1993b). The SNARE superfamily is comprised of at least 24 known yeast members and more than 35 proteins in mammals (Bock et al., 2001). The SNARE hypothesis is proposed to explain how membrane proteins can traffic specifically from one compartment to the other; each type of transport organelle carries a v-SNARE and binds to particular t-SNARE on the apposing membrane. SNARE proteins can function in multiple trafficking steps, some members can substitute for other family members, and multiple SNAREs are found at any particular compartment (Brandhorst et al., 2006; Darsow et al., 1997; Gerrard et al., 2000; Liu and Barlowe, 2002; Thorngren et al., 2004).

Most of the SNARE family of proteins are from the SNAP (Synaptosomal-associated protein of 25KD), VAMP (vesicle-associated membrane proteins), or the syntaxin subfamilies. They all vary widely in size and structure, but they all share one characteristic in that they contain a SNARE motif (Bock et al., 2001). The SNARE motif contains 60-70 amino acids (aa) that include eight heptad repeats typical for coiled coils. Most SNAREs have a C-terminal transmembrane
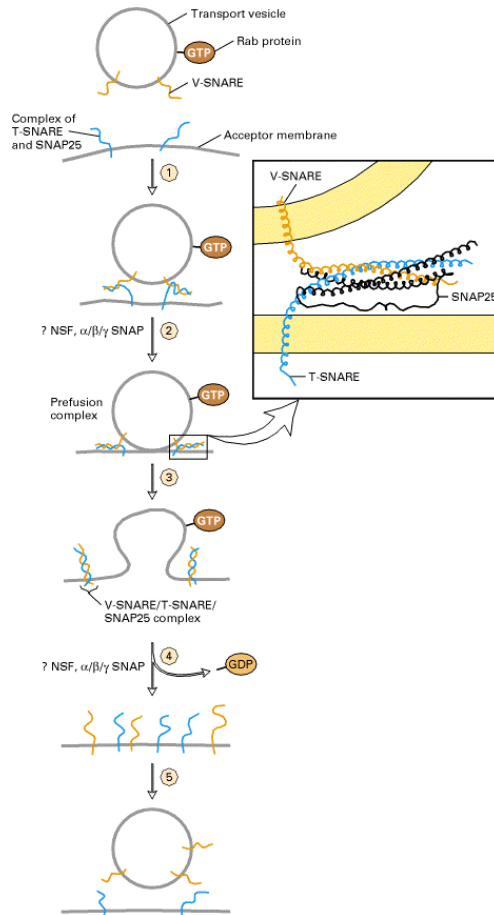
Figure 7. The SNARE complex. Formation of the SNARE complex probably requires interaction between the helical domains of SNAP-25, VAMP, and syntaxin. Immediately following complex formation, apposing membranes fuse *(Molecular Biology of the Cell*, 4th Edition).

domain (syntaxins and VAMPs), and others have a post-translational modification that anchors them to the membrane (SNAP).

As membranes fuse, SNARE proteins on opposite membranes associate into a core complex. It is mediated by SNAP motifs and when they come into contact, large conformational changes result. A SNARE complex is formed by the interplay of four SNAP motifs in parallel direction; two of the motifs are supplied by a SNAP protein, one by syntaxin, and the last by VAMP (Figure 7; Fasshauer et al., 1997; Sutton et al., 1998). Assembly is thought to proceed in a zipper-like fashion as SNAREs in the apposing membranes pull the membranes closer together and form an extremely stable complex (Hanson et al., 1997; Lin and Scheller, 1997). Other regulators, such as NSF and soluble NSF- attachment proteins are responsible for the subsequent disassembly of the SNARE complex (Sollner et al., 1993a). *In vitro* data show that SNAREs are not predetermined in their appropriate binding partners. There appears to be some degree of promiscuity, as some SNARE complexes are more stable than others (Fasshauer et al., 1997; McNew et al., 2000; Parlati et al., 2002; Paumet et al., 2002; Yang et al., 1999). To date, it is under debate in the specificity of SNARE complex formation in various membrane fusion events and exactly which proteins bind with one another *in vivo*. Important questions remain on the complex's function: Is the complex involved in only recognition of the apposing membranes? Or, is it required for the entire fusion event? SNARE proteins on one membrane may or may not encode in themselves which SNAREs they bind on the opposite membrane. Also, SNAREs may or may not be confined to

specific compartment(s). Even though enormous progress has been made, many important questions remain.

Not surprisingly, numerous accessory and regulatory proteins to SNAREs have been identified. Tethers link fusing membranes before SNARE protein interaction (Guo et al., 1999). These include COG complexes and golgins of the Golgi apparatus and EEA1 of early endosomes (Christoforidis et al., 1999; Whyte and Munro, 2003). Tethers function with Rab family GTPases to promote the initial association of fusing membranes. Just as with SNARE proteins, multiple Rabs have been identified at different steps of trafficking (Jahn et al., 2004). As we have now begun to understand, SNAREs, tethers, and Rabs all have critical roles in the intricate details of membrane fusion and protein trafficking within the cell.

SNAP-25

SNAP-25 is most well understood for its role in neurotransmitter exocytosis. It is a member of the same family of proteins as SNAP-23 (Ravichandran et al., 1996) and SNAP-29 (Steegmaier et al., 1998). Classically, SNAP-25 is thought to be predominantly neuronal, whereas the other two members are their supposed non-neuronal counterparts. Important for our studies, there is an emerging field of evidence that shows SNAP-25 functions in intracellular membrane trafficking of non-neuronal cell types.

SNAP-25 is a hydrophilic protein that consists of 206 aa (Figure 8). Palmitic fatty acid groups are added to four central cysteine residues, and it is

SNAP-25

A

CCCC

1   42   45   90   157   205

B

1   191   298
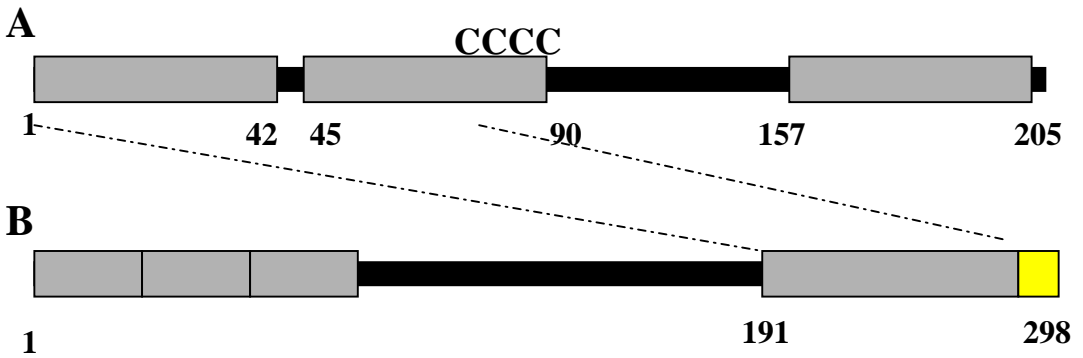
syntaxin 4

Figure 8. SNAP-25 and syntaxin 4 structure. SNAP-25 (**A**) is a protein of 206 aa, while syntaxin 4 (**B**) consists of 298 aa. The four cysteine residues are labeled on SNAP-25 and are the sites for palmitoylation. Amino acids 2-82 are responsible for syntaxin binding, while the N-terminal 107 aa of syntaxin 4 are required for SNAP-25 association. The areas in gray are intramolecular coiled coil regions of the proteins. The yellow region of syntaxin 4 is the transmembrane domain. These coiled coil regions of SNAP-25 and syntaxin 4 bind together, along with VAMP, and form the stable SNARE complex.

responsible for the association of SNAP-25 with membranes (Loranger and Linder, 2002). The amino and carboxy-terminal domains of SNAP-25 form an intramolecular coiled coil complex that associates with a VAMP and a syntaxin family member during membrane fusion (Chapman et al., 1994). An important tool in studying SNAP-25 function was the identification of botulinum neurotoxins A and E as SNAP-25 cleaving compounds. They have been beneficial in the studies of SNARE function in membrane fusion as a way to study SNAP-25 loss-of-function (Sakaba et al., 2005). SNAP-25 consists of 8 exons, and alternative splicing of exon 5 creates two variants, SNAP-25A and B. SNAP-25B is the predominant adult isoform (Bark et al., 1995).

Even though more than 35 mammalian SNARE proteins exist, only a few are involved in exocytosis and the rapid release of neurotransmitters. SNAP-25 was identified from neuronal specific mRNA, and with functions in neurons being the most well-studied. For neurotransmitter release to occur, cytosolic synaptic vesicles (SV) must dock and fuse with the presynaptic membrane after nerve membrane depolymerization. VAMP 1 and 2 are integral SV proteins, while syntaxins 1 and 2 are bound to the presynaptic membrane. SNAP-25 associates with syntaxins at the cytosolic face of the nerve membrane. SNAP-25, VAMP, and syntaxins provide the four coiled-coils to form the SNARE complex during membrane fusion (Montecucco et al., 2005). With only a few SNAREs identified as being functional components of neurotransmitter release, other SNARE proteins have also shown to be critical for intracellular membrane fusion events in other cell types.

SNAP-23 is considered to be the predominant non-neuronal SNAP family member (Ravichandran et al., 1996), but additional studies identify SNAP-25 as also being important for intracellular vesicle organelle fusion events. Outside of the brain and neurons, SNAP-25 has been identified in gastric parietal cells (Karvar et al., 2002), osteoblasts (Bhangu et al., 2003), melanocytes (Scott and Zhoa, 2001), adipocytes (Jagadish et al., 1996), cells from kidney collecting ducts (Shukla et al., 2001), and relevant for our studies, in myocytes of the heart and muscle (Jagadish et al., 1996; Ma et al., 2005; Sevilla et al., 1997). More importantly, these studies describe intracellular SNAP-25 localization and function as being important for vesicle trafficking.

SNAP-25 is mostly located at the membrane in neurons, but a large intracellular pool of SNAP-25 still exists. SNAP-25 has been localized to sorting vesicles (SVs), along with the early endosomes marker EEA1 (Waite et al., 1998), indicating a function in proper trafficking and localization of neurotransmitter carrying SVs to the plasma membrane (Selak et al., 2004; Walch-Solimena et al., 1995). Reports also state that SNAP-25 binds directly with the Hrs protein in the early endosomes of neuronal cells (Komada and Soriano, 1999; Sun et al., 2003; Tsujimoto et al., 1999). Hrs has been shown to interact with numerous proteins implicated in membrane trafficking (Bean et al., 2000; Bean et al., 1997; Chin et al., 2001). The SNAP-25-Hrs association appears to be a negative regulator of homotypic early endosome fusion (Sun et al., 2003). Studies show that SNAP-25 has a role in vesicle trafficking from the plasma membrane to the sorting and recycling endosomes, and finally to the

Golgi in an ARF6-endocytic pathway (Aikawa et al., 2006; Prekeris et al., 1998). Critical for our studies, SNAP-25 was shown to associate with Rab11a and TGN38, markers for the recycling endosomes and *trans*-Golgi network (TGN), respectively. The role of SNAP-25 in exocytosis of neurotransmitters remains the most well studied and understood aspect of SNAP-25 function, but it is becoming evident that SNAP-25 also has a significant role in intracellular membrane and vesicle trafficking.

Our laboratory has created the conditional *Lek1* allele for protein ablation in a tissue specific and/or temporal manner (Chapter V). Therefore, results obtained from other knock-out studies conducted with Lek1 interacting proteins are beneficial in postulating the roles Lek1 may have in given molecular pathways. The SNAP-25 knock-out mouse was created by Washbourne et al (2001) that gave intriguing results for SNAP-25 function during mouse brain development. Knock-out mutant mice were not observed at birth, and as a result, they examined E17.5-18.5 mice. At this stage, mice were in a tucked position, were smaller in size, and failed in both spontaneous movement and reflexes in response to stimuli. The heart and other organs appeared normal. From their data, they concluded that SNAP-25 was not required for nerve-outgrowth and stimulus independent neurotransmitter release, which are functions dependent on the SNARE complex during membrane fusion events in the brain. Brain development proceeded normally, and the phenotypes were observed in fetus musculature, which are tissues that we have also shown to contain high levels of Lek1 expression. They speculate other SNARE proteins may be able to

compensate for SNAP-25 loss-of-function in the brain. They conclude that SNAP-25 is critical for evoked action potential synaptic transmission.

Syntaxin 4

Syntaxin 4 is one of 15 identified syntaxin proteins (Hong, 2005). It is a 298 aa protein, and along with SNAP-25, it is a SNARE protein involved in membrane docking and fusion. The syntaxin 1A and 1B isoforms were originally identified by their interaction with the synaptic vesicle protein synaptotagmin (Bennett et al., 1992). Soon, numerous non-neuronal family members were identified in various secretory and endosomal pathways (Bock et al., 2001). Syntaxins localize to multiple intracellular membranes functioning at membrane fusion interfaces (Figure 9). Syntaxin 4 was originally identified as being a cell-surface syntaxin (Bennett et al., 1993), but more recently has been identified in intracellular locations, such as Rab11a positive vesicles (Band et al., 2002; Torrejon-Escribano et al., 2002). All syntaxin proteins, except syntaxin 11, are transmembrane proteins anchored by C-terminal tails. The proteins contain several hydrophobic regions that form coiled coil structures. Most importantly for our studies, the ~60 aa long coiled coil SNARE region closest to the transmembrane domain is responsible for syntaxin association with SNAP-25 (Figure 6; Weimbs et al., 1997).

Through their SNARE domains, syntaxins and SNAP-25 proteins form t-SNARE complexes at target membranes. The t-SNARE complexes then interact
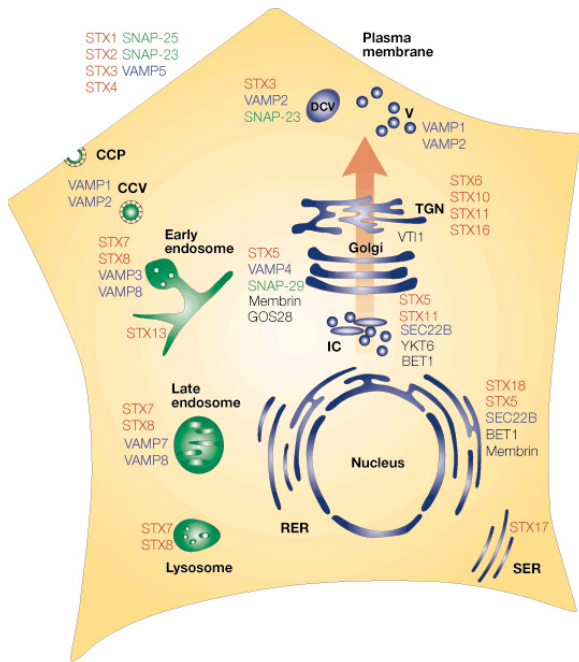
Figure 9. Syntaxin localization. Syntaxin family proteins (labeled STX) are found at multiple compartments throughout the cell. Syntaxin 4 was initially characterized by its localization at the cell membrane, but has been since localized to intracellular compartments. This is a diagram demonstrating the complexity of defining syntaxin localization, because they do function in multiple pathways. Not only does syntaxin 4 function at the cell surface, but it also localizes to intracellular Rab11a positive endosomes. Figure adapted from Chen et al., 2001.

with VAMP family members at vesicle membranes (Sollner et al., 1993b). Together they form the four coiled-coil helices of the SNARE complex.

Syntaxin 4 localizes to the basal lateral membrane in pancreatic β- cells and Madin-Darby kidney cells and is involved in plasma membrane trafficking from the *trans*-Golgi network (TGN) to basal lateral surface (Spurlin and Thurmond, 2006). Relevant for this document, syntaxin 4 has been localized to Rab11a positive recycling endosomes in NRK cells, a myeloma cell line (Band et al., 2002). Interestingly, it is a cell line that also highly expresses Lek1 (unpublished data).

GLUT4 vesicle fusion with the plasma membrane is an instrumental step in insulin-regulated glucose internalization and transport. In response to elevated blood sugar levels, the pancreas secretes insulin. Insulin-responsive tissues, suchas the liver, muscle, and adipose tissue, then internalize glucose in an insulin-dependent manner (Figure 10). GLUT4 primarily functions in cells that are responsive to insulin stimulation, namely adipocytes and muscle cells (Charron et al., 1989; James et al., 1989). In a non-induced environment, GLUT4 is localized to intracellular pools (Birnbaum, 1989; James et al., 1988; James et al., 1989). After a meal, GLUT4 positive vesicles quickly redistribute to the plasma membrane to function in uptake of glucose.

Data suggest the existence of an insulin-sensitive compartment: the GLUT4 storage vesicle (GSV). Exactly how these GSVs traffic to the cell membrane is under debate. Under basal conditions, the GSV pool colocalizes with TGN markers, and once stimulated, GSVs traffic to the cell membrane and
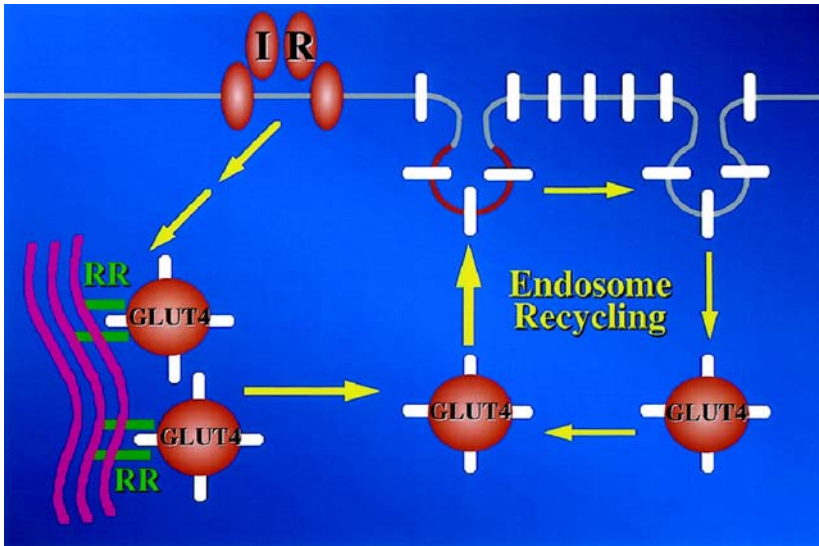
Figure 10. GLUT4 trafficking. Insulin released by β-cells stimulates an increase in muscle and adipocyte glucose uptake. Insulin binds to the cell surface Insulin Receptor (IR) that initiates a signaling cascade which results in trafficking of GLUT4 vesicles to the cell membrane. This catalyzes the uptake of glucose into the cell, and GLUT4 is recycled through intracellular membrane compartments. Rab11a is implicated in the recycling of GLUT4. Figure adapted from Pessin et al., 1999.

localize with VAMP2. Markers for early and late endosomes and lysosomes are largely devoid from the GSVs (Martin et al., 1998). Compartments that contribute to GSVs and the timing of such events in GSV trafficking are not well understood. There is evidence that once these specialized compartments arise from the TGN, GLUT4 cycles through recycling endosomes under basal conditions until they localize to the plasma membrane upon insulin stimulation (Bryant et al., 2002). An alternative model states that GSVs develop in a post-endosomal compartment derived from Rab11a-positive recycling endosomes (Malide et al., 1997; Zeigerer et al., 2002). What has become clear is that the Rab11a plasma membrane recycling pathway is important in GLUT4 trafficking (Kessler et al., 2000; Larance et al., 2005; Millar et al., 1999; Uhlig et al., 2005).

The importance of syntaxin 4 in vesicular transport is exemplified by the regulation of GLUT4-containing vesicles in skeletal muscle, cardiomyocytes, and adipose tissue after insulin stimulation (Bryant et al., 2002; Pessin et al., 1999). Of the non-neuronal syntaxin proteins, syntaxin 4 is the only member that interacts with VAMP2 (Cheatham et al., 1996; Kawanishi et al., 2000; Martin et al., 1998; Randhawa et al., 2000), and it is the VAMP family member implicated in GLUT4 trafficking to the cell surface. 3T3-L1 adipocyte treatment with dominant-negative peptides significantly reduces GLUT4 trafficking and glucose uptake. These data support that syntaxin 4 is the major syntaxin protein to function in insulin-dependent GLUT4 trafficking (Cheatham et al., 1996; Macaulay et al., 1997; Olson et al., 1997; Tellam et al., 1997; Volchuk et al., 1996).

The syntaxin 4 knock-out mouse line has been created (Yang et al., 2001). Similarly to the SNAP-25 knock-out, homozygous syntaxin 4 mutants are embryonic lethal, therefore experiments utilizing heterozygous mutants were examined. As with SNAP-25 mutant mice, the phenotypes in syntaxin 4 mutants were observed in muscle tissues. Surprisingly, no defects in insulin stimulated GLUT4 trafficking were seen in adipose tissue, a major reservoir of syntaxin 4 and model of its function (Charron et al., 1989; James et al., 1988; James et al., 1989). In contrast, a 50% reduction in skeletal muscle glucose transport was observed, while GLUT4 trafficking was also significantly reduced (Yang et al., 2001). These data show that no other SNARE proteins appear able to compensate for knock-down of syntaxin 4. Therefore, protein levels may be critical for glucose uptake in myocytes. Even with reduced levels of syntaxin 4 in heterozygous mutants, adipocytes appear to function. There still may be sufficient amounts of syntaxin 4 for glucose uptake in mutant cells, or another SNARE may replace lost syntaxin 4 function.

Interestingly, Spurin et al (2006) also performed studies utilizing pancreatic β-cells from heterozygous syntaxin 4 mutant mice, a cell type that has high syntaxin 4 expression (Jacobsson et al., 1994). Results from their experiments demonstrate that syntaxin 4 also functions in insulin release from β-cells. There are two phases to insulin release. First phase release is the membrane fusion of insulin containing granules already at the cell surface. Second phase incorporates insulin release from intracellular pools that need to be trafficked to the cell surface. Data show that mutant cells had a 50% reduction

in first phase insulin release, and a trend in reduction in second phase insulin secretion was also found. The most intriguing aspect of these studies, in relationship to our work with Lek1, is that β-cells also express high levels of SNAP-25 (Sadoul et al., 1995). Both SNAP-25 and syntaxin 4 have known interactions with cytLEK1 (Chapters II and III).

From the data that will be presented in the following chapters, we are able to make the following hypotheses on Lek1 function. Through the interaction of Lek1 with SNAP-25 and syntaxin 4, Lek1 is the first identified bridge that links recycling endosomes with the MT network. We have also created the conditional Lek1 allele, which has given intriguing results. It appears that ablating Lek1 functon early in heart development alters cardiomyocyte proliferation. These data support a role for the protein as a regulator of proliferation and differentiation. Mutant hearts also have a defect in electroconductivity. Therefore, we postulate that protein trafficking and recycling to the cell membrane may be affected in mutant cardiomyocytes. We show that Lek1 may be a critical regulator of protein trafficking and of proliferation and/or differentiation during heart development.

CHAPTER II

CYTLEK1 IS A REGULATOR OF PLASMA MEMBRANE RECYCLING
THROUGH ITS INTERACTION WITH SNAP-25

Introduction

The trafficking of proteins between organelles and the plasma membrane is mediated by transport vesicles that originate from a series of budding and fusion events between donor membranes and acceptor membranes. Vesicle docking and fusion is regulated, in part, by SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors), a class of coiled-coil proteins (Chen and Scheller, 2001; Jahn and Sudhof, 1999; Sollner et al., 1993b). SNARE proteins form coiled-coil aggregates that help link two opposing membranes for fusion (Chen and Scheller, 2001; Jahn and Sudhof, 1999; Sollner et al., 1993b). Endosome membrane fusion is also dependent on SNAREs (Braell, 1987; Gruenberg et al., 1989; Mullock et al., 2001; Salzman and Maxfield, 1988). The SNARE protein SNAP-25 (Synaptosomal-associated protein of 25 KD) is a member of this complex and participates in vesicle membrane docking and fusion. A role for SNAP-25 in membrane fusion of early endosomes has been previously documented, as disruption of SNAP-25 inhibits early endosome fusion (Braun et al., 2004; Sun et al., 2003). But, functions in other endosomal pathways, such as the recycling pathway, have not been well established.

In mammalian cells, the plasma membrane recycling system is critical in the maintenance and regulation of membrane proteins. Pumps, channels, receptors, and other membrane proteins are delivered to and removed from the membrane through this system. Studies have established that along with SNARE proteins, the Rab GTPase family is critical in this process. This family contains over 50 protein members and has been implicated in the formation, targeting, and fusion of transport vesicles (Casanova et al., 1999; Novick and Zerial, 1997; Ullrich et al., 1996; Wang et al., 2000). One member, Rab11a, is important in transferrin (Tf) receptor recycling through the perinuclear recycling system in nonpolarized cells (Green et al., 1997; Ren et al., 1998; Ullrich et al., 1996).

The Tf receptor is a glycoprotein that associates with the cell membrane and is critical in the regulation of iron uptake in a variety of cell types. Iron is indispensable for life in both heme and non-heme iron proteins in a wide range of cellular processes, including oxygen transport, electron transport, DNA synthesis, and nitrogen fixation. With the exception of mature erythrocytes and some terminally differentiated cells, Tf receptor is expressed in all cells but at varying degrees. Cells that express the highest levels of protein include immature erythrocytes, placental tissue, and rapidly proliferating cells (reviewed in Ponka and Lok, 1999). The Tf receptor is 85 KD and contains 760 aa, and each binds one Tf molecule. Diferric Tf has the greatest affinity for the receptor, monoferric Tf an intermediate affinity, and apotranferrin has the least (Young et al., 1984). A model of tranferrin-iron uptake is depicted in Figure 11. Once internalized, early
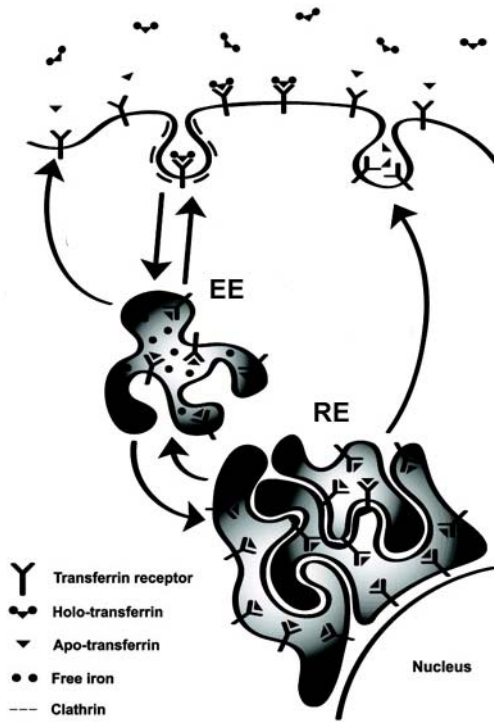
Figure 11. Transferrin uptake. In the first step, diferric Tf preferentially binds to the Tf receptor. Bound receptors then cluster into clathrin-coated pits. Second, the Tf receptor complexes are internalized. Third, iron is released from the receptor in endosomes by a temperature and energy-dependent process, which requires the lowering of pH within the vesicles. Tf receptor and apotransferrin are then recycled to the plasma membrane, with Rab11a and recycling endosomes (RE) having roles in the process. Figure adapted from Magadan et al., 2006.

(Jin and Snider, 1993), late (Sakai et al., 1998), and recycling endosomes (Ullrich et al., 1996) have all been shown to contain intracellular pools of the internalized Tf receptor complex. It remains unclear how the different intracellular pools vary in function. Rabs 4 (Daro et al., 1996), 5 (Bucci et al., 1992), 11 (Ullrich et al., 1996), and 22 (Magadan et al., 2006) have functions in endocytosis of the complex. Rab11a participates in recycling of the apotransferrin and the receptor from the perinuclear recycling endosome network to the plasma membrane.

Rab11a also regulates transcytosis and apical recycling of polymeric IgA receptor through the apical recycling system in polarized cells (Casanova et al., 1999; Wang et al., 2000). Furthermore, Rab proteins play a well-established role in docking of vesicles to their target compartment and in vesicle association with the actin cytoskeleton (Apodaca et al., 1994; Lapierre et al., 2001; Ullrich et al., 1996).

Previous studies have identified a number of Rab11a interacting proteins, one of which is myosin Vb, an unconventional myosin that is implicated as a motor protein for the transit of vesicles out of the plasma membrane recycling endosome pathway (Lapierre et al., 2001; Reck-Peterson et al., 2000). This is of particular interest because expression of a myosin Vb-tail chimera, which lacks the myosin motor and neck domains, colocalizes with Rab11a in perinuclear vesicles in HeLa cells and causes retardation of Tf trafficking, a model of plasma membrane recycling (Hales et al., 2002; Lapierre et al., 2001). Similar to transfection with myosin Vb chimeras, the expression of Rab11a mutants and truncations of Rab11a-interacting proteins block exit of Tf from the recycling

endosome vesicles (Hales et al., 2002; Junutula et al., 2004; Lapierre et al., 2001; Lindsay and McCaffrey, 2002; Ren et al., 1998).

Our laboratory has discovered Lek1, a relatively large protein of over 300 KD, which is a member of the LEK family of proteins (Goodwin et al., 1999; Mancini et al., 1995; Pabon-Pena et al., 1999). These proteins share similar structures that include numerous leucine zippers, a central spectrin repeat, an atypical Rb binding domain, and a nuclear localization sequence domain in its C-terminus (Ashe et al., 2004; Dees et al., 2000; Goodwin et al., 1999; Pabon-Pena et al., 1999). Even though the LEK family of proteins displays similar homology, they contain divergent domains and have varying expression patterns and functions.

Lek1 undergoes post-translational cleavage that produces two peptides: a C- terminal peptide that immediately localizes to the nucleus, termed nucLEK1, and an N- terminal peptide named cytLEK1 that distributes throughout the cytoplasm (Ashe et al., 2004; Soukoulis et al., 2005). Until now, studies on Lek1 function have focused on two areas: the role of nucLEK1 in cell division and differentiation (Ashe et al., 2004; Goodwin et al., 1999; Papadimou et al., 2005), and the function of cytLEK1 in regulation of cell shape through its association with Nde1 (formally NudE) and the microtubule network (Soukoulis et al., 2005). Important to the current study, Nde1 has been shown to bind Lis1 and dynein (Faulkner, 2000; Morris and Xiang, 2000; Smith, 2000). Both Lis1 and dynein interact with the microtubule network through the Lis1 pathway regulating membrane trafficking, organelle positioning, migration, and mitosis (Banks and

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Heald, 2001; Gibbons, 1996; Terada et al., 1998). Our laboratory has previously shown that dominant-negative protein expression and morpholino suppression of cytLEK1 function severely alters cell shape by interfering with the microtubule network (Soukoulis et al., 2005). Together, these data indicate a role of cytLEK1 with the Lis1 pathway and the microtubule network. However, the functions of cytLEK1, the Lis1 pathway, and the microtubule network in membrane trafficking and organelle positioning remain poorly understood.

In an effort to further define cytLEK1 function, the highly coiled N- terminal portion of cytLEK1 was used in a Y2H screen to identify novel interacting proteins. One of the binding proteins identified was SNAP-25. This interaction was consistent with the hypothesis that cytLEK1 plays a role in the dynamics of the cytoskeleton and in membrane trafficking. In the present study, we define the interaction domains within cytLEK1 and SNAP-25 that are responsible for association between the two proteins. Immunofluorescence and immunoprecipitation studies demonstrate that both transiently expressed and endogenous cytLEK1 and SNAP-25 proteins interact in a complex that also includes Rab11a, and myosin Vb, which are partners in plasma membrane recycling. The SNAP-25 interacting SNARE proteins vesicle-associated membrane protein 2 (VAMP2) and syntaxin 4 were also identified in this complex. Finally, we show that disruption of cytLEK1 function inhibits Tf trafficking, a model for plasma membrane recycling. Taken together with our previous data, the present study suggests that cytLEK1 provides a critical link

between recycling endosomes and the microtubule network through its association with SNAP-25.

Materials and Methods

Yeast Two-Hybrid Screen

The N-terminus of cytLEK1 (aa 1-689) was PCR amplified using a full-length cytLEK1 clone (aa 1-2210) containing restriction sites and ligated into pGBKT7 for use in the Matchmaker Y2H System 3 (BD Biosciences Clontech). The bait containing yeast were mated with a yeast strain pretransformed with a whole mouse embryonic day 17.5 cDNA library. Yeast colonies that survived on Quadruple Dropout Medium (QDO; SD/-Ade/-His/-Leu/-Trp/X-a-Gal) and exhibited lacZ expression were subjected to further testing. Colonies were then streaked several times to ensure plasmid segregation. Library plasmids were isolated and the inserts sequenced by the Vanderbilt Sequencing Core Facility and identified using NCBI Blast (Altschul et al., 1990). For each identified protein product, false positive tests involving empty vector and random protein matings were conducted to eliminate spurious interactions according to the manufacturer's recommendations.

Cell Culture and Transfection

COS-7, NIH 3T3, and C2C12 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium supplemented with 10, 10, and 20% FBS respectively,

100 ug/mL penicillin/streptomycin, and L- glutamine, in a 5% CO_2 atmosphere at 37^0C. Cells were grown to 50-75% confluency and transfected with DNA using FuGENE 6 (Roche) according to the manufacturer's recommendations.

Immunostaining and Microscopy

For transient protein experiments, cells were grown on glass chamber slides and transfected 24 h after passage. Cells for transient and endogenous studies were gently washed with 1X PBS and fixed with either Histochoice or 4% paraformaldehyde for 20 min. Cells stained for γ-tubulin were fixed with methanol at -20^oC for 15 mins. Cells were washed with 1X PBS, permeabilized with 0.25% Triton X-100 in 1X PBS for 10 min, and blocked for at least 1 h in 2% BSA in 1X PBS. Primary antibodies were diluted in 1% BSA and incubated overnight at 4^oC. Cells were washed 3 times in 1X PBS; secondary antibodies were added for 1 h at room temperature. Cells were again washed 3 times with 1X PBS and coverslips mounted with AquaPoly/Mount (PolySciences). Cells were visualized by fluorescence microscopy with an AX70 (Olympus), or for confocal analysis, with an LSM510 (Ziess) microscope. Images were captured and processed using Magnafire (Olympus) and Photoshop (Adobe). For deconvolution analysis, confocal Z stacks (0.5 um optical thickness) were utilized, using a blind 3D deconvolutional algorithm (AutoQuant Imaging). All images of control and experimental cells were processed identically.

Coimmunoprecipitation Using Transient Transfections

COS-7 cells were grown on 10 cm plates; proteins were harvested 48 h post transfection. The ProFound Mammalian c-Myc Tag Co-IP Kit (Pierce) was utilized according to manufacturer's protocol. Briefly, cells were washed once with ice-cold TBS, incubated with M-Per Extraction Reagent (Pierce) containing protease inhibitor (Sigma), and centrifuged at 16,000 X g for 20 min at $4^{o}C$. Lysate protein concentration of the supernatant was determined using a bicinchoninic acid solution assay (Pierce). 100 µg total lysate was incubated for 2 h at $4^{o}C$ with 10 µl anti-c-myc agarose slurry with gentle shaking at $4^{o}C$. Columns were washed 3 times with 1X TBS-Tween. Protein was eluted with 2X non-reducing sample buffer (Pierce) at $95^{o}C$ for 5 min. To reduce proteins for SDS-PAGE analysis and western blot analysis, 2 µl 2-mercaptoethanol was added. Ten µl of total lysate supernatant was used to confirm protein expression. Blots were developed using NBT-BCIP (Roche) and scanned into digital images (Hewlet-Packard).

Deletional Analysis

The cytLEK1 5'LCR (aa 1-689) and SNAP-25 yeast deletion constructs were created using a PCR approach and transformed into AH109 and Y187 yeast, respectively, for matings. The 5' LCR was further truncated into the N-terminal 5'LSD. Deletion constructs were created that combined various regions of these domains as shown in Fig. 12A and B. Colonies were grown on QDO Medium and tested lacZ expression to determine viable interactions. In order to

confirm results by coimmunoprecipitation in mammalian cells, the relevant cytLEK1 and SNAP-25 yeast plasmid inserts were cloned into the pCMV-myc and EGFP-C3 expression vectors (BD Biosciences Clontech) and used for transfection studies in COS-7 cells. Truncations of cytLEK1 appear in Fig. 10A and B as follows: aa 1-689, 1-540, 1-474, 1-364, 1-170, 171-689, 365-689, and 171-364. Truncations of SNAP-25 are as follows: aa 1-207, 1-102, 103-207, 1-75, 1-27, 20-102, 28-102, and 15-75.

Coimmunoprecipitation of Endogenous Protein Complexes Containing cytLEK1

NIH 3T3 cells were lysed with Nonidet P-40 buffer with gentle sonication. Whole cell lysates were recovered and samples containing 2-3 mg total protein were precleared with GammaBind Plus Sepharose (Amersham Biosciences) for 20 min at 4°C with gentle rotation. Cell lysates were collected and incubated overnight with 3 μg of a monoclonal SNAP-25 antibody (Sigma). GammaBind Plus Sepharose was added to bind the antibody-protein complex. Beads were washed 3 times with cold 1X PBS and proteins were eluted with Laemmli sample buffer at a boiling temperature for 5 min. Proteins were resolved on a 6% SDS-PAGE gel and analyzed by Western blot analysis.

Nocodazole Treatment

Cells were transfected with the appropriate plasmids and exposed to 100 μM nocodazole (Sigma) for 30 min at 4°C in the appropriate serum conditions (Soukoulis et al., 2005). Cells were then immunostained as described above.

Morpholino (MO) Antisense Oligomer Treatment

Methods used and the production and use of MO that specifically inhibits production and accumulation Lek1 have been previously reported (Ashe et al., 2004; Soukoulis et al., 2005).

Tf Trafficking

For Tf internalization studies, COS-7 cells were cotransfected with the binding domains of cytLEK1 and SNAP-25. 5'LSD was ligated into pVenus (Ibata et al., 2002) and SNAP-25 was ligated into pCerulean (Rizzo et al., 2004); both gifts from Dr. Piston, Vanderbilt Univeresity). Cells coexpressing both proteins could be analyzed. 24 h post transfection, cells were serum starved for 2 h with DMEM containing 0.2% BSA at 37^oC in CO_2. Cells were then incubated for 30 min with serum media containing 50ug/ml Alexa-633 Tf (Molecular Probes) at 4^oC to allow binding (Time-0). Labeled Tf was then allowed to internalize for 5, 10, and 20 min. Cells were then washed with 1XPBS, trypsinized, and resupended. The fluorescence intensity of cell-associated Alexa-633 Tf was measured by flow cytometry utilizing a BD LSRII (BD Biosciences; Vanderbilt Flow Cytometry Core). The mean intensity of each cell population (5000 cells) was recorded at each time point. The intensity of Alexa-633-conjugated Tf was gated by expression of Venus and Cerulean in cotransfected cells. Control mock transfected cells expressed EGFP (Clontech). The mean flourescense intensity was compared between cotransfected and mock transfected cell populations.

For the Tf recycling analysis, MO and Standard Control (SC) cell populations were allowed to bind and internalize labeled Tf for 30 min (described above). After internalization (Time-0), pulse labeled Tf was chased by addition of normal DMEM containing 10% FBS and analyzed at 5, 10, and 20 min after labeled Tf internalization. The mean fluorescence intensity was compared between MO and SC cell populations

Antibodies

cytLEK1, Rab11a, and myosin Vb antibodies were previously described (Lapierre et al., 2001; Soukoulis et al., 2005). Anti-cytLEK1 specificity has been tested by immune peptide competition and by selective loss of reactivity in conditional knockout of the *Lek1* gene in the developing mouse heart (Pooley and Bader, manuscript in preparation). Also, screening of lambda GT11 libraries with this antiserum identified only Lek1 transcripts (Pabon-Pena and Bader, unpublished data). SNAP-25, syntaxin 4, and γ-tubulin antibodies were obtained from Sigma. Golgin, p58, and giantin antibodies were obtained from Molecular Probes. α-myc and α-GFP antibodies were obtained from BD Bioscience. VAMP2 and VAMP3 antibodies were purchased from StressGen, and VAMP8 antibody was from Abcam. Alexa Fluor 488- and 568- conjugated secondary antibodies were utilized (Molecular Probes). For triple labeled immunofluorescence studies, polyclonal anti-myc (Novus) was directly labeled with the Zenon Alexa-647 labeling kit (Molecular Probes). Alkaline phosphatase-conjugated secondary antibodies for western blot were purchased from Sigma.

Results

Identification of SNAP-25 as a cytLEK1 interacting protein

A Y2H screen was used to identify novel cytLEK1 binding partners and further characterize cytLEK1 function. The region chosen as bait to screen an embryonic whole mouse cDNA library consisted of the N-terminal most 689 aa of cytLEK1 beginning at the translation start site (base pairs 1-2067; termed 5' LCR for cyt**L**EK **C**oil **R**egion; Figure 12). A PROSITE domain search of this region identified a highly coiled structure with a leucine zipper (Rutkowski et al., 1989). From the Y2H screen, four independent clones were found to contain the full coding sequence of SNAP-25, and all clones passed the false screening process. No other region of cytLEK1 tested thus far has shown interaction with SNAP-25. Since we have previously demonstrated that cytLEK1 has a function with the microtubule network (Soukoulis et al., 2005), and SNAP-25 is important for vesicular transport (Braun et al., 2004; Sun et al., 2003), we pursued this protein interaction to test whether cytLEK1 is involved in membrane trafficking.

Identification of cytLEK1 and SNAP-25 binding domains

In order to define the domain within the 5'LCR region of cytLEK1 that associates with SNAP-25, we employed a Y2H approach. A series of truncations of the 5'LCR region revealed a minimal region of cytLEK1 that was sufficient to bind the full-length SNAP-25. We termed this binding region as 5'LSD, for cyt**L**EK **S**NAP-25 Binding **D**omain (aa 1-474; Figure 12A-B). While all constructs
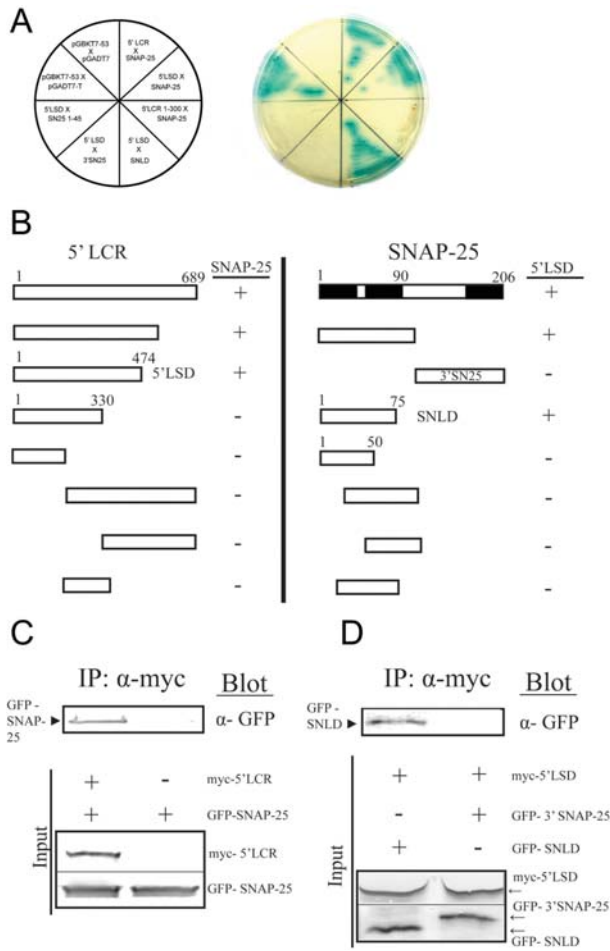
Figure 12. Identification of cytLEK1/SNAP-25 interaction and characterization of binding domains. A) Our initial Y2H screen identified SNAP-25 as having a direct interaction with cytLEK1. 5'LCR was used as bait and associates with SNAP-25. A series of truncations were constructed by PCR and then transformed into appropriate yeast strains. Yeast were then grown and plated on QDO medium. Positive associations grew and exhibited blue color upon Gal testing. As a control, growth was indicated by yeast transformed with pGBKT7-53 and pGADT7-T. The negative control utilized yeast expressing pGBTK-53 and the empty vector pGADT7. Positive cytLEK1 and SNAP-25 interactions must contain the 5'LSD and SNLD regions of the proteins to associate and grow on QDO medium. **B)** This is a summary of the 5'LCR deletion constructs that were tested for interaction with full-length SNAP-25 by Y2H analysis. (+) indicates interaction between the constructs. The N-terminal 474 aa of cytLEK1, 5'LSD, is required and sufficient for association with SNAP-25. A similar deletion series was constructed with SNAP-25 sequences. The N-terminal 75 aa, SNLD, was found to be required and sufficient for cytLEK1 binding. **C)** COS-7 cells were transfected with 5'LCR and GFP-SNAP-25 or with GFP-SNAP-25 alone. An immunoprecipitation was conducted with α-myc antibody, and blots were probed with α-GFP antibody. Input lanes show transfected protein expression in the lysate. GFP-SNAP-25 was precipitated in the presence of 5'LCR, but not without 5'LCR. **D)** COS-7 cells were transfected with both 5'LSD and GFP-SNLD, and GFP-SNLD was found to immunoprecipitate with 5'LSD. As a negative control, cells were transfected with 5'LSD and GFP-3'SN25, which demonstrates no interaction between the proteins. Thus, we have identified the 5'LSD of cytLEK1 and the SNLD of SNAP-25 as being required for association.

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containing this region were found to bind SNAP-25 in yeast matings, further truncations of 5'LSD eliminated all interactions with full length SNAP-25 (Figure 12A-B). Therefore, we determined that 5'LSD was critical for cytLEK1/SNAP-25 interaction. Of note, 5'LSD association does not appear to extend to all members of the SNAP family of proteins, as SNAP-23 did not interact with 5'LSD in Y2H analysis.

Next, we analyzed the region within SNAP-25 that was responsible for cytLEK1 interaction. SNAP-25 deletion studies revealed that the N-terminal 75 aa of the protein, termed **SN**AP-25 **L**ek1 binding **D**omain (SNLD), were sufficient and required for the interaction of the 5'LSD domain of cytLEK1 (Figure 12A-B). Further truncations of SNLD eliminated all protein interactions. Interestingly, this binding region within SNAP-25 contains two coil domains critical for its interactions with VAMP/Synaptobrevin and syntaxin (Chapman et al., 1994). Both VAMP/Synaptobrevin and Syntaxin are important for membrane docking and fusion (Chapman et al., 1994; Hong, 2005; Stoichevska et al., 2003).

To determine whether cytLEK1 and SNAP-25 interact within mammalian cells, COS-7 cells were then cotransfected with both 5'LCR and a GFP-SNAP-25 fusion construct. As seen in Figure 12C, coimmunoprecipitations of GFP-SNAP-25 revealed interaction with myc-tagged 5'LCR. Control experiments demonstrated no precipitation of SNAP-25. In order to confirm the interacting domains, we performed coimmunoprecipitation analyses with the minimal interacting domain, 5'LSD, and either the GFP-SNLD or the 3' domain of SNAP-25, termed GFP-3'SN25. While interaction was confirmed for 5'LSD and GFP-

SNLD, GFP-3'SN25 did not form a complex with the 5'LSD of Lek1 (Figure 12D). These results demonstrate that the 5'LSD region of cytLEK1 is required for SNAP-25 interaction and confirm our Y2H results.

Endogenous cytLEK1 colocalizes and associates with its interacting partner SNAP-25 in murine cells

We next examined the endogenous colocalization and association of cytLEK1 and SNAP-25. Cell lines previously shown to express both cytLEK1 (Soukoulis et al., 2005) and SNAP-25 (Sevilla et al., 1997) were used in these studies. As seen in confocal and deconvolution images in Figure 13, there was significant colocalization of cytLEK1 with SNAP-25 in NIH 3T3 fibroblast and C2C12 myoblast cells. Images show a strong overlap of intense perinuclear distribution of the proteins, with further colocalization extending away from the nucleus. Cytoplasmic distribution of SNAP-25 has been previously described (Aikawa et al., 2006; Blasi et al., 1995; Catsicas et al., 2002; Kataoka et al., 2000; Sun et al., 2003; Yan et al., 2003). Our data reveal that the colocalization of the proteins is not absolute in these cell lines, as overlap in staining was greatest surrounding the nucleus and became less apparent in the cell periphery. Since both endogenous proteins have multiple and varied functions, absolute colocalization was not expected. The staining pattern seen in these cell lines was not an artifact, as colabeling studies with other markers, such as the cytoplasmic proteins β-catenin and Bves, showed no significant colocalization (data not shown). Even though SNAP-25 is considered most predominantly neuronal in
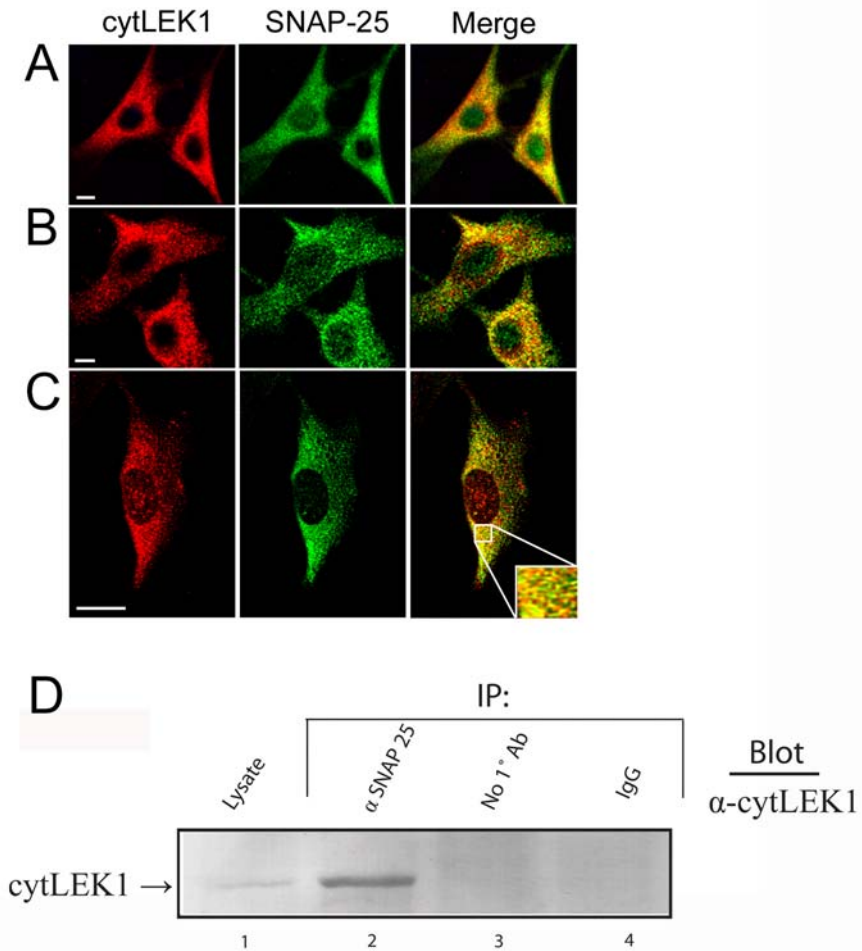
Figure 13. Endogenous cytLEK1 and SNAP-25 colocalize in murine cells. CytLEK1
expression is shown in red, while SNAP-25 is shown in green. **A)** CytLEK1 and SNAP-25
demonstrated significant overlap in expression throughout the cytoplasm in NIH 3T3
fibroblasts (merge). They share a high degree of colocalization in the perinuclear region, but
SNAP-25 has a broader distribution extending into the cell periphery. **B)** C2C12 myoblasts
demonstrated a similar distribution pattern to that seen in the NIH 3T3 fibroblasts. **C)**
Deconvolution analysis of proteins in C2C12 myoblasts was conducted to show a high degree
of colocalization. All images are from confocal microscopy. (Bar, 10 μm). **D)** CytLEK1 forms
an endogenous complex with SNAP-25. Endogenous protein complexes were analyzed using
NIH 3T3 cell lysates for coimmunoprecipitation analysis with α -SNAP-25 antibody,
Sepharose beads alone, or IgG antibody alone. After precipitation, elution, and western
blotting, the blot was probed with α-cytLEK1 antibody. **Lane 1** demonstrates the presence of
cytLEK1 in the lysate. **Lane 2** shows that cytLEK1 precipitates with SNAP-25. **Lanes 3 and
4** demonstrate the lack of precipitation with beads and non-immune IgG.

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expression, numerous non-neuronal cell types have been documented that express SNAP-25 (Bhangu et al., 2003; Jagadish et al., 1996; Karvar et al., 2002; Macaulay et al., 1997; Scott and Zhoa, 2001).

We next tested whether endogenous cytLEK1/SNAP-25 complexes could be isolated from cells. A series of coimmunoprecipitation studies, with the same NIH 3T3 cell line that demonstrated immunofluorescent colocalization, was conducted with an antibody previously used to recover SNAP-25 and its interacting partners (Kolk et al., 2000). As seen in Figure 13D (lane 2), SNAP-25 forms an endogenous complex containing cytLEK1. In contrast, neither Sepharose beads nor α-IgG antibodies alone were able to precipitate cytLEK1 (Figure 13D, lanes 3 and 4). Thus, along with our genetic, biochemical, and transient protein localization and interaction studies, we demonstrated that cytLEK1 and SNAP-25 associate and form an endogenous complex.

5'LSD redistributes with SNAP-25 expression

Our previous data showed that the 5'LSD of cytLEK1 and SNAP-25 interact at a biochemical level. We next confirmed that, similar to the endogenous proteins, the transfected protein constructs colocalized in mammalian cells. Immunochemical reagents used in this study do not detect endogenous cytLEK1 or SNAP-25 in COS-7 cells. COS-7 cells were transfected with either 5'LSD or SNAP-25. In cells expressing 5'LSD alone, a cytoplasmic localization with a distinct punctate perinuclear distribution was observed (Figure 14A). Cells transfected with GFP-SNAP-25 also demonstrated a perinuclear distribution, in
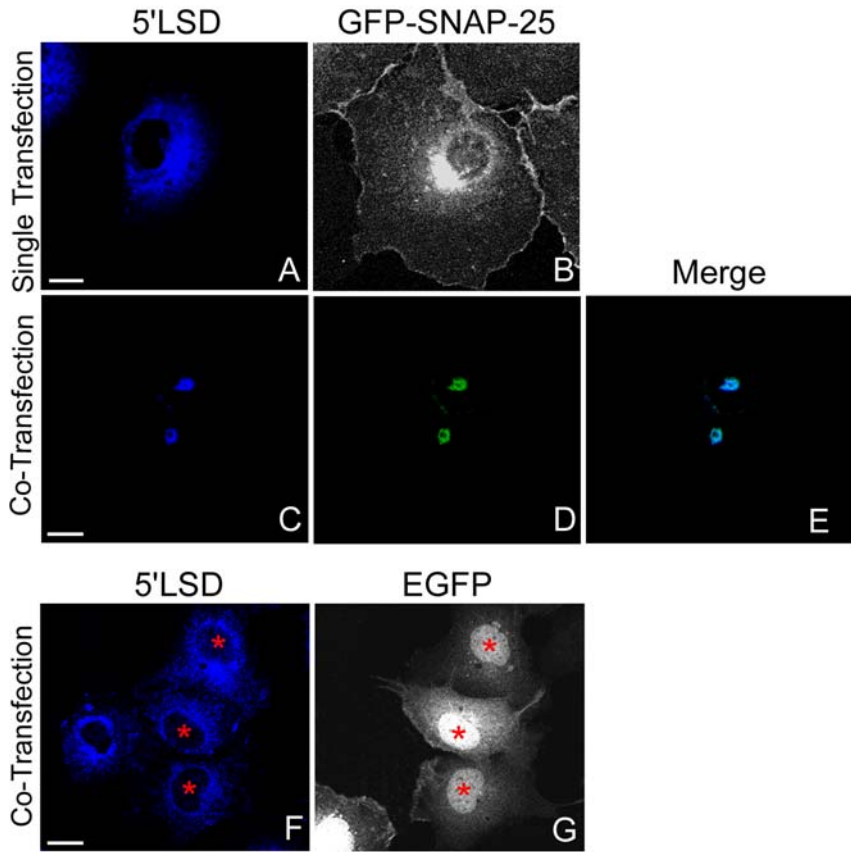
Figure 14. Transfected 5'LSD and GFP-SNAP-25 distribution in COS-7 cells. COS-7 cells were transfected individually with either 5'LSD or GFP-SNAP-25 **(A and B)**, or cotransfected with both constructs **(C-E)**. α-myc immunostaining (blue) is shown in **A** and **C**. GFP fluorescence (green) is observed in **B** and **D**. **A)** Cells singly transfected with 5'LSD demonstrated a high perinuclear distribution. **B)** GFP-SNAP-25 expressing cells also showed a cytoplasmic distribution with high levels of fluorescence observed in the perinuclear region and at the cell periphery. **(C-E)** Cotransfected cells demonstrated relocalization and redistribution of both proteins to a perinuclear ring with extensive overlap seen in the merged image **(E)**. Control cells show no redistribution of 5'LSD with EGFP expression (F-G). (*) denotes cotransfected cells. All images are from confocal microscopy. (Bar, 5 um)

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addition to high levels of expression at the cell periphery (Figure 14B). This pattern of SNAP-25 overexpression has been reported previously (Xiao et al., 2004).

Interestingly, when COS-7 cells were cotransfected with 5'LSD and GFP-SNAP-25, a dramatic redistribution of transiently expressed protein localization was observed. Immunoreactivity of 5'LSD overlapped extensively with that of GFP-SNAP-25 at a distinct perinuclear focus (Figure 14, C-E). Importantly, this overlap was not observed when EGFP was cotransfected with 5'LSD, as EGFP remained expressed throughout the cytoplasm and nucleus (Debily et al., 2004) with minimal colocalization and no redistribution of 5'LSD distribution (Figure 14, F-G). These findings demonstrate a specific interaction between 5'LSD and GFP-SNAP-25, which is not a consequence of simple protein overexpression.

5'LSD and SNAP-25 interact with components of the recycling endosomal pathway

Coexpression of 5'LSD and GFP-SNAP-25 demonstrated an intense overlap in a perinuclear locus. This perinuclear localization is similar to the pattern seen in HeLa cells transiently expressing either the myosin Vb-tail or a truncated form of the plasma membrane recycling endosome associated Rab11-family interacting protein 2 (Hales et al., 2002; Lapierre et al., 2001). To determine whether components of the endosomal recycling pathway were also present in the 5'LSD/GFP-SNAP-25 complex, we assessed the distribution of Rab11a in cotransfected cells (Green et al., 1997; Ullrich et al., 1996). As seen in Figure 15A, redistribution of Rab11a to the same perinuclear region in 5'LSD and
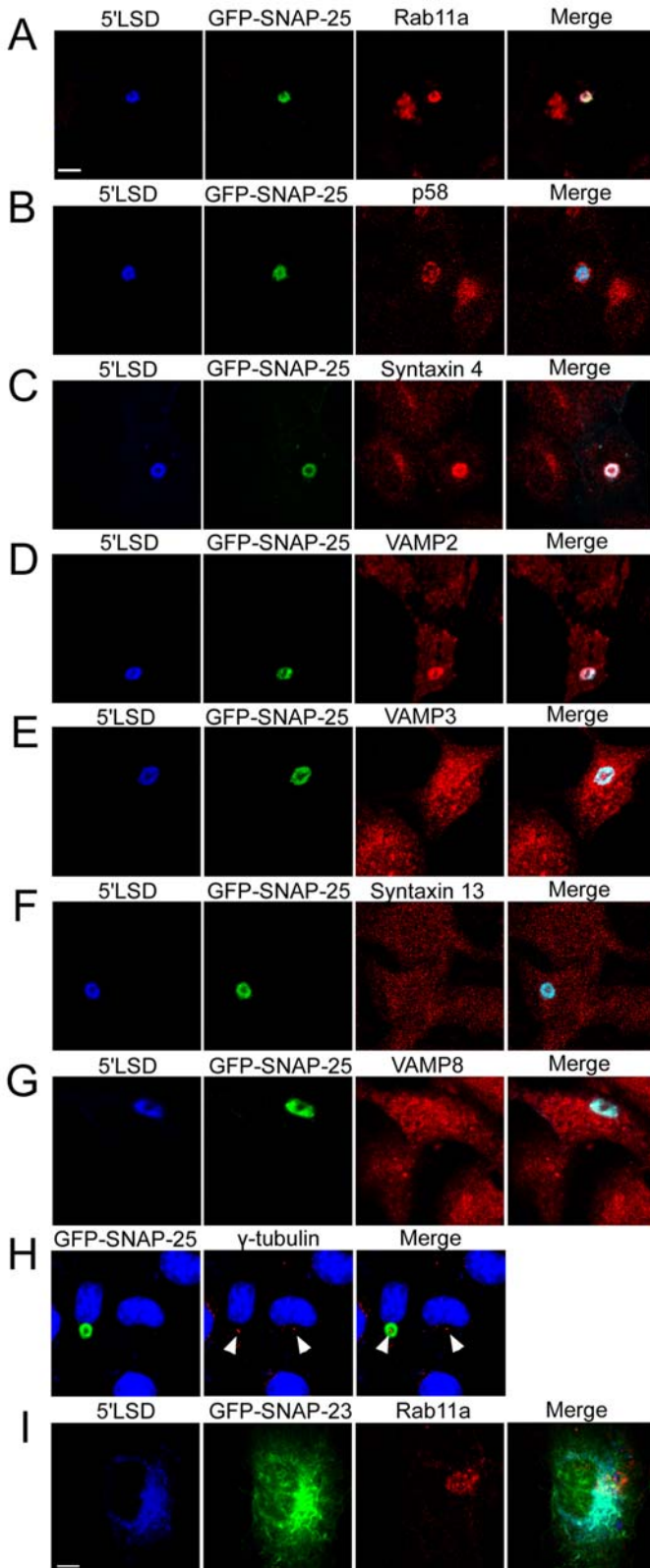
Figure 15. Rab11a colocalization with 5'LSD and GFP-SNAP-25.

Figure 15– cont. COS-7 cells were cotransfected with 5'LSD and GFP-SNAP-25 or GFP-SNAP-23. In **A-G**, cells were triple imaged as labeled with α -myc (blue), GFP fluorescence (green), and as labeled in red. Cells coexpressing 5'LSD and GFP-SNAP-25 colacalize to a perinuclear foci. **A)** Cells co-expressing 5'LSD and GFP-SNAP-25 showed a high degree of localization with endogenous Rab11a. **B)** p58, a Golgi marker, does not colocalize in cotransfected cells, but there was redistribution of the protein that is excluded from the perinuclear focus of the transfected proteins. **C-D)** Both endogenous Syntaxin 4 and VAMP2 localize and have high expression at the perinuclear focus containing 5'LSD and GFP-SNAP-25. **E-G)** VAMP3, Syntaxin 13, and VAMP8 do not show strong localization of the proteins at the perinuclear focus. **H)** Cotransfection of 5'LSD and GFP-SNAP-25 relocalize around the centrosome, as indicated by γ-tubulin staining in red. Dapi staining is indicated in blue. **I)** As a control, GFP-SNAP-23 was cotransfected with 5'LSD. Interstingly, the trasfected proteins did not redistribute to a perinuclear focus as observed with cotransfection of SNAP-25 and 5'LSD, and there was no dramatic redistribuation of endogenous Rab11a (red). Images in **A-G**, and **I** were taken by confocal microscopy. (Bar, 4 μm)

GFP-SNAP-25 coexpressing cells was readily observed. In order to test whether this phenotype was specific for 5'LSD and SNAP-25 interaction, we tested overexpression of 5'LSD and SNAP-23. Interestingly, coexpression of the two proteins did not form the tight perinuclear focus and did not redistribute endogenous Rab11a into that structure (Figure 15I). Analysis of colocalization with the Golgi showed minimal colocalization with the transfected proteins at the perinuclear focus, here shown with the Golgi marker p58 (Figure 15B). It is of interest to note though, that Golgi proteins showed redistribution to a position adjacent to and at the center of the perinuclear 5'LSD and GFP-SNAP-25 locus. As indicated by γ-tubulin staining, the 5'LSD and GFP-SNAP-25 ring focus encircled the centrosome (Figure 15H).

Other SNARE proteins have been shown to be associated with recycling endosomes. The vesicular SNARE proteins VAMP2, VAMP3, syntaxin 4, and syntaxin 13 have all been reported to localize in Rab11a containing recycling endosomes (Band et al., 2002; Calhoun and Goldenring, 1997; McMahon et al., 1993; Prekeris et al., 1998). We next examined whether these proteins were also in the perinuclear focus in cotransfected cells. We examined and detected both VAMP2 and Syntaxin 4 at the perinuclear focus in cells coexpressing 5'LSD and GFP-SNAP-25 (Figure 15, C-D). Surprisingly, neither endogenous VAMP3 nor Syntaxin 13 protein expression redistributed with coexpression of the transfected proteins (Figure 15, E-F). As an internal control, VAMP8, shown to be expressed in early endosomes (Nagamatsu et al., 2001), was examined for localization at

the perinuclear focus and was not found to be redistributed to the 5'LSD/GFP-SNAP-25 focus (Figure 15G).

To test whether Rab11a is contained within the same 5'LSD/GFP-SNAP-25 complex, whole protein lysates from cotransfected COS-7 cells were collected and analyzed. Lysates containing transfected 5'LSD and GFP-SNAP-25 proteins were probed for Rab11a and were subsequently found to contain endogenous Rab11a in the 5'LSD/GFP-SNAP-25 complex (Figure 16). Myosin Vb, a key regulator of Rab11a-containing recycling vesicles (Lapierre et al., 2001), was also found in the complex (Figure 16). Notably, further Y2H analyses showed no direct interaction between 5'LSD and either Rab11a or myosin Vb, therefore suggesting an indirect association between these proteins and cytLEK1. The membrane bound SNARE protein VAMP2 has also been reported to be present on Rab11 containing vesicles (Calhoun and Goldenring, 1997). We coimmunoprecipitated the same lysates and identified VAMP2 to be associated in the complex (Figure 16). From these data, we have thus characterized critical proteins in the complex that links 5'LSD with recycling endosomes.

The 5'LSD/GFP-SNAP25 complex is formed independent of the microtubule network

Previous studies have shown that endosomal recycling is dependent on an intact microtubule network and that vesicles are dispersed upon microtubule disruption with the depolymerizing agent nocodazole (Apodaca et al., 1994; Casanova et al., 1999; Lapierre et al., 2001). Gross alteration of microtubule
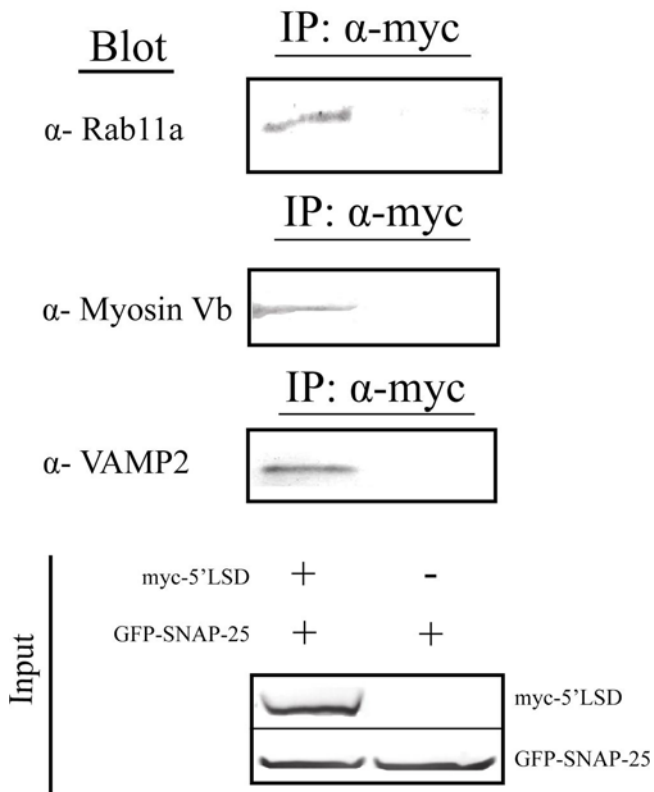
Figure 16. Rab11a, myosin Vb, and VAMP2 are in the same complex as 5'LSD/GFP-SNAP-25. COS-7 cells were cotransfected with 5'LSD and GFP-SNAP-25 or transfected with GFP-SNAP-25 alone, and lysates were obtained. Immunoprecipitations were conducted utilizing α-myc antibody, and the blots were probed with either α-Rab11a, α-myosin Vb, or α-VAMP2 antisera to detect endogenous proteins. Input lanes show presence of transfected proteins in the lysate. Precipitation of 5'LSD/GFP-SNAP-25 demonstated the presence of Rab11a, myosin Vb, and VAMP in the complex, but neither Rab11a, myosin Vb, nor VAMP2 immunoprecipitate in lysates expressing GFP-SNAP-25 alone.

network organization after cotransfection of 5'LSD and GFP-SNAP-25 was not observed (Figure 17). Interestingly, 5'LSD/GFP-SNAP-25 coexpressing cells did not demonstrate redistribution of the complex after challenge with nocodazole (Figure 17, D-F). Therefore, we postulate that the recycling complex is stable and is independent of the microtubule network after disruption with the microtubule-depolymerizing agent. Immunostaining for Rab11a in cotransfected cells showed that the recycling vesicle protein continued to associate at the distinct perinuclear focus after treatment with nocodazole (Figure 17F, arrow). In nontransfected cells, nocodazole treatment resulted in a diffuse distribution of Rab11a throughout the cytoplasm (Figure 17F, arrowhead), which is similar to that seen in MDCK cells after disruption of the microtubule network (Casanova et al., 1999). Since 5'LSD does not contain the Nde1 binding domain, we propose that the lack of redistribution of the vesicle components in cells expressing 5'LSD and GFP-SNAP-25 was due, in part, to the inability of 5'LSD to interact with the microtubule network. These results suggest that the 5'LSD/SNAP-25/Rab11a/myosin Vb/VAMP2/Syntaxin 4 containing complex in cotransfected cells is not associated with the microtubule system. Furthermore, the expression of 5'LSD and GFP-SNAP-25 results in a redistribution of the recycling endosome pathway. Therefore, it would be expected that endosomal recycling would be altered.
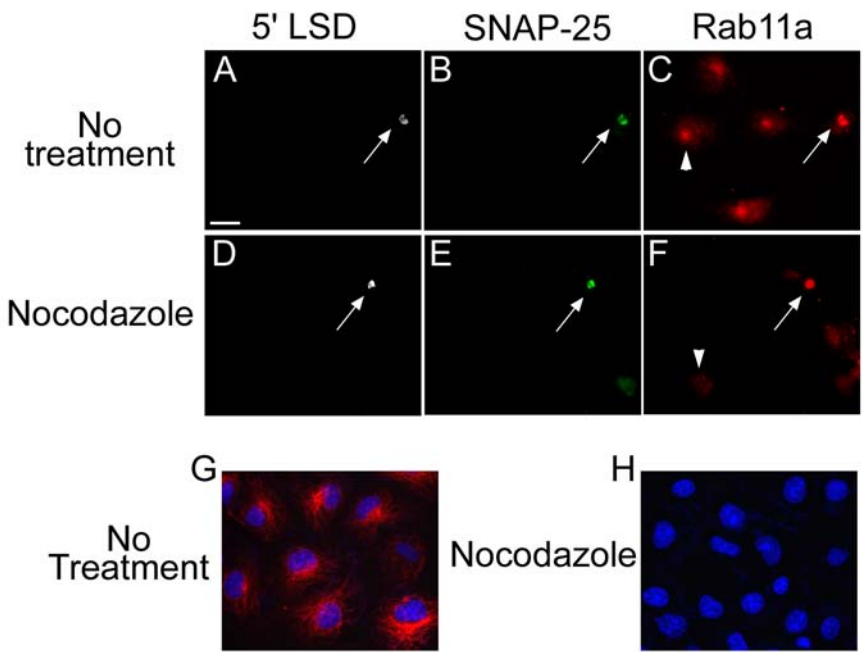
Figure 17. Morphological effects of cotransfected COS-7 cells. Cells were cotransfected with 5'LSD and GFP-SNAP-25, as previously shown (**A-C**), or treated with nocodazole for 30 min (**D-F**). 5'LSD is immunostained with α-myc antibody in white, GFP fluorescence in green, and α-Rablla is indicated in red. Cells demonstrated colocalization of 5'LSD/GFP-SNAP-25/Rab11a with no treatment as expected (**A-C**), but cotransfected cells treated with nocodazole also showed no redistribution of any of the proteins (**D-F**). Nontransfected cells in panel **F** showed a dispersion of Rab11a throughout the cytoplasm (arrowhead). Cotransfected cells are indicated with arrows. **G-H**) Cells were cotransfected with 5'LSD and GFP-SNAP-25 as previously shown. Cells were then preextracted and stained for β-tubulin (red) to visualize the microtubule network. Cells never demonstrated 5'LSD nor GFP-SNAP-25 localization after preextraction. Therefore, the perinuclear focus that contains 5'LSD and GFP-SNAP-25 in cotransfected cells is washed out in the soluble fraction and is not bound to the microtubule network. DAPI is in blue. (Bar, 10 μm)

cytLEK1 functions in Tf recycling

Tf receptor trafficking is known to depend on the plasma membrane recycling pathway (Bilan et al., 2004; Ghosh et al., 1994; Sonnichsen et al., 2000). Disruption of the recycling endosomal pathway by mutants of Rab11a, Rab11a-FIP2, and myosin Vb inhibits Tf recycling (Hales et al., 2002; Lapierre et al., 2001; Ren et al., 1998). In order to determine whether disruption of cytLEK1 function by expression of 5'LSD and GFP-SNAP-25 inhibits vesicle transport, Tf recycling was examined in cotransfected COS-7 cells by utilizing flow cytometry.

Cells were cotransfected and cells expressing both 5'LSD and SNAP-25 were analyzed for Tf uptake (Figure 18A). After a 30 min time period allowing Alexa-633 labeled Tf to bind the cells, the amounts of internalized labeled Tf were measured at 5, 10, and 20 min time points. Tf uptake is diminished in coexpressing cells, as they demonstrated ~13% reduction in labeled Tf internalization at all timepoints compared to control cells. The rates of recycling were not affected in cotransfected cells which mirrors the results of Nakamura et al. (2005) in Tf internalization. Taken together, the coexpression of the binding partners 5'LSD and SNAP-25 forms a dominant-negative complex and results in the redistribution of recycling endosome network and an inability of the cells to recycle Tf properly.

In order to further define the function of cytLEK1 and determine whether the protein alone has a role in Tf recycling, we examined Lek1 knock-down by MO antisense oligomers in NIH 3T3 fibroblasts (Figure 18B). We have previously
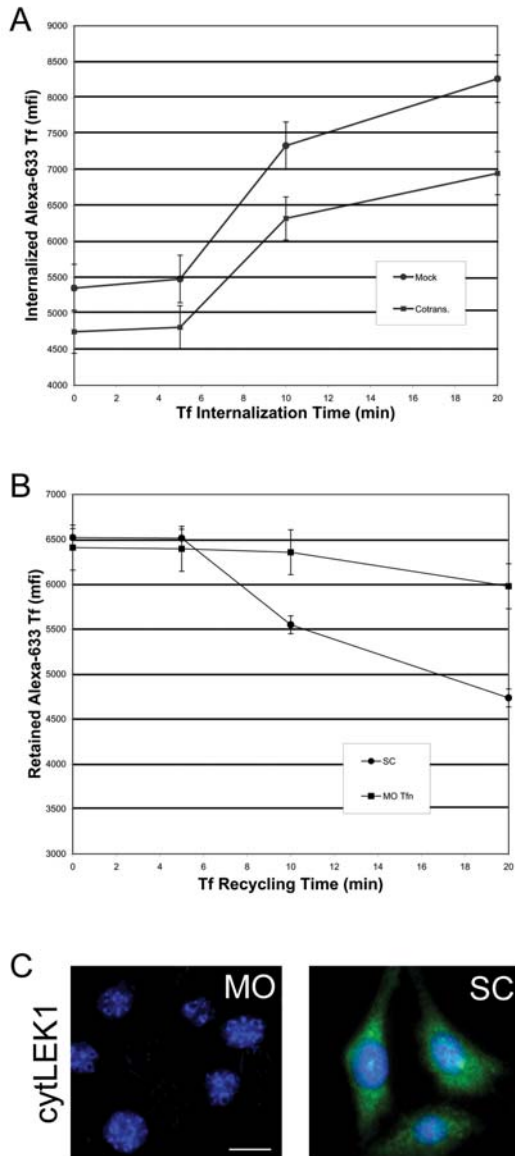
Figure 18. cytLEK1 functions in Tf recycling. A) Cos-7 cells were cotransfected with 5'LSD and SNAP-25 (squares) or with the vector only expressing EGFP (circles) and allowed to bind Alexa-633 Tf for 30 min at 4°C. After labeled Tf was allowed to bind the cells (Time-0), internalized, labeled Tf was measured in cells at 5, 10, and 20 min time points post binding. Cotransfected and mock transfected cells were analyzed by flow cytometry and measured for mean fluorescence intensity. **B)** Lek1 MO oligomers were utilized to knock-down protein expression in NIH 3T3 fibroblasts. SC oligomers were used as controls. Cells were examined 48 h after treatment. Cells were allowed to internalize labeled Tf and then chased with unlabeled Tf. Mean fluoresnce intensity was measured by flow cytometry in MO and SC treated cells at 0, 5, 10, and 20 min after Alexa-633 Tf internalization. MO treated cells demonstrate a significant decrease in rate of Tf recycling. After a 20 min chase period, Lek1 knock-down cells retain 26% more labeled Tf than SC cells. Data are means $\pm$ SE from three independent experiments. (*) ANOVA , $P<0.01$ vs. control. **C)** Lek1-specific MO treated cells demonstrate a significant, but not complete, knock-down of endogenous cytLEK1 compared to SC treated cells. (Bar, 10 μm)

confirmed the effectiveness and specificity of this Lek1 knock-down technology (Ashe et al., 2004; Soukoulis et al., 2005). Briefly, knock-down cells and standard control (SC) cells were allowed to bind Alexa-633 Tf for 30 min and then allowed to internalize labeled Tf for 30 min. After the internalization of labeled Tf, media containing unlabelled Tf was added to the cells (Time-0). Flow cytometry was used to measure the levels of Alexa-633 Tf retained in knock-down and SC cell populations at 5, 10, and 20 min after internalization. As expected, Lek1 knock-down cells recycled labeled Tf at a significantly slower rate than SC cells and had a higher level of labeled Tf retained in the cells. These results further demonstrate that cytLEK1 function is critical for endosome recycling.

Discussion

Lek1 is a member of a family of proteins that exhibits functional diversity, demonstrating roles in regulation of the cell cycle, myocyte differentiation, and microtubule dynamics. The human family member Mitosin/CENP-F has been shown to associate with the kinetochore, and its localization pattern is dependent on the cell cycle and also appears to play a role in cell division (Liao et al., 1995; Mancini et al., 1995). The chicken protein CMF1 has a function in chick myocyte differentiation in the developing embryo (Dees et al., 2000; Pabon-Pena et al., 1999; Wei et al., 1996). The mouse family member, Lek1 has a role in cell division and differentiation (Ashe et al., 2004; Dees et al., 2005). Recently, Lek1 has been implicated in specification of the cardiac lineage from embryonic stem cells (Papadimou et al., 2005). Of interest for the current study, we have recently

identified cytLEK1 as a Nde1 binding protein (Soukoulis et al., 2005). Nde1 is a member of the Lis1 pathway and has been shown to associate with the microtubule network. Lek1 knock-down and dominant-negative experiments have profound effects on the microtubule network and cell morphology (Soukoulis et al., 2005).

We have identified SNAP-25, a member of the SNARE family, as a novel cytLEK1 interacting protein. Expression of 5'LSD of cytLEK1 and SNAP-25 leads to the redistribution of the endosomal recycling system with relocalization of Rab11a, myosin Vb, and the membrane associated SNARE proteins VAMP2 and Syntaxin 4 into a perinuclear focus. SNAP-25 is well established in its direct interaction with VAMP2 (Chapman et al., 1994; Jahn and Sudhof, 1999). To date, most work has concentrated on VAMP3 and syntaxin 13 as being SNARE proteins localized to recycling endosomes. Data has been reported linking VAMP2 and syntaxin 4 as being proteins localized to recycling endosomes (Band et al., 2002; Calhoun and Goldenring, 1997). We have now identified VAMP2 and syntaxin 4 as being SNARE proteins in recycling endosomes in COS-7 cells. Emerging data continues to identify multiple SNAREs operating in trafficking steps and interacting with multiple protein complexes. Thus, characterizing SNARE complexes is critical to understand regulation of vesicle trafficking.

An important link between the Lis1 pathway and recycling endosomes has now been identified. Cotransfected cells were studied since it established a stable protein complex that acts as a dominant-negative in COS-7 cells. Our data phenocopies previous patterns reported in transfection studies of dominant-

negative myosin Vb-tail and the mutant Rab11-FIP2 (129-512) (Hales et al., 2002; Lapierre et al., 2001), further implicating a role for cytLEK1 in the regulation of vesicular transport. Because studies have shown that docking and fusion of vesicle membranes within endosomal pathways are dependent on SNARE proteins (Foletti et al., 1999; Kodrik et al., 1998; Liang et al., 2004a; Mullock et al., 2001), cytLEK1 association with SNAP-25 predicts a function in the recycling pathway. Additionally, the microtubule network has been shown in the regulation of plasma membrane recycling (Apodaca et al., 1994; Casanova et al., 1999), yet proteins responsible for vesicle interaction with microtubules remain largely unknown. Our data indicate that cytLEK1 belongs to a new class of proteins that link recycling vesicles with the microtubule network and has implications for regulation of endosomal trafficking in a broad spectrum of developmental and cell biological processes.

Identification of cytLEK1 and SNAP-25 interaction provides a physical link between recycling endosomes and the microtubule network

The microtubule network is important in plasma membrane recycling (De Brabander et al., 1988; Gibbons, 1996; Lapierre et al., 2001; Sakai, 1991). These studies have established that depolymerization of the microtubule cytoskeleton by nocodazole treatment disperses the recycling system (Apodaca et al., 1994; Hales et al., 2002; Lapierre et al., 2001). Matanis et al. (2003) identified Bicaudal-D as the link between microtubules and Rab6a positive vesicles, but proteins regulating plasma membrane recycling through the microtubule network remain

more obscure. Additional protein regulators of vesicle/microtubule association likely exist.

From our data, we postulate that expression of 5'LSD, which lacks the Nde1 binding domain and therefore does not interact with the microtubule network, results in separating the 5'LSD/GFP-SNAP-25/Rab11a/myosin Vb/VAMP2/Syntaxin 4 perinuclear complex from the microtubule cytoskeleton. 5'LSD would represent a dominant-negative form of cytLEK1 that alters its function in vesicle recycling. As seen in Figure 17, treatment of cotransfected COS-7 cells with nocodazole has no noticeable redistribution of Rab11a as compared to wild-type cells. This is in contrast to Lapierre et al. (2001), where myosin Vb and Rab11a positive vesicles partially dispersed after nocodazole treatment, suggesting that an intact microtubule network was needed for recycling endosome function and movement. We demonstrate that the perinuclear complex is independent of the microtubule network and is part of the soluble fraction of cells, further implicating a role for cytLEK1 in recycling endosome trafficking. It is interesting to note that the cytLEK1 binding partner Nde1 influences microtubule based Golgi trafficking, demonstrating that the Lis1 pathway is involved in organelle transport (Liang et al., 2004b). We propose that cytLEK1 may be the bridge between Rab11a-containing recycling vesicles and the microtubule network through cytLEK1 binding to both SNAP-25 and Nde1.

5'LSD and SNAP-25 expression disrupt protein recycling

Once we established cytLEK1 as a possible bridge between recycling vesicles and the microtubule network, we tested the effects of 5'LSD on Rab11a and Tf recycling. Expression of 5'LSD and GFP-SNAP-25 leads to relocalization of the Rab11a-containing vesicles into a perinuclear focus. As seen in Figure 18, Tf can enter transfected cells, but at significantly reduced amounts. We postulate that there is a reduction of Tf receptor at the cell surface, but Tf can still be internalized by early endosomes (Sheff et al., 2002). The retardation of Tf recycling has also been observed when dominant-negative constructs of Rab11a or its binding partners are expressed in nonpolarized cells (Hales et al., 2002; Lapierre et al., 2001; Mammoto et al., 1999; Valetti et al., 1999; Zeng et al., 1999). Furthermore, knock-down of Lek1 expression significantly reduces Tf recycling and exit from the cell (Figure 18B). Our data demonstrate that expression of the SNAP-25 binding domain, 5'LSD, alters endosomal recycling, placing cytLEK1 as an essential member in an established recycling process. Therefore, we postulate that cytLEK1 and its association with an intact microtubule network are vital for recycling endosome trafficking.

To date, our studies demonstrate that cytLEK1 associates with both SNAP-25 and Nde1. Whereas previous studies have established a link between Rab11a-positive recycling endosomes and the actin cytoskeleton through association with myosin Vb, no proteins responsible for the interaction between recycling endosomes and the microtubule network have yet to be identified. The present data demonstrate that cytLEK1, SNAP-25, Rab11a, myosin Vb, VAMP2,

and syntaxin 4 can form a complex in association with plasma membrane recycling vesicles. Supporting our studies, Calhoun et al. (1997) and Peng et al. (1997) have also identified the SNARE protein VAMP2 in Rab11a containing recycling endosomes, while Band et al. (2002) has characterized syntaxin 4 at Rab11a positive endosomes. Therefore, we propose as a model that this complex acts as a bridge for recycling vesicles to the microtubule network through the ability of cytLEK1 to bind SNAP-25 and Nde1. cytLEK1 is a newly identified protein that links recycling endosomes with the Nde1/Lis1 pathway and the microtubule network. Similarly, the myosin Vb/Rab11a complex would bridge to the actin cytoskeleton. Thus, we have defined a multi-protein complex coordinating the dynamic interaction of recycling system membranes with both the microtubule and actin cytoskeleton.

CHAPTER III

CYTLEK1 FUNCTIONS WITH SYNTAXIN 4 IN PLASMA MEMBRANE
TRAFFICKING

Introduction

Plasma membrane trafficking is mediated by a series of budding and fusion events between donor and acceptor membranes. The SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) family of proteins is responsible, at least in part, for regulation of intracellular membrane events. Apposing SNARE proteins, which consist of vesicle associated proteins (VAMPs), syntaxins, and synaptosomal-associated protein of 25KD (SNAP-25), form coiled-coil aggregates that are important in regulating membrane fusion events. Plasma membrane trafficking between cellular compartments is critical for localization of proteins to the cell surface (Chen and Scheller, 2001; Jahn and Sudhof, 1999; Sollner et al., 1993b). Trafficking of plasma membranes between the Golgi and recycling endosome network is implicated but not well described (Mallard et al., 2002; Wilcke et al., 2000). The regulators of this process are largely unknown.

Syntaxin 4 is a SNARE protein that is localized to the plasma membrane and is also found in intracellular compartments, such as recycling endosomes (Bajohrs et al., 2005; Band et al., 2002). Plasma membrane trafficking to the cell surface involves the SNARE family proteins, but little is known about the potential

function of syntaxin 4 at recycling endosomes. Interestingly, even though no function has been identified for syntaxin 4 at the Golgi, transfected syntaxin 4 is found in that compartment (Takuma et al., 2002). Rab11a, a marker of recycling endosomes, functions in plasma membrane recycling in both polarized and nonpolarized cell lines (Ullrich et al., 1996; Urbe et al., 1993). Also, plasma membrane recycling endosomes have been shown to contain SNAP-25 and syntaxin 4 (Aikawa et al., 2006; Band et al., 2002; Pooley et al., 2006).

The importance of syntaxins in vesicular transport is exemplified by the regulation of GLUT4-containing vesicles in skeletal muscle, cardiomyocytes, and adipose tissue after insulin stimulation (Bryant et al., 2002; Pessin et al., 1999). In these cell types, fusion of GLUT4 vesicles at the cell membrane is the key step in insulin-regulated glucose transport and is mediated by SNARE proteins (see Chapter I). Both syntaxin 4 and VAMP2 localize to GLUT4-positive vesicles and regulate trafficking to the cell membrane (Cain et al., 1992; Martin et al., 1996; Pessin et al., 1999; Volchuk et al., 1996). Even though most studies have concentrated on the function of SNAP-23 in GLUT4 trafficking, SNAP-25 has been isolated from GLUT4 positive cells (Jagadish et al., 1996), however a potential role for SNAP-25 in GLUT4 trafficking remains obscure. Rab11a also localizes in GLUT4-positive vesicles in insulin responsive tissues (Kessler et al., 2000; Larance et al., 2005; Millar et al., 1999; Uhlig et al., 2005). The interaction of this system with the cytoskeleton and its significance are not well understood.

Lek1 is the murine member of the LEK/CENPF/mitosin family of proteins (Goodwin et al., 1999). This family of proteins displays sequence homology, yet

each family member contains unique domains and have varying expression patterns in cells and organisms (Ashe et al., 2004; Dees et al., 2000; Goodwin et al., 1999; Liao et al., 1995; Pabon-Pena et al., 2000; Zhu et al., 1995a; Zhu et al., 1995b). Lek1 is a relatively large protein that contains 2998 amino acids (aa; NCBI accesion number DQ642022). There is a post-translational modification that produces the N-terminal peptide, cytLEK1, which is distributed throughout the cytoplasm (Pooley et al., 2006; Soukoulis et al., 2005). Recently, we have described cytLEK1 interaction with Nde1 of the Lis1 pathway (Soukoulis et al., 2005). In turn, Nde1 interacts with Lis1 and dynein to regulate the microtubule network (Faulkner, 2000; Morris and Xiang, 2000; Smith, 2000). The association of Nde1, Lis1, and dynein through the Lis1 pathway have critical roles regulating membrane trafficking, positioning, cell migration, and mitosis (Banks and Heald, 2001; Gibbons, 1996; Terada et al., 1998). Important for the current study, the Lis1 pathway functions with the Golgi network and in membrane trafficking (Kondratova et al., 2005; Liang et al., 2004b). By utilizing dominant-negative protein expression and Morpholino (MO) induced suppression of Lek1 expression, we have demonstrated that altering cytLEK1 function severely alters the microtubule network (Soukoulis et al., 2005). The role of cytLEK1 in membrane trafficking remains unresolved.

Our most recent data demonstrates that cytLEK1 physically associates with SNAP-25, and together these proteins complex with Rab11a, myosin Vb and VAMP2. Furthermore, disruption of endogenous Lek1 function by dominant-negative protein expression or protein knock-down severely retards the recycling

endosome network and transferrin trafficking (Pooley et al., 2006). We postulate that cytLEK1 establishes a link between recycling endosomes and the microtubule network through its ability to bind both Nde1 and SNAP-25 (Pooley et al., 2006; Soukoulis et al., 2005). However, the functions of cytLEK1, SNAP-25, Nde1, and the microtubule network in membrane trafficking and organelle positioning remain poorly understood.

In the current study, we demonstrate that syntaxin 4 and cytLEK1 physically interact. These data are consistent with the hypothesis that cytLEK1 plays a role in the dynamic regulation of plasma membrane trafficking through its association with Nde1, the microtubule network, and SNARE proteins. Using genetic, immunolocalization, and immunoprecipitation studies, we demonstrate that both transiently expressed and endogenous cytLEK1 directly associates with syntaxin 4 at recycling endosomes. This complex also contains VAMP2 and SNAP-25. Finally, we show that disruption of Lek1 function inhibits GLUT4 trafficking, a model of syntaxin 4 function in membrane trafficking, thus demonstrating the essential nature of syntaxin 4-cytLEK1 interaction. The present study suggests that cytLEK1 provides a physical link between syntaxin 4-positive membranes and the microtubule network, and that these interactions are critical in membrane trafficking.

Materials and Methods

Yeast Two-Hybrid Screen

The screen was previously described in Pooley et al., 2006. Briefly, The N-terminus of cytLEK1 (aa 1-689) was utilized in the Matchmaker Y2H System 3 (BD Biosciences Clontech). Library plasmids were isolated from yeast colonies that survived on Quadruple Dropout Medium (QDO; SD/-Ade/-His/-Leu/-Trp/X-a-Gal) and exhibited lacZ expression. The inserts were then sequenced by the Vanderbilt Sequencing Core Facility and identified using NCBI Blast (Altschul et al., 1990). For each identified protein product, false positive tests involving empty vector and random protein matings were conducted to eliminate spurious interactions according to the manufacturer's recommendations.

Cell Culture and Transfection

COS-7, 3T3-L1, and C2C12 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium supplemented with 10, 10, and 20% FBS respectively, 100 ug/mL penicillin/streptomycin, and L- glutamine, in a 5% CO_2 atmosphere at 37^oC. 3T3-L1 preadipocytes were differentiated by treatment with insulin, dexamethasone, and isobutylmethlxanthine as previously described (Frost and Lane, 1985), and cells were used for experimentation 9-12 days after initiation of differentiation. For transfection, cells were grown to 50-75% confluency and transfected with DNA using FuGENE 6 (Roche) according to the manufacturer's

recommendations. Full-length cytLEK1 was constructed by placing the N-terminal 2210 aa with a FLAG-tag into pCI-neo (Promega).

Immunostaining and Microscopy

Cells for transient and endogenous studies were gently washed with 1X PBS and fixed with either 4% paraformaldehyde, to visualize endogenous proteins, methanol, to visualize transient protein, or Histochoice (Amresco), to visualize the microtubule network, for 20 min. Cells were washed with 1X PBS, permeabilized with 0.25% Triton X-100 in 1X PBS for 10 min, and blocked for at least 1 h in 2% BSA in 1X PBS at room temperature. Primary antibodies were incubated overnight at 4°C. Cells were then washed 3 times in 1X PBS and secondary antibodies were added for 1 h at room temperature. Cells were again washed 3 times with 1X PBS and coverslips mounted with AquaPoly/Mount (PolySciences). Cells were visualized by fluorescence microscopy with an AX70 (Olympus), or for confocal analysis, with a LSM510 (Ziess) microscope. Images were captured and processed using Magnafire (Olympus) and Photoshop (Adobe). All images of control and experimental cells were processed identically.

Coimmunoprecipitation Using Transient Transfections

COS-7 cells were grown on 10 cm plates; proteins were harvested 48 h post transfection. The ProFound Mammalian c-Myc Tag Co-IP Kit (Pierce) was utilized according to the manufacturer's protocol. Briefly, cells were washed once with ice-cold TBS, incubated with M-Per Extraction Reagent (Pierce)

containing protease inhibitor (Sigma), and centrifuged at 16,000 X g for 20 min at 4°C. Lysate protein concentration of the supernatant was determined using a bicinchoninic acid solution assay (Pierce). 100 µg total lysate was incubated for 2 h at 4°C with 10 µl anti-c-myc agarose slurry with gentle shaking at 4°C. Columns were washed 3 times with 1X TBS-Tween. Protein was eluted with 2X non-reducing sample buffer (Pierce) at 95°C for 5 min. To reduce proteins for SDS-PAGE analysis and Western blot analysis, 2 µl 2-mercaptoethanol was added. Ten µl of total lysate supernatant was used to confirm protein expression. Blots were developed using NBT-BCIP (Roche) and scanned into digital images (Hewlett-Packard).

Coimmunoprecipitation of Endogenous Protein Complexes Containing cytLEK1

C2C12 cells were lysed with Nonidet P-40 buffer with gentle sonication. Whole cell lysates were recovered and samples containing 2-3 mg total protein were precleared with GammaBind Plus Sepharose (Amersham Biosciences) for 20 min with gentle rotation at 4°C. Cell lysates were collected and incubated overnight with 3 µg of a polyclonal syntaxin 4 antibody (Sigma). GammaBind Plus Sepharose was added to bind the antibody-protein complex. Beads were washed 3 times with cold 1X PBS and proteins were eluted with Laemmli sample buffer at a boiling temperature for 5 min. Proteins were resolved on a 6% SDS-PAGE gel and analyzed by Western blot analysis. Twenty µg of protein lysate was loaded to visualize cytLEK1 in whole cell lysate.

MO Antisense Oligomer Treatment

Production of and methods utilizing MO to specifically knockdown endogenous Lek1 have been previously reported (Ashe et al., 2004; Pooley et al., 2006; Soukoulis et al., 2005).

2-Deoxy-D-Glucose Transport Assay

48 h after MO addition, 3T3-L1 were starved of serum for 1 h. The cells were then incubated with 100nM insulin in KRH buffer for 20 min. Glucose transport was initiated by addition of 0.5 mM 2-deoxy-D-[1,2-^{3}H] glucose (0.25μCi). After 10 min, transport was terminated by washing the cells 3X with cold KRH buffer. Cells were then solubilized with 0.5% SDS, and the incorporated radioactivity was measured by liquid scintillation counting. All quantitative data are representative of three separate experiments conducted over three days, each with n=6-8. A one sample Student's t-test was used after normalization to standard control (SC) cell populations.

Antibodies

cytLEK1 and Rab11a (a gift from Dr. James Goldenring, Vanderbilt University) antibodies were previously described (Pooley et al., 2006; Soukoulis et al., 2005). SNAP-25, syntaxin 4, and β-tubulin antibodies were obtained from Sigma. Golgin-97 was obtained from Molecular Probes. Syntaxin 4, EEA1, α-myc and α-GFP antibodies were obtained from BD Bioscience. VAMP2 and VAMP3 antibodies were purchased from StressGen. Alexa Fluor 488- and 568-

conjugated secondary antibodies were utilized (Molecular Probes). For triple labeled immunofluorescence studies, polyclonal anti-myc (Novus) was directly labeled with the Zenon Alexa-647 labeling kit (Molecular Probes). Alkaline phosphatase-conjugated secondary antibodies for Western blot were also purchased from Sigma.

Results

Identification of syntaxin 4 as a cytLEK1 interacting protein

Syntaxin 4 was identified as a cytLEK1 interacting protein using 5' LCR as bait. Both syntaxin 4 and the previously identified cytLEK1 interacter SNAP-25 are members of the SNARE family of proteins (Chen and Scheller, 2001; Jahn and Sudhof, 1999; Sollner et al., 1993b). Also utilizing Y2H analysis, 5'LSD (aa 1-474) was found to be the minimal domain of cytLEK1 required for syntaxin 4 interaction (Figure 19), just as it was for SNAP-25 (Pooley et al., 2006). Since we have previously established interactions of cytLEK1 with the microtubule network (Soukoulis et al., 2005) and SNAP-25 in the membrane trafficking system (Pooley et al., 2006; Soukoulis et al., 2005), the association of cytLEK1-syntaxin 4 was investigated further.
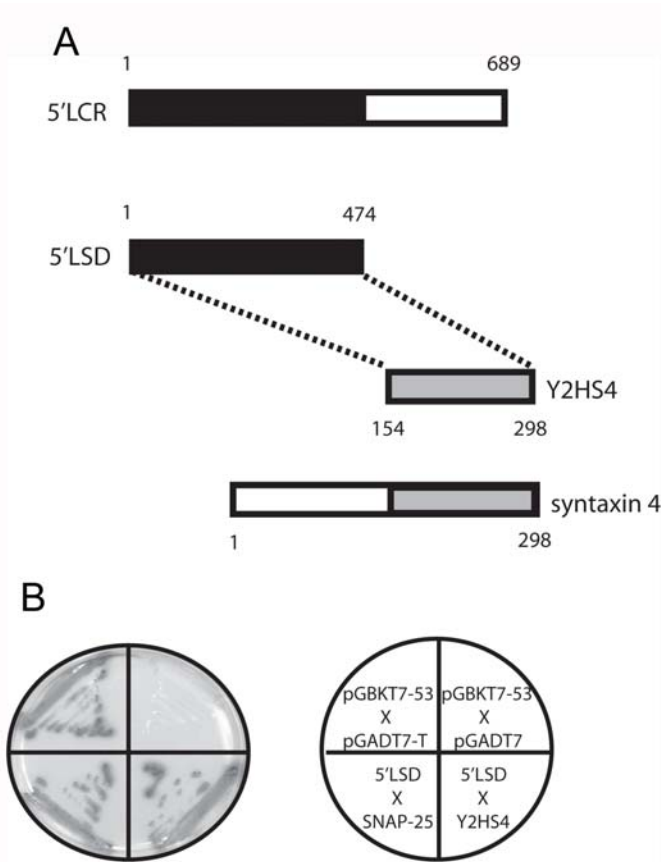
Figure 19. Identification of syntaxin 4 as cytLEK1 interacting protein. A) A Y2H screen was conducted and described previously [Pooley, 2006 #42]. One of the interacting proteins with 5'LCR, the N-terminal 689 aa of cytLEK1, was identified as syntaxin 4. The plasmid was isolated from a colony that survived on QDO media and subsequently sequenced. The resulting sequence was identified as the C-terminal 144 aa of syntaxin 4, termed Y2HS4. The C-terminal 474 aa of cytLEK1, 5'LSD, was further characterized as being the region of cytLEK1 sufficient for syntaxin 4 interaction by Y2H. **B)** Positive associations grew on QDO media and exhibited blue color upon β-Gal testing. As a positive control, growth was indicated by yeast transformed with pGBKT7-53 and pGADT7-T. Also used as a positive control, 5'LSD interacts with SNAP-25 as seen before [Pooley, 2006 #42]. The negative control utilized yeast expressing pGBTK-53 and the empty vector pGADT7 and demonstrated no growth on the media. The test interaction clearly demonstrates that 5'LSD does associate with the Y2HS4 portion of syntaxin 4 in yeast.

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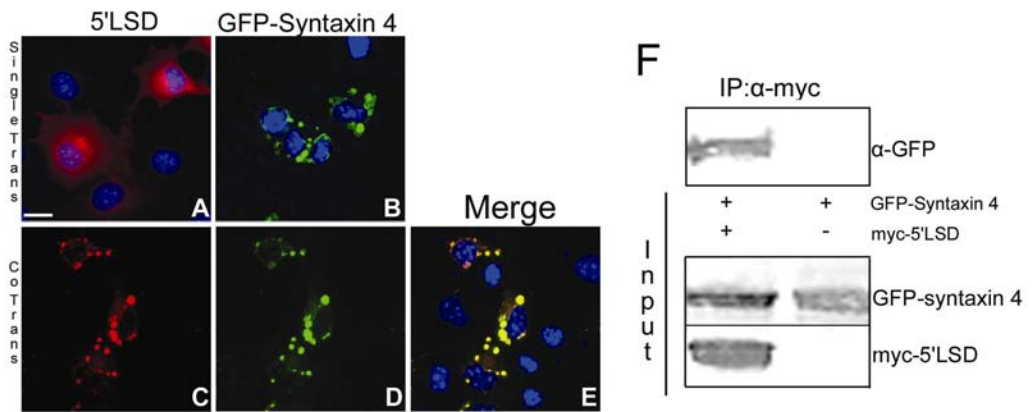
Figure 20. Transfected 5'LSD redistributes in COS-7 cells expressing GFP-syntaxin 4.
COS-7 cells singly transfected with 5'LSD **(A)** show a cytoplasmic distribution of the protein
with a high perinuclear distribution. Cells singly tansfected with GFP-syntaxin 4 **(B)** show a
significantly different distribution at defined foci throughout the cell. When cells are
cotransfected with syntaxin 4 and 5'LSD **(C-E)**, syntaxin 4 remained at the multiple
intracellular foci, but 5'LSD demonstrated a significant redistribution to the same foci occupied
by GFP-syntaxin 4 as seen in the merged image **(E)**. 5'LSD is indicated in red, while GFP-
syntaxin 4 is shown in green. Dapi (blue) is used to visualize the nucleus. **F)** COS-7 cells were
transfected with 5'LSD and GFP-syntaxin or with GFP-syntaxin 4 alone for a negative control.
An immunoprecipitation was conducted with α-myc antibody, and blots were probed with α-
GFP antibody. Input lanes show transfected protein expression in the lysate. GFP-syntaxin 4
was precipitated in the presence of 5'LSD. The control shows there was no spurious binding of
GFP-syntaxin 4 to the beads.

Transient protein expression reveals cytLEK1-syntaxin 4 interaction

We next examined cytLEK1-syntaxin 4 protein association and localization in COS-7 cells. As we reported previously (Pooley et al., 2006), 5'LSD overexpression in COS-7 cells localizes to the perinuclear region of cells and extends more diffusely to the cell periphery (Figure 20A). Overexpression of chimeric GFP-syntaxin 4 localizes to multiple foci located throughout the cell (Figure 20B). Interestingly, in cells expressing both proteins, 5'LSD redistributed to GFP-syntaxin 4-positive foci (Figure 20C-E). These data suggest a direct association between cytLEK1 and syntaxin 4.

To determine whether the two proteins interacted within these cells, a coimmunoprecipitation was conducted with lysates containing transiently expressed 5'LSD and GFP-syntaxin 4 proteins. As seen in Figure 20F, we were able to co-precipitate GFP-syntaxin 4 utilizing 5'LSD, while control experiments demonstrated no spurious GFP-syntaxin 4 association with the beads. Taken together, these results demonstrate that the 5'LSD region of cytLEK1 is responsible for syntaxin 4 association and confirm our Y2H results.

Endogenous cytLEK1 and syntaxin 4 associate in mammalian cells

Figure 21A demonstrates the association of cytLEK1 with the microtubule network. We next examined the endogenous colocalization and expression of cytLEK1 and syntaxin 4 in murine cell lines. Initially, C2C12 myoblasts were utilized since both cytLEK1 and syntaxin 4 are expressed in these cells (Pooley et al., 2006; Soukoulis et al., 2005; Tortorella and Pilch, 2002). As seen in Figure
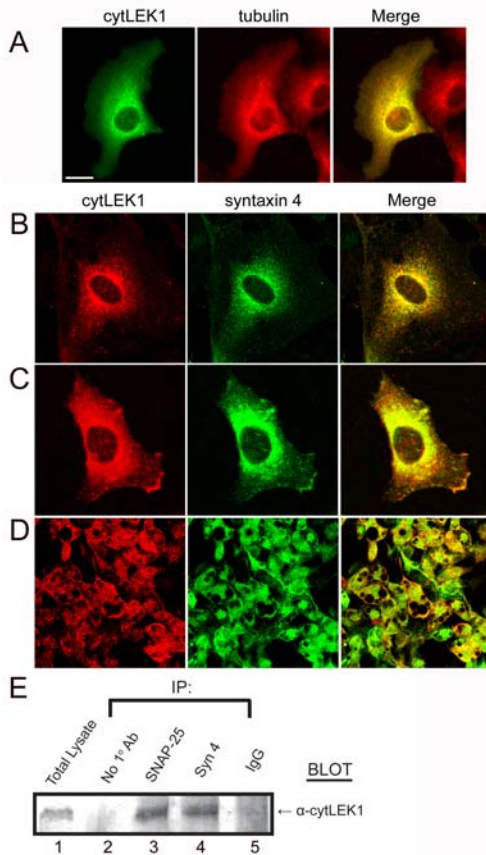
Figure 21. Endogenous cytLEK1 and syntaxin 4 colocalize in murine cells. A) COS-7 cells were transfected with full-length cytLEK1 and immunolabeling was conducted. A transfected cell is labeled with FLAG and demonstrates a fibrous distribution of cytLEK1 (green) when processed in Histochoice. β-tubulin in the transfected cell is labeled in red. The merged image shows a significant colocalization of cytLEK1 on the microtubule network, therefore supporting a role for cytLEK1 with the microtubule network. Cells could not be pre-extracted, as too much immunoreactivity was lost after processing. **B)** CytLEK1 and syntaxin 4 demonstrated significant overlap in expression throughout the cytoplasm in C2C12 myoblasts (merge). They share a high degree of colocalization in the perinuclear region, but cytLEK1 has a broader distribution extending further into the cell periphery. **C)** 3T3-L1 preadipocytes also demonstrate a similar expression patterns of cytLEK1 and syntaxin 4 to that seen in C2C12 cells. Interestingly, both endogenous proteins demonstrate a higher expression pattern at the cell periphery than that seen in C2C12 myoblasts. **D)** 3T3-L1 cells were differentiated as described in methods. Both endogenous cytLEK1 and syntaxin 4 appear to have high localization throughout the cells. All images are from confocal microscopy. (Bar, 10 μm). **E)** CytLEK1 forms an endogenous complex with syntaxin 4. Endogenous protein complexes were analyzed using C2C12 cell lysates for coimmunoprecipitation analysis with, Sepharose beads alone, α-SNAP-25 antibody, syntaxin 4 antiserum, or IgG antibody alone. After precipitation, elution, and western blotting, the blot was probed with α-cytLEK1 antibody. **Lane 1** demonstrates the presence of cytLEK1 in the lysate. **Lane 2** demonstrates the absence of precipitation with beads alone. **Lane 3** is a positive control that demonstrates cytLEK1 precipitation with SNAP-25. **Lane 4** shows that cytLEK1 precipitates with syntaxin 4. **Lanes 5** demonstrate the lack of precipitation with non-immune IgG.

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21B, confocal analysis demonstrated significant colocalization in the perinuclear region of the cell extending into the cell periphery. In this cell line, overlap was not absolute, as the staining pattern of cytLEK1 extended further in the cell periphery than that of syntaxin 4. This is to be expected, because both proteins have been shown to bind other proteins and function in multiple pathways.

Analysis of syntaxin 4 function has been established in 3T3-L1 apipocytes (Cain et al., 1992; Pessin et al., 1999; Volchuk et al., 1996). Therefore, in prelude to our functional studies of their interaction, we next examined for coexpression and colocalization of cytLEK1 and syntaxin 4 in this cell line. The intense perinuclear staining and colocalization of both cytLEK1 and syntaxin 4 mirrored the cytoplasmic expression observed in C2C12 cells, but both proteins demonstrated more significant staining at the cell periphery as compared to C2C12 myoblasts (Figure 21C). Interestingly, differentiated 3T3-L1 adipocytes demonstrated a high degree of endogenous cytLEK1 and syntaxin 4 throughout the cell with significant colocalization (Figure 21D). Protein expression is broader in the differentiated cells and appears to have a higher distribution throughout the cell extending to the cell periphery. It should be noted that previous studies of 3T3 cells have also demonstrated the cytoplasmic localization of syntaxin 4 as observed here (Band et al., 2002).

Next, we probed for the presence of endogenous complexes containing both cytLEK1 and syntaxin 4 using immunoprecipitation in lysates from C2C12 cells. As seen in Figure 21E, lane 4, cytLEK1 was co-precipitated with syntaxin 4, while beads only (lane 2) and non-immune IgG (lane 5) were negative. As a

positive control (lane 3), cytLEK1 was co-precipitated from the same lysate using an antiserum against the syntaxin 4 binding partner SNAP-25 (Pooley et al., 2006). Taken together, these data support the hypothesis that cytLEK1 and syntaxin 4 interact endogenously.

Overexpression of 5'LSD and syntaxin 4 redistributes proteins of the membrane trafficking network

The current data demonstrate that cytLEK1 and syntaxin 4 interact, while previous studies have determined that exogenously-expressed syntaxins accumulate in the Golgi (James et al., 2004; Rowe et al., 1999; Takuma et al., 2002; Washbourne et al., 2001). We confirmed this result utililizing the *trans*-Golgi network (TGN) marker golgin-97 in COS-7 cells cotransfected with GFP-syntaxin 4 and 5'LSD (Figure 22A).

We have previously demonstrated that VAMP2 forms a complex with 5'LSD and SNAP-25 (Pooley et al., 2006). VAMP2 is also well-documented for its role with syntaxin 4 in intracellular vesicle trafficking (Chamberlain and Gould, 2002; Cheatham et al., 1996; Kawanishi et al., 2000; Martin et al., 1996). Therefore, we examined whether VAMP2 localized to the TGN with 5'LSD and syntaxin 4. As seen in Figure 22B, VAMP2 localized with the transfected proteins at the TGN. VAMP3 is also a syntaxin 4 binding protein, but appears to have a role with endosomal populations (Galli et al., 1994; Kay et al., 2006; Mallard et al., 2002; McMahon et al., 1993; Polgar and Reed, 2003). Interestingly,
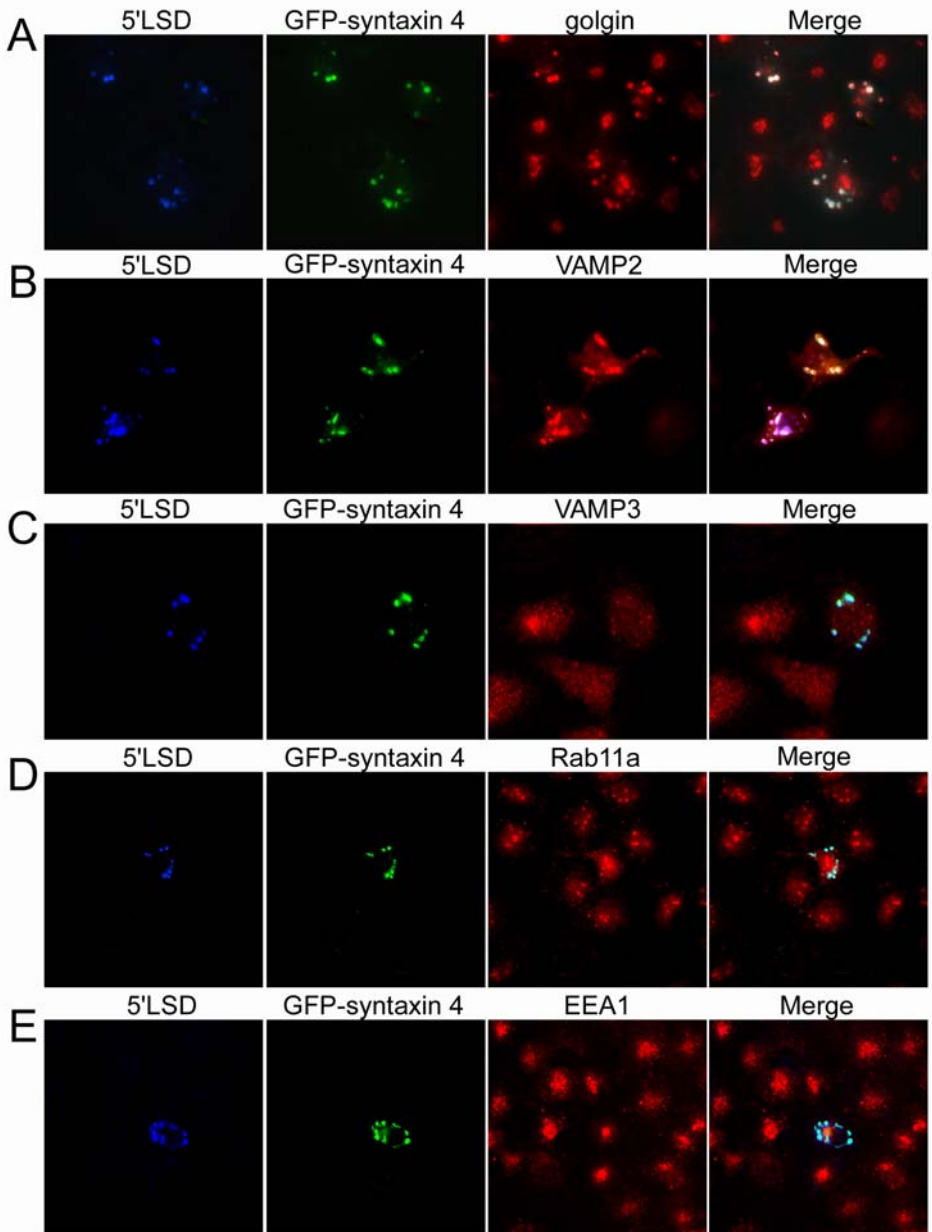
Figure 22. Transient 5'LSD and syntaxin 4 colocalize at the TGN in COS-7 cells. COS-7 cells were cotransfected with 5'LSD and GFP-syntaxin 4, and only those expressing both transient proteins were analyzed. **A)** 5'LSD and GFP-syntaxin 4 colocalize with the TGN marker golgin-97. **B)** VAMP2 also demonstrates a high degree of colocalization with 5'LSD and GFP-syntaxin 4. **C)** VAMP3 does not demonstrate any significant redistribution to 5'LSD-GFP-syntaxin 4 foci. **D)** Rab11a, a marker of recycling endosomes, does not demonstrate noticeable colocalization. **E)** Early endosomes, stained by EEA1, also show no significant relocalization with 5'LSD-GFP-syntaxin 4 foci. Therefore, the 5'LSD-GFP-syntaxin 4 complex is specific for localization at the TGN. 5'LSD staining is indicated in blue, GFP- syntaxin 4 in green, and the third marker, as indicated, is in red (Bar, 10 μm).

immunostaining for VAMP3 showed no discernable relocalization to the TGN foci (Figure 22C).

We next examined whether this localization was specific to Golgi or was the result of nonspecific accumulation of plasma membrane networks. Therefore, we probed for localization of Rab11a, a Rab GTPase protein marker of the recycling endosome (Uhlig et al., 2005; Urbe et al., 1993). It should be noted that expression of 5'LSD and SNAP-25 results in accumulation in recycling endosomes (Pooley et al., 2006). Interestingly, Rab11a was not redistributed to the Golgi with transfection of 5'LSD and syntaxin 4 (Figure 22D) and early endosomes were not co-localized with the 5'LSD-GFP-syntaxin 4 foci (Figure 22E). Thus, co-expression of syntaxin 4 and its cytLEK1 binding domain results in their accumulation specifically in the Golgi but does not result in redistribution of other endosomal compartments.

Endogenous cytLEK1 redistributes with overexpression of syntaxin 4

Co-expression of exogenous syntaxin 4 and the syntaxin 4 binding domain of cytLEK1 results in accumulation in the TGN. We next determined whether endogenous cytLEK1 localization was influenced by overexpression of GFP-syntaxin 4. As seen in Figure 23A, in cells expressing the syntaxin 4 chimera, endogenous cytLEK1 overlapped significantly with transiently expressed syntaxin 4 (Figure 23A). In fact, most endogenous cytLEK1 expression appeared to be at the syntaxin 4 foci. In addition, endogenous SNAP-25 redistributed and colocalized at the GFP-syntaxin 4 foci, just as its binding partner cytLEK1 (Figure
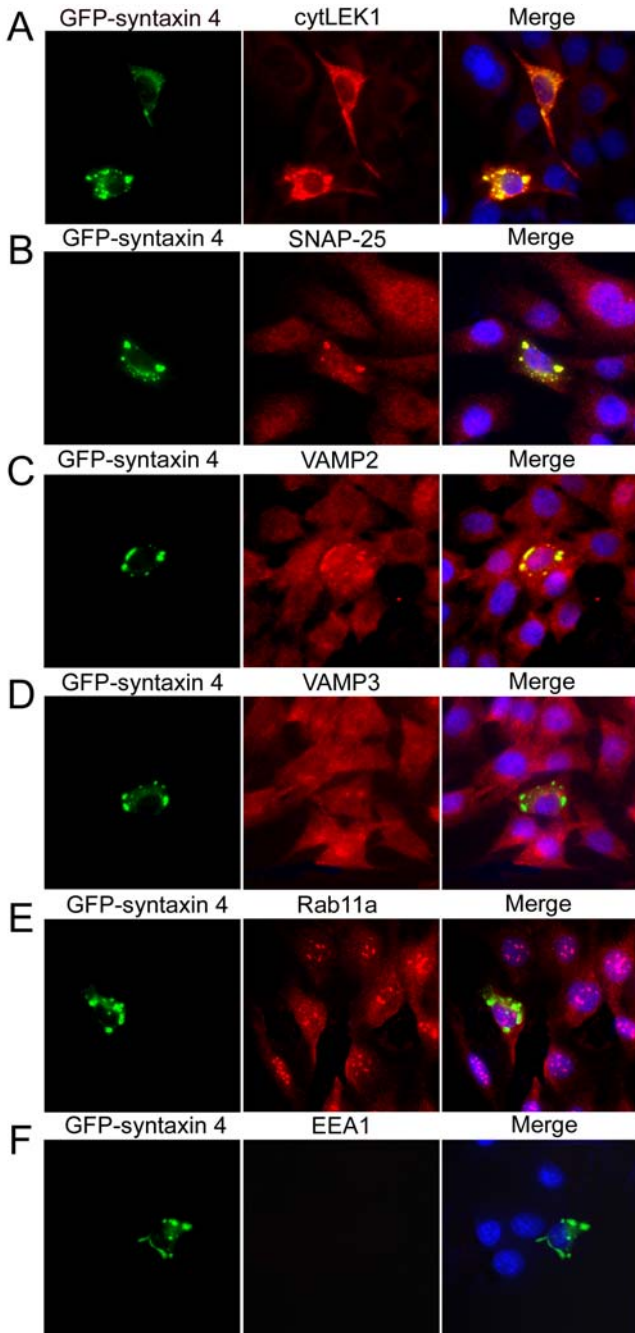
Figure 23. Endogenous cytLEK1, SNAP-25, and VAMP2 colocalize with GFP-syntaxin 4.
C2C12 myoblasts were tansfected with GFP-syntaxin 4 and analyzed for endogenous protein
localization. **A)** CytLEK1 demonstrated significant colocalization at the foci that contained
GFP-syntaxin 4. **B-C)** Endogenous SNAP-25 and VAMP2 demonstrate localization with GFP-
syntaxin 4 **D)** VAMP3 does not demonstrate any significant relocalization with expression of
GFP-syntaxin 4. **E-F)** Rab11a, a marker for recycling endosomes, and EEA1, a marker for early
endosomes, do not redistribute with transient syntaxin 4 expression, demonstrating specific
localization to the TGN. GFP-syntaxin 4 is shown in green, while the endogenous markers are
in red. Nuclei are visualized with Dapi (blue) (Bar, 10 μm).

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23B). Interestingly, endogenous VAMP2 was also localized at the GFP-syntaxin 4 foci (Figure 23C) while VAMP3, a SNARE protein functionally implicated with syntaxin 4 (Olson et al., 1997), did not (Figure 23D). Again, Rab11a did not show any noticeable relocalization or colocalization with the transient syntaxin 4 expression (Figure 23E). Overexpression of syntaxin 4 also had no notable effect on the early endosome network as seen by Figure 23F. Therefore, we conclude that overexpression of the syntaxin 4 chimera results in accumulation in the Golgi network and concomitant redistribution of cytLEK1, VAMP2, and SNAP-25. No noticeable redistribution of recycling and early endosome networks was observed.

5'LSD accumulates with endogenous SNAP-25 and syntaxin 4

Previous and current data show that 5'LSD, the syntaxin 4 binding domain of cytLEK1, interacts with both syntaxin 4 and SNAP-25 (Pooley et al., 2006). Here, studies overexpressing 5'LSD and its binding partners show that 5'LSD accumulates at either the TGN or at recycling endosomes (Figures 22 and 23). We next examined distribution of 5'LSD in singly transfected C2C12 myoblasts.

As seen in Figure 24, 5'LSD localizes throughout the cell, but it also has unique expression patterns at multiple distinct perinuclear foci. Overexpressed 5'LSD and SNAP-25 proteins accumulated at a single perinuclear focus resulting in the collapse and redistribution of the recycling endosome network (Pooley et al., 2006). Since syntaxin 4 also localizes to the same recycling endosome perinuclear focus as 5'LSD and SNAP-25, we examined endogenous syntaxin 4

localization in cells expressing only 5'LSD. Syntaxin 4 showed significant localization at the foci. Transiently expressed 5'LSD also colocalized with endogenous SNAP-25 (Figure 24B), a cytLEK1 binding partner (Pooley et al., 2006).

Next, the subcellular compartment(s) that 5'LSD localizes to were further characterized. Since 5'LSD localizes to the TGN in cells overexpressing syntaxin 4 (Figure 24), and 5'LSD colocalizes with transiently expressed SNAP-25 at recycling endosomes (Pooley et al., 2006), we tested TGN and Rab11a localization at cells expressing only 5'LSD. Interestingly, examination of markers for both the TGN and recycling endosomes demonstrated expression at the 5'LSD foci. 5'LSD accumulated with both markers for the TGN and the recycling endosome compartments. 5'LSD also colocalized with cytLEK1 binding partners syntaxin 4 and SNAP-25, along with VAMP2. As demonstrated in previous experiments, EEA1 and VAMP3 did not localize with 5'LSD (data not shown). Together, these data demonstrate cytLEK1 association with syntaxin 4, SNAP-25, and VAMP2 at distinct subcellular compartments that function in plasma membrane trafficking.

Lek1 knock-down inhibits glucose transport

Syntaxin 4 has a critical role in GLUT4 trafficking in insulin responsive tissues (Cain et al., 1992; Martin et al., 1998; Pessin et al., 1999; Volchuk et al., 1996). Insulin stimulates the translocation of intracellular GLUT4 vesicle pools to the plasma membrane in target tissues, which include cardiac and skeletal
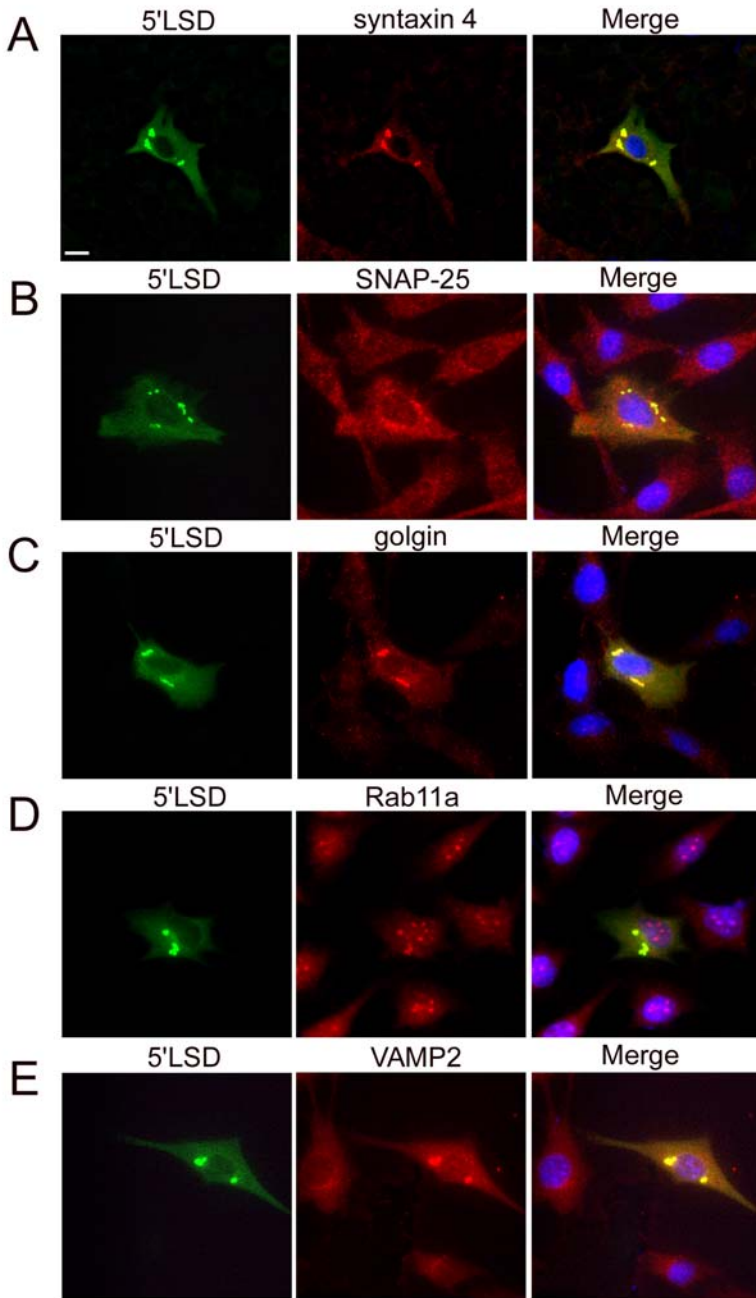
Figure 24. 5'LSD localizes to foci containing both TGN and recycling endosomes. 5'LSD was transfected into C2C12 cells and marker for SNAREs, TGN, and recycling endosomes were immunolabelled. **A)** Syntaxin 4 did colocalize with 5'LSD. **B-E)** SNAP-25, golgin, Rab11a, and VAMP2 did colocalize with 5'LSD. 5'LSD immunofluorescence is shown in green, while endogenous markers are in red. DAPI was used to visualize the nuclei (blue) (Bar, 10 μm).

muscle and adipose tissue. Activation of the insulin receptors triggers a large increase of GLUT4 vesicle trafficking and exocytosis as compared to basal conditions. cytLEK1 is now known to form a complex with two proteins that function in GLUT4 trafficking, syntaxin 4 and Rab11a (Kessler et al., 2000; Larance et al., 2005; Millar et al., 1999; Uhlig et al., 2005). In order to test Lek1 function in GLUT4 trafficking, we utilized the 2-Deoxy-D-Glucose transport assay (Kawanishi et al., 2000). Briefly, 3T3-L1 cells were differentiated, and Lek1 function was inhibited using MO anti-sense oligomers, a method previously described by our group (Ashe et al., 2004; Pooley et al., 2006; Soukoulis et al., 2005). Radio-labeled binding and internalization within the cell population was then measured. As expected, depletion of Lek1 significantly reduces GLUT4 trafficking, as a ~53% reduction of 2-deoxy-D- [1,2-^{3}H] glucose at the cell surface (p<0.01) was observed (Figure 25). All values were normalized against the SC cell populations since experiments were conducted on three consecutive days. These results further demonstrate that cytLEK1 function is critical for membrane trafficking.

Discussion

We have identified a novel interaction between cytLEK1 and syntaxin 4. cytLEK1 also interacts with Nde1, a dynein and microtubule associated protein, and the SNARE protein SNAP-25. Taken together, these studies support our hypothesis that cytLEK1 is a regulator of and link between recycling endosomes and the microtubule network. Most importantly, depleting endogenous Lek1 severely
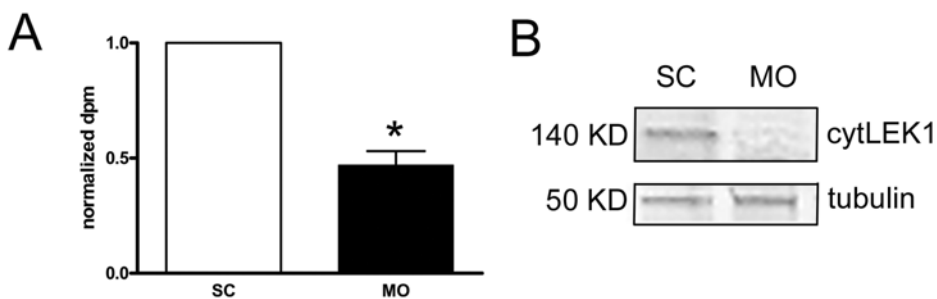
Figure 25. Depletion of LEK1 alters GLUT4 trafficking. A) 3T3-L1 apipocytes were differentiated and depleted of Lek1 by addition of MO. 48 h post MO addition, cells were processed according to methods. Normalized to SC cell populations, cells with MO addition had a two-fold decrease in radio-labelled glucose trafficking to the plasma membrane as counted by disintegration per minute (dpm). Data are normalized against the SC counts and are shown as means $\pm$ SE from three independent experiments. (*) one sample Student's t-test, p<0.01 vs. SC control. **B)** Also shown previously [Pooley, 2006 #42; Soukoulis, 2005 #41; Ashe, 2004 #46], MO addition is specific to cytLEK1 depletion as demonstrated by Western blot.

affects GLUT4 trafficking, a model pathway used to study syntaxin 4 function in membrane trafficking. To date, no Lek1 family proteins have demonstrated roles in vesicle trafficking, and these data are the first to reveal a potentially critical property for this family in regulation of plasma membrane trafficking.

The LEK family has a role in microtubule-based processes

The Lek1/CENP-F/mitosin family has demonstrated roles in cell cycle, division, and differentiation through microtubule based function (Ashe et al., 2004; Dees et al., 2000; Pooley et al., 2006; Rattner et al., 1993; Soukoulis et al., 2005; Zhu et al., 1995b). The human CENP-F/mitosin protein has been shown to bind the kinetochore and is highly expressed at the G_1/S boundary (Zhu et al., 1995a; Zhu et al., 1997; Zhu et al., 1995b). The kinetochore is located at the centromere of the chromosome and serves as the site for microtubule spindle attachment during mitosis (Cleveland et al., 2003; Rieder and Salmon, 1998). Dynein, a microtubule-based motor, is also important for chromosome positioning and segregation (Cleveland et al., 2003; Heald and Walczak, 2000; Sharp et al., 2000). Silencing of CENP-F/mitosin results in misalignment of chromosomes during mitosis and premature cell death (Yang et al., 2005), thus defining a role for this protein in microtubule based processes. Mouse Lek1 has a defined association with a regulator of dynein and microtubules (Soukoulis et al., 2005). Taken together, the LEK family of proteins has diverse roles in organelle dynamics associated with microtubule-based processes. While it is clear that LEK family proteins are involved in organelle positioning and movement, to date,

the interacting proteins and regulators of such functions are largely unknown. Through a series of experiments to identify interacting proteins, we have determined that cytLEK1 provides a connection between the MT network and recycling endosomes.

cytLEK1 associates with syntaxin 4

Here, we determined through genetic, biochemical, and immunochemical data, that cytLEK1 has a direct interaction with syntaxin 4. Previous data have shown that cytLEK1 and SNAP-25 interact through the 5'LSD domain of cytLEK1. We now demonstrate that both transiently expressed 5'LSD and endogenous cytLEK1 associate with syntaxin 4 using biochemical and cytological analyses. In cotransfection studies, 5'LSD and syntaxin 4 colocalized specifically to the TGN, as markers for other cellular and endosomal compartments did not demonstrate significant colocalization. It should be noted that syntaxin proteins localize to the TGN in overexpression studies (James et al., 2004; Rowe et al., 1999; Takuma et al., 2002; Washbourne et al., 2001), and these current data are in accord with previous studies. VAMP2, but not VAMP3, also localized to these foci.

Studies performed in C2C12 myoblasts with single transfections corroborated our previous data that syntaxin 4 localized with cytLEK1 at recycling endosomes. Additionally, GFP-syntaxin 4 colocalized with endogenous cytLEK1, SNAP-25 and VAMP2 at the TGN. In turn, transient 5'LSD colocalized with syntaxin 4, SNAP-25, and VAMP2. We now show that cytLEK1 directly binds

both SNARE proteins SNAP-25 and syntaxin 4, and VAMP2 appears to be the prominent VAMP family member in this complex.

cytLEK1-syntaxin 4 interaction is critical in regulation of the recycling endosome network

cytLEK1 binds syntaxin 4 at recycling endosomes. Interestingly, the region of the protein responsible for this interaction is the same highly coiled binding region of cytLEK1 that associates with syntaxin 4 and SNAP-25. Here, we demonstrate that both endogenous and transiently expressed cytLEK1 colocalize with its transfected binding partner syntaxin 4. Rab11a, a protein cytLEK1 has been shown to associate with, at least indirectly, is a well-defined Rab GTPase that regulates plasma membrane recycling and delivery of proteins from the TGN to the plasma membrane. Rab11a has also been localized to GLUT4 positive vesicles (Kessler et al., 2000; Larance et al., 2005; Millar et al., 1999; Uhlig et al., 2005). Importantly, 5'LSD transfection results in a redistribution of SNAP-25, VAMP2, Rab11a, and syntaxin 4 to the same foci as 5'LSD, indicating that this complex of proteins function at recycling endosomes. These data support our previous finding that this complex forms at the recycling endosome network. VAMP2 has also been identified at the Golgi apparatus (Chamberlain and Gould, 2002; Cheatham et al., 1996; Kawanishi et al., 2000; Martin et al., 1998), and a function for Rab11a at the TGN has been described (Chen and Wandinger-Ness, 2001; Crespo et al., 2004). This is most interesting when considering that 5'LSD localizes not only with Rab11a, but also golgin-97.

We propose that cytLEK1 is a key regulator of plasma membrane recycling. Since 5'LSD colocalizes with both the recycling marker Rab11a and the TGN marker golgin-97, cytLEK1 may be an important regulator of plasma membrane trafficking between these two compartments. Through the ability of cytLEK1 to bind Nde1 and at least two SNARE proteins directly, it may be a critical link for plasma membrane trafficking along the microtubule network.

To test the significance of cytLEK1-syntaxin 4 association, we used a GLUT4 translocation assay as a model of plasma membrane trafficking. Syntaxin 4 is a SNARE protein that functions in GLUT4 vesicle trafficking. By depleting cells of Lek1, there was a significant decrease in the amount of labeled glucose in the cells. Not only does cytLEK1 localize with syntaxin 4 in the recycling endosome network, but our data demonstrate that cytLEK1 has a key role in this syntaxin 4 dependent pathway. We have now established roles for cytLEK1 in membrane trafficking. cytLEK1 has now been shown to have a function in two plasma membrane trafficking processes in the cell, the plasma membrane recycling system of transporting transferrin (Pooley et al., 2006), and GLUT4 vesicle trafficking (present data). It remains to be determined whether cytLEK1 is specific to regulation of plasma membrane trafficking through recycling endosomes, or if it is a general regulator of vesicle movement along the microtubule network. In either case, the current study reveals an entirely new property of cytLEK1, and in addition places it in a molecular pathway through its interaction with multiple SNARE proteins. The identification of cytLEK1 as a

regulator of plasma membrane recycling is an entirely new property for any of the LEK family of proteins.

CHAPTER IV

THE OTHER CYTLEK1 BINDING PARTNERS

Introduction

Lek1 undergoes a post-translational modification that produces nucLEK1 and cytLEK1 peptides (Chapter I). Initial studies on nucLEK1 localization demonstrate that the protein is in proliferating cells of the developing embryo. Since nucLEK1 is highly expressed in the developing heart, we focused on its role during cardiomyocyte proliferation and differentiation (Ashe et al., 2004; Goodwin et al., 1999). Specifically, Lek1 mRNA was detected in the heart from E9.5 and downregulated after neonatal day 4 and was not found in the adult (Goodwin et al., 1999). Coincidently, this is the same time when cardiomyocytes cease proliferation and undergo terminal differentiation. Future studies showed that nucLEK1, along with CENP-F/Mitosin and CMF1, binds to Rb family proteins (Rb, p107, and p130), which are critical for cellular proliferation and differentiation (Ashe et al., 2004; Redkar et al., 2002; Zhu et al., 1995b). Critical *in vitro* and *in vivo* data show that disruption and depletion of Lek1 inhibits myocyte proliferation and differentiation (Ashe et al., 2004; Dees et al., 2000; Wei et al., 1996). These data support a function for Lek1 as a regulator of cell proliferation and differentiation through its interaction, at least in part, with Rb proteins.

Our most recent data also show that the C-domain of cytLEK1 is responsible for interaction with Nde1, a member of the Lis1 pathway (see Chapter I). Proteins that are members of the Lis1 pathway function in dynein-mediated processes of the MT network. Depletion of Lek1 causes a severe collapse of the MT network, further indicating a cytLEK1 function with the cytoskeleton (Soukoulis et al., 2005). We reported that cytLEK1 functions with the MT network through its interaction with Nde1. At that time, out of 2998 aa, we had functional data that accounted for less than 10% of Lek1. Therefore, we postulated that more unique associations exist through the other noncharacterized regions of the protein.

In order to further characterize the function of cytLEK1 in molecular pathways, we developed the conditional *Lek1* knock-out mouse (Chaper IV). Due to the large size of the *Lek1* gene, we utilized the Cre-loxP system to delete the first five exons of *Lek1* specifically in the heart. Postulating logical hypotheses on what phenotypes may result after ablating *Lek1* in the heart and to give further insight into the function of the deleted region, we utilized this peptide as bait for a Y2H screen to identify novel Lek1 binding proteins. The bait was constructed to contain only the first five exons of *Lek1*, termed 5'LSD. Placing Lek1 within a pathway(s) in which this domain is responsible for Lek1 function is critical to characterize the conditional *Lek1* knock-out and to determine its role in development.

Through this Y2H screen, we identified and characterized two novel Lek1 binding partners, SNAP-25 (Chapter II) and syntaxin 4 (Chapter III). Along with

these two proteins, the screen identified six other proteins that may associate with 5'LSD of Lek1. Characterizing these interactions is critical in our goal of understanding Lek1 function in its entirety. Results may clarify the roles of Lek1 in proliferation and differentiation or its function with trafficking and the MT network. They may also demonstrate Lek1 function(s) in a completely new manner.

Materials and Methods

Yeast Two-Hybrid Screen

The Y2H screen was conducted utilizing the Matchmaker Y2H System 3 (BD Biosciences Clontech). The bait construct, 5' LSD, was constructed by PCR amplifying aa 1-474 from a full-length cytLEK clone (aa 1-2210). 5'LSD was cloned into the EcoRI/BamHI sites of the pGBKT7 vector. The bait plasmid was then transformed into AH109 yeast according to the manufacturer's instructions. The AH109 yeast containing the bait vector were then mated to Y187 yeast containing a pretransformed whole mouse embryonic day 11 cDNA library. The matings were grown at 30°C overnight with gentle agitation (50 rpm). Mated colonies were plated onto QDO media (SD/-Ade/-His/-Leu/-Trp) and grown for 4 days at 30°C. Positive and negative controls were also conducted according to manufacturer's instructions. Colonies that grew on QDO were replica plated on QDO-α-gal to retest positive associations. Blue colonies were then streaked for segregation, and only blue colonies were analyzed. Bait and prey vectors were

isolated from positive yeast colonies utilizing the Yeastmaker Yeast Plasmid Isolation Kit (BD Biosciences Clontech). Plamids were then transformed into XL-1 Blue *E.coli* (Stratagene), isolated, and then sequenced at the Vanderbilt University Sequencing Core Facility. The resulting sequences were identified using NCBI Blast (Altschul et al., 1990). To confirm and validate the interactions, each resultant vector was then put through a false positive screening process. Briefly, they were mated with yeast containing empty vectors or random proteins to examine for spurious protein interactions. Clones that only grew with the original bait construct containing 5'LSD were further analyzed.

Cloning of *hook2*

The mouse *hook2* full-length cDNA clone was obtained from Invitrogen (clone # 4211990). A PCR product was constructed utilizing primers that amplified the entire *hook* mRNA and then ligated the resulting product into the HindIII/KPN sites of pEGFP-C3 (BD Bioscences Clontech).

5' primer: TATAAAGCTTATGAGTGTGGACAAGGCCGAG.

3'primer: TATAGGTACCGTGCTTGTCAGTGGGGCG.

Cell Culture

COS-7 and NIH 3T3 fibroblast cells (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum, 100 µg/ml penicillin/streptomycin, and L-glutamine. Cells were maintained in 5% CO_2 at 37^oC.

Cell Transfections

COS-7 and NIH3T3 fibroblasts were grown to 60-70% confluency and transfected using FuGENE6 (Roche) as per the manufacturer's recommendations. For one four-well slide: FuGENE was added to a 1.5 ml eppendorf tube containing 100 µl OptiMEM (Gibco) and gently mixed. One µg of DNA was then added and mixed. It was then incubated at room temperature for 20 min. Twenty-five µl of DNA mixture was added per well containing fresh medium. Cells were allowed to grow for at least 24 h before processing.

Immunocytochemistry and Microscopy

Cells were grown on glass chamber slides (Nalge Nunc), gently washed with 1X PBS, and fixed with Histochoice (Amresco) for 20 min. After fixation, cells were washed three times with 1X PBS, permeabilized with 0.25% Triton X-100 in PBS for 10 min., and blocked with 2% BSA in PBS for at least one h. Primary antibodies were applied overnight at 4°C. Cells were again washed three times in PBS. Secondary antibodies were added for one h at room temperature. Cells were washed three times with PBS and cover slips were attached to the slide with Aqua Poly/Mount (PolySciences). Cells were visualized by fluorescence microscopy on an AX70 (Olympus). Digital images were captured identically using Magnafire (Optronics) and processed identically with adobe Photoshop software.

Antibodies

Monoclonal α-p50 antibody was purchased from BD Bioscience, while polyclonal myc antiserum was obtained from Novus, and monoclonal α-myc antibody was acquired from Clontech.

Results

Lek1 functions in proliferation and differentiation, and it associates with the MT network through its Nde1 interaction (see Chapter I). To further define the function of Lek1 in these processes and to discover novel pathways for Lek1function, a Y2H screen was utilized. The region of cytLEK1, 5'LSD, was chosen because it is the same region that is being ablated in the *Lek1* knock-out mouse, and a function for this region was unknown. It is predicted to have two leucine zippers in this region of cytLEK1, structures that generally play a role in protein-protein and protein-DNA interactions (O'Shea et al., 1989). 5'LSD consisted of the N-terminal 474 aa of cytLEK1 and was used as bait to screen a whole mouse embryonic day 11 cDNA library. This screen yielded eight clones that passed the false screening process.

Dynamitin/p50

Dynamitin/p50 is a subunit protein of the dynactin complex. Eleven different complex subunits have been identified in the complex, with some subunits appearing more than once. Dynactin is composed of 20 individual polypepetide subunits (Figure 26). Dynactin is a complex that is required for
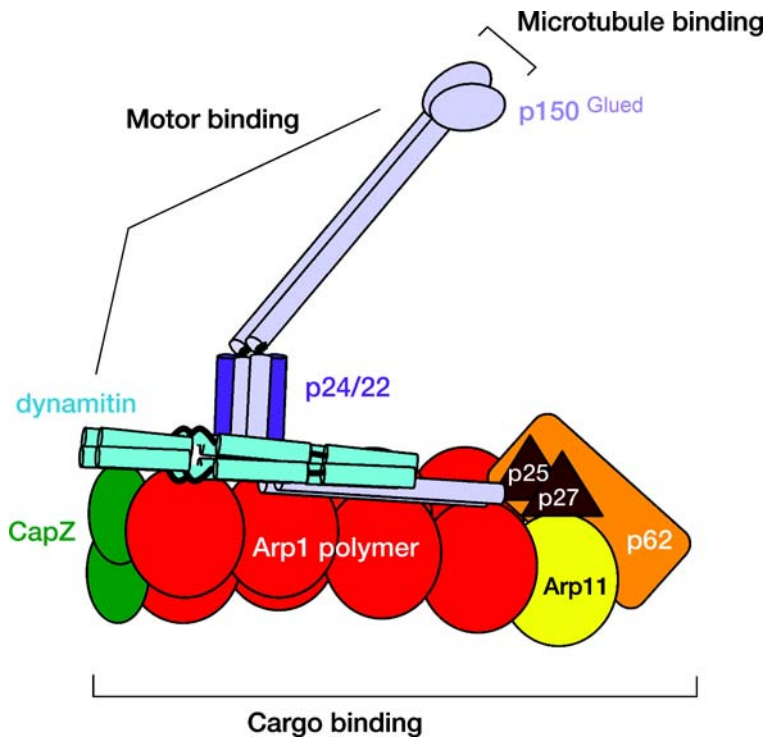
Figure 26. Dynactin. Schematic illustrating the approximate location of the 11 unique
subunits of the dynactin complex, including the p50/dynamitin protein. Note that some
subunits appear more than once. Figure from Schroer et al., 2004.

most, if not all, dynein functions. They are considered to be intimate partners in MT based functions. Studies indicate that dynactin participates in a wide range of functions with cellular structure, many of which are roles in transporting cargos by the dynein motor. It appears that movement of cargo by dynein requires dynactin as the bridge between dynein and the cargo. Dynactin is also required for mitosis and cell proliferation by its association with centrosomes (Quintyne et al., 1999). Each dynactin molecule contains four p50 subunits. It appears to have a central role in linking the cargo-binding region of the dynactin complex with the dynein and microtubule-binding arm of the dynactin molecule (Figure 26). The p50 protein localization has been shown at the organelles, centrosomes, and kinetochores (Karki and Holzbaur, 1999). Since cytLEK1 and dynactin function with the MT network and are involved in organelle transport, preliminary studies were done examining cytLEK1 and p50 colocalization.

Three different mammalian cell lines were utilized to examine endogenous cytLEK1 and p50 localization: C2C12 myoblast, NIH3T3 fibroblas, and purified primary neonatal day 4 myocytes (Goodwin et al., 1999; Pooley et al., 2006; Soukoulis et al., 2005). They shared similar distribution patterns in each cell line. In all three cell types, cytLEK1 had a high of cytoplasmic localization, while p50 also had a similar distribution. In merged images (Figure 27), the proteins share a high degree of colocalization in cells. It is also of great interest to note that in C2C12 cells, both proteins demonstrated a speckled nuclear distribution. The p50 protein has a known function and associates with kinetochores (Echeverri et al., 1996). Another LEK family member, CENP-F/Mitosin, has a described
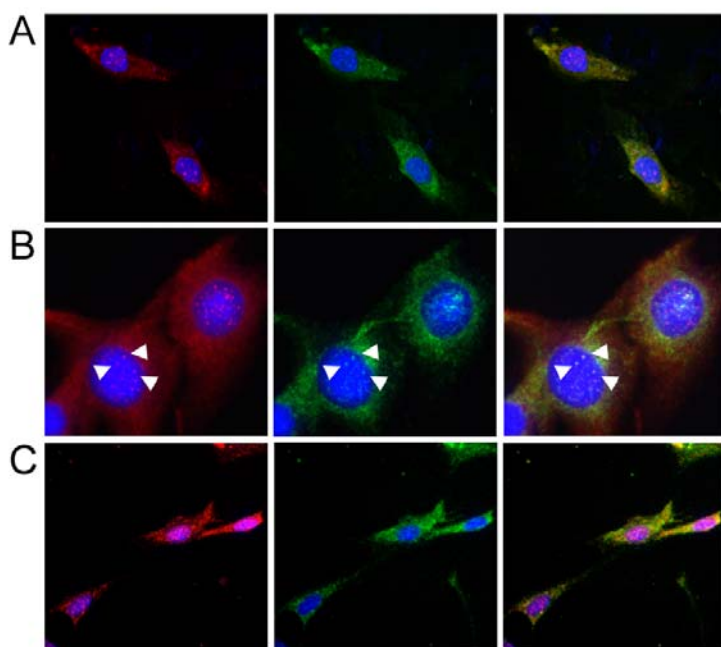
Figure 27. cytLEK1 and p50 endogenous protein localization. As shown previously in Chapters II and III, cytLEK1 (green) has a cytoplasmic distribution with high perinuclear localization. There is a strong overlap of p50 (red) staining demonstrated by the merged images. C2C12 myoblast (A), NIH3T3 fibroblast (B), and primary myoblast (C) cells were examined for colocalization of endogenous proteins. Interestingly, NIH3T3 cells show endogenous protein localization of both cytLEK1 and p50 in an area in the nucleus that could be at the kinetochores (arrowheads).

interaction with the kinetochore (Zhu, 1999). There may also be a population of cytLEK1 that resides at the kinetochore (Soukoulis and Bader, unpublished data), thus defining their localization and function with the kinetochore is critical in characterizing this association. cytLEK1 and p50 have both been separately shown to function in cell division and to have roles in organelle transport and MT based processes while displaying similar colocalization. Further characterization of the cytLEK1-p50 interaction will give great dividends in defining cytLEK1 functions with the cytoskeleton.

Hook2

The Hook family of proteins is an emerging class of proteins that bind microtubules and membranes. Studies on the *D. melanogaster* homolog indicate a function for the protein in multivesicular body trafficking (Kramer and Phistry, 1996; Kramer and Phistry, 1999; Sunio et al., 1999), while the *C. elegans* homolog appears to function in attachment of the centrosome to the nucleus (Malone et al., 2003). The three human members, Hook1-3, have conserved N-termini that bind MTs, while the C-termini are more divergent and contain the vesicle membrane-binding domain. Hook1 interacts with Rab proteins and functions in routing proteins through the endosomes network. Hook3 has been shown to affect Golgi stabilization and distribution (Walenta et al., 2001). No published studies of Hook2 function exist at this time. Because of similar characteristics shared with cytLEK1 in MT association and function in vesicle transport, preliminary overexpression studies were conducted with Hook2.

Hook2 was identified as a cytLEK1 interacting protein in our Y2H screen. COS-7 cells were utilized to examine exogenous expression and localization of transient 5'LSD and GFP-hook2. As seen in previously published reports (Pooley et al., 2006), 5'LSD expression has a high perinuclear expression pattern and extends into the cell periphery. Interestingly, the Hook2 chimera had a pinpoint localization at the centrosome. In cells that were cotransfected with 5'LSD and GFP-hook2, there was an obvious and dramatic relocalization of 5'LSD to the same focus that contained GFP-hook2 (Figure 28), and both proteins localize at the centrosome (Moynihan and Bader, unpublished data). Interestingly, it appears that 5'LSD expression is actually less in cotransfected cells compared to singly 5'LSD transfected cells. This is only visualized by immunofluorescence to date, but further characterization will be conducted. Our genetic and transient protein expression data support a direct cytLEK1-Hook2 interaction, making studies defining the function of this association essential.

Discussion

Even though all the results here are preliminary, it is worthwhile to further speculate and discuss the significance of our Y2H results. Examining these associations and characterizing their possible interactions is fundamental in understanding cytLEK1 and its function in different molecular pathways. It is essential that we understand cytEK1 function, since the protein appears to be important for basic cell processes, such as cell proliferation and differentiation and intracellular movement of proteins.
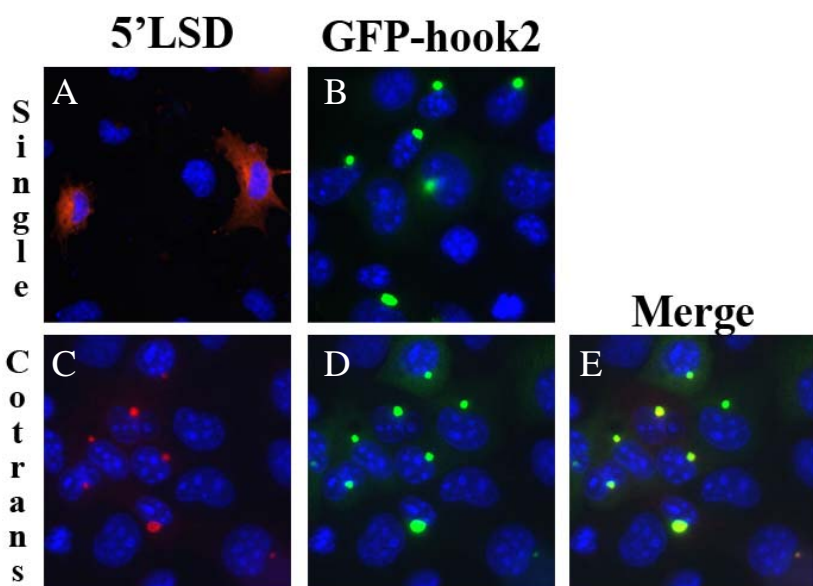
Figure 28. Coexpression of transient 5'LSD and GFP-hook2. COS-7 cells were singly transfected **(A-B)** or cotransfected with 5'LSD and GFP-hook2 **(C-E)**. As seen, 5'LSD (red) has a cytoplasmic distribution **(A)**, while GFP-hook2 (green) has a pinpoint localization in the area of the centrosome **(B)**. In cotransfected cells **(C-E)**, 5'LSD has a dramatic relocalization to the same foci as GFP-hook2. We have identified this focus as being at the centrosome (Moynihan and Bader, unpublished data).

cytLEK1-p50 interaction

Endogenous cytLEK1 and p50 have a similar distribution in mammalian cells and appear to have significant colocalization. Dynactin is a multimeric complex that contains 11 different subunits, one of which is p50. The p50 protein is 551 aa and forms an α-helical structure that appears to be the critical protein link by interacting with itself and the dynactin subunits p150glued, p24/22, and Arp 1 rod. The middle portion of p150glued interacts with MT based motors, while p24/22 and Arp 1 are important in cargo recognition (reviewed in Schroer, 2004). Arp1, or Actin related protein, can hydrolyze ATP and form filaments (Bingham and Schroer, 1999). In relation to cytLEK1, Arp1 binds spectrin-family proteins (Holleran et al., 2001), which allows one to speculate that this association between cytLEK1 and dynactin could be a mechanism utilized by dynein to bind a variety of subcellular structures, refer to model in Figure 26.

Subcellular localization of dynactin has shown that it is present in many different regions of the mammalian cell. Dynactin has distinct localization at centrosomes and spindle pole bodies, similar to that seen with cytLEK1 (Clark and Merer, 1992; Gill et al., 1991; Paschal et al., 1993; Soukoulis et al., 2005), and Soukoulis and Bader, unpublished data). Studies indicate that dynactin governs microtubule anchoring during mitosis. Cells with overexpression of the p50 subunit or other dynactin subunits displayed irregular spindle morphology. As a result, dynactin was mislocalized at the centrosome, and the MTs were in unfocused arrays (Quintyne et al., 1999). Centrosomal component transport was also inhibited after disruption of the dynactin complex (Quintyne et al., 1999).

Along with dynactin localization at the centrosome and spindle appparatus, p50 is located specifically at the kinetochore. The protein associates with the kinetochore proteins Rod, Zw10, and Zwilch, which are essential for mitotic checkpoint signaling (Karess, 2005). CLIP-170, another protein associated with dynactin, relocalizes to and functions in spindle-kinetochore attachment during the onset of mitosis (Tanenbaum et al., 2006). Dynactin also has been localized to the cell cortex. It is speculated that dynactin's binding of microtubules allows a force to be generated that orients the spindles in the proper arrangement (Busson et al., 1998). Dynactin is an integral regulator of mitosis in its key roles with dynamics of centrosomes, spindles, and kinetochores.

Pertinent to our studies, dynactin has been localized at a number of intracellular membranes, because most organelles, if not all, are capable of microtubule-based movement. As would be expected, there are a number of studies describing dynactin regulation of organelle trafficking. Originally identified as a kinetochore protein during mitosis and a p50 binding protein, ZW10 is localized at the ER and throughout the cytoplasm during interphase (Hirose et al., 2004). Overexpression and knockdown assays of ZW10 revealed its involvement in membrane trafficking between the endoplasmic reticulum and Golgi. We have also described a role for cytLEK1 in membrane trafficking at the recycling endosomes network (see Chapters II and III). Along with dynactin localization at the Golgi, dynactin has been localized to numerous other organelle structures. Overexpression of p50 causes accumulation of Golgi, early and late endosomes, and lysosomal structures at the cell periphery. Therefore, these

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results indicate that p50 and dynactin are required for proper plus-ended transport of organelles (Burkhardt et al., 1997; Deacon et al., 1997; Presley et al., 1997; Valetti et al., 1999). These studies may indicate that cytLEK1 not only functions in recycling endosome movement, but it is a more broad regulator of intracellular movement. Alternatively, p50-cytLEK1 interaction may have a critical role specifically in plasma membrane recycling.

Protein members of the Lis1 pathway bind the dynein heavy chain through Nde1 and also the dynactin complex indirectly (Sasaki et al., 2000; Smith, 2000). Nde1, a cytLEK1 binding protein (see Chapter I), has been localized to the cell cortex and centrosomes, where it has roles in mitosis and spindle assembly. Studies conducted in *A. nidulans* indicate a direct interaction of Nde1 with the Arp1 subunit of dynactin. However, direct or indirect associations between Nde1 and the dynactin complex are lacking. cytLEK1 may provide the first identified link between the p50 subunit of dynactin and Nde1.

A number of factors are responsible for recruitment of dynactin and dynein to Golgi membranes. The Arp1 subunit binds directly with the Golgi-specific spectrin β-III spectrin. Rab6 appears to regulate the binding of dynactin to the TGN. BICD, a binding partner of Rab6, also has the ability to bind p50, implying a regulatory mechanism of dynactin localization and function at the Golgi (Matanis et al., 2002; Young et al., 2005). These proteins assist in tethering the dynactin-dynein motor to the MT network. Similarly, Rab7 is proposed to be the link between dyanctin and endosomes (Cantalupo et al., 2001). It is now quite evident that dynactin is a key regulator of organelle and membrane transport of

several intracellular pathways along the MT network. These data show that p50 and dynactin have a broad role in organelle movement. cytLEK1 has an emerging role with SNAP-25 and Rab11a in the recycling endosome pathway. Therefore, future studies need to address whether or not cytLEK1 has a broad role in membrane transport throughout the cell or if it is specific to recycling endosomes. cytLEK1 may be the key regulator of p50 and dynactin association with the plasma membrane recycling pathway.

cytLEK1-hook2 interaction

Hook2 is a member of an emerging family of proteins that associates with the MT network and membranous cargo, which are thought to be nonmotor MT linkers for cellular components. There are three mammalian hook homologs that have nearly identical N-termini but differ in their C-terminal cargo binding domains. The central region consists of a coiled-coil domain, similar to cytLEK1 and its other binding partners. Interestingly, northern blot analysis reveals that Hook1 is found only in the testes. RT-PCR shows *hook1* transcript expression at very low levels in adult tissues. *Hook1* maps to chromosome 4 in the mouse and is in the same locus as the previously identified *azh* mutation (Mendoza-Lujambio et al., 2002). The *azh/azh* mouse is a mutant that displays abnormal sperm head morphology and looping at the midpiece of sperm flagellum (Cole et al., 1988; Meistrich et al., 1990). RT-PCR analysis shows that deletion of exons 10 and 11 from the 22 exon *hook1* gene results in a premature stop codon and then translation of a non-functional protein. Hook1 is located at MT structures located

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throughout the developing spermatids. From these studies, it is speculated that Hook1 is responsible for MT and associated protein localization in the differentiating spermatid. Truncation of the wild-type protein results in altered sperm morphology and may be a cause of male infertility (Mendoza-Lujambio et al., 2002). *In vitro* cell culture data show that Hook1 localizes to unidentified discrete punctuate subcellular structures that appear to be associated with MTs (Walenta et al., 2001).

Hook3 is a more ubiquitous protein than Hook1. It is expressed in all tissues, 293 cells, human, and monkey cells tested thus far. Examination of Hook3 localization in Hep2 cells shows that it has a perinuclear distribution and colocalizes significantly with Golgi and the microtubule organizing center. Similar to all Hook proteins, Hook3 binds MTs, and MT integrity is required for proper localization of Hook3. Overexpression of Hook3 disrupts the Golgi complex (Walenta et al., 2001).

Hook2 is also a widely expressed family member, as it is expressed in all rodent tissues tested. It is the family member that we identified in our Y2H screen. Interestingly, protein expression peaks in the developing brain at a time when most neuronal precursors cease division and undergo differentiation. This is in striking similarity to what is observed with cytLEK1 expression in the developing heart. Lek1 is expressed at high levels during cardiomyocyte proliferation and is drastically down-regulated as cells terminally differentiate (Ashe et al., 2004; Goodwin et al., 1999). Hook2 protein is distributed throughout the cytoplasm, but has more intense staining at the centrosome. Y2H analysis

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utilizing Hook2 as bait identified the centrosomal protein CEP110. Furthermore, overexpression analyses demonstrated altered centrosome localization and function and retarded MT aster formation (Personal communication, H. Kramer).

As one can see, the overriding theme of the Hook family's functions appears to be organelle transport along the MT network. In separate studies, we have identified a cytLEK1 function with the MT network and a role for cytLEK1 in vesicular transport in recycling endosomes (Pooley et al., 2006; Soukoulis et al., 2005). Similar to Hook2, cytLEK1 also has a high localization in a focus juxtaposed to the nucleus that coincides with centrosome localization (Soukoulis et al., 2005; Soukoulis and Bader, unpublished data). The interaction between cytLEK1 and Hook2 needs to be further characterized for obvious reasons. We identified Hook2 in our Y2H screen as a cytLEK1 interacting protein demonstrating interaction between the proteins, while both have a similar protein subcellular localization patterns at the centrosome. It could be speculated that Hook2 and cytLEK1 regulate function at the centrosome. In separate studies, overexpressing proteins phenocopy one another in the retardation of microtubule polymerization at the centrosome (Soukoulis et al., 2005; personal communication, Helmut Kramer). Further clarification is needed as to whether cytLEK1-Hook interaction is specific for only Hook2, or if other Hook members are able to associate with cytLEK1.

Other proteins identified in the Y2H screen

Along with p50 and Hook2, the Y2H screen identified four other proteins as preliminary cytLEK1 interacting proteins. Because no further data has been generated characterizing the association, they will only be briefly discussed. Keratins, identified in the screen, are members of the intermediate filament family of proteins. Keratins have been shown to exist in high copy numbers in pretransformed libraries and have been shown to be prone to giving false-positive results in Y2H. As a result, cytLEK1-keratin interaction is unlikely, as previous colcalization experiments have shown sparse colocalization between the proteins (Pooley, Soukoulis, and Bader, unpublished data).

Oligophrenin was identified and is a protein that encodes a RhoGAP protein involved in X-linked mental retardation (Zanni, 2005). It may modulate Rho GTPase activity in neuronal morphogenesis by regulating the actin cytoskeleton. The only common characteristic that oligophrenin shares with cytLEK1 is its association with the cytoskeleton, however oligophrenin has only been identified to interact with actin. To date, cytLEK1 has no known function with actin.

Structural maintenance of chromosome (SMC) proteins are chromosomal GTPases that function in chromosome organization, segregation, and dynamics, especially during mitosis. SMC4 was also identified in our Y2H screen as a cytLEK1 interacting protein. It forms a condensing complex with other accessory proteins that bind to chromosome arms during mitosis and help maintain chromosome architecture. Emerging data suggests that SMC proteins play a vital

role in assembling centromeric heterochromatin and orienting sister kinetochores to allow spindle attachment (Losada and Hirano, 2005). Even though cytLEK1 and SMC4 have different localization patterns and are quite divergent in their known functions, there may be a population of cytLEK1 that localizes to the kinetochore (Soukoulis and Bader, unpublished data). cytLEK1 may bind SMC4 at the kinetochore during mitosis and help in spindle attachment, a MT-based process.

Lastly, laminin B1 was recognized in the Y2H screen. Since laminins are part of the extracellular matrix, a functional role with cytLEK1 appears obscure and should not be pursued. We hypothesize that this is not a true interaction and may be a false-positive from our screen.

The N-terminal portion of cytLEK1 used for the bait construct in our Y2H screen contains two leucine zippers. Leucine zippers are generic motifs that drive protein-protein interaction (O'Shea et al., 1989). It is logical for us to characterize cytLEK1 functions with proteins that have a similar expression pattern, subcellular localization, and demonstrated roles in shared cellular processes (i.e. interaction with the MT network and/or involvement in vesiclar transport, since these are two pathways of known cytLEK1 function). Hook2 and p50 have well-defined functions with the MT network and in vesicle trafficking and positioning. Therefore, it would be of most interest for us to pursue these interactions.

According to published reports, it appears that keratin, oligophrenin, and SMC4 have no known function with the cytoskeleton. It may be that these proteins are inherently "sticky" and had a spurious interaction with our bait

construct, even though they passed a false-positive screen and appear to be legitimate cytLEK1 interacting proteins according to our initial Y2H analysis. It should also be noted that we have done multiple Y2H screens with various regions of cytLEK1, and these proteins were never identified in other screens.

To date, it appears that cytLEK1 may have multiple functions with the microtubule network. It is possible that the C-domain of cytLEK1 binds MTs while the more N-terminal 5'LSD arm of cytLEK1 associates with proteins that link to the MT network. cytLEK1 has a broad expression pattern with a high expression pattern around the nucleus. We have demonstrated that cytLEK1 is at the kinetochore, the centrosome, and with recycling endosomes. cytLEK1 may in fact interact with numerous proteins at given organelles, and the underlying role of cytLEK1 is in associating structures with the MT network. It may be a protein that is a general regulator of association between organelles and the MT network, or it may be specific to certain organelles (for example, recycling endosomes and the kinetochore). I propose that cytLEK1 may be a general link for organelles to the cytoskeleton.

CHAPTER V

DEVELOPMENT OF THE CONDITIONAL *LEK1*
KNOCK-OUT ALLELE

Introduction

To date, study of the LEK family of proteins has remained confined to overexpression and knock-down studies with exogenous factors, such as with RNAi and Morpholino oligomers. Overexpression of dominant-negative proteins in chicken cell lines demonstrates a CMF1 function in muscle cell proliferation and differentiation (Dees et al., 2000; Wei et al., 1996). The human CENP-F/mitosin associates with the kinetochore and mitotic spindles in a cell cycle dependent manner (Liao et al., 1995; Zhu et al., 1995a; Zhu et al., 1995b). Several studies have examined CENP-F/mitosin knock-down in human cell lines utilizing RNAi. All studies have varying results and conclusions, but knock-down of CENP-F/mitosin implicate roles for the protein in kinetochore assembly, regulation of chromosome behavior, and control of the spindle assembly checkpoint during mitosis (reviewed in Varis et al., 2006). While RNAi is a useful tool in studying protein knock-down in cell lines, critical *in vivo* studies utilizing protein ablation are completely lacking, particularly in organogenesis.

The Cre-loxP system is a widely used tool for genetic tailoring of the mouse genome. Cre recombinase from the P1 bacteriophage efficiently recombines DNA located between consensus 34 base pair recognition loxP sites.

By utilizing this system, DNA can be altered in such a way that it becomes inverted, excised, or recombined between two different DNA templates. It can then be manipulated so the gene of interest is activated or inactivated after recombination. Numerous transgenic mouse lines have been created that express Cre recombinase in tissue and temporal specific manners. One is now able to create global knock-out or conditional knock-outs depending on which Cre line is utilized. Conditional lines are especially useful when a traditional knock-out results in early embryonic-lethality preventing study of later functions, or when the gene of interest has roles in multiple tissues (reviewed in Kwan, 2002; Nagy, 2000).

No studies have been conducted on any of the LEK proteins that examine complete loss-of-function. Groups have used RNAi and Morpholino technology with some success. The major drawbacks with these technologies is that one never knows if the protein is completely diminished in every cell, and they cannot be utilized *in vivo* in mice. The holy grail of knock-down/knock-out technology is the creation of a conditional allele of the gene of interest, and in our case, Lek1. We have now created the conditional *Lek1* allele and initial studies characterizing the conditional knock-out in the heart have been completed.

Our pilot studies utilizing the *Lek1$^{loxP/loxP}$* mouse line have utilized the cardiomyocyte specific cardiac troponin-T Cre line (cTnT-Cre) to conditionally ablate Lek1 function specifically in cardiomyocytes early in heart organogenesis. Our pilot studies indicate a "small heart" phenotype that includes a reduction in

myocyte layer thickness and overall heart size in mutant hearts with significant alterations in their functional capabilities.

Materials and Methods

Labeling of Probes for BAC Screening

The Rediprime II Random Prime Labeling System was obtained from Amersham. After PCR amplification of the Lek1 fragment used as a template for the probe, 25ng template DNA was diluted in 45 µl of TE. The DNA was denatured at $95^{o}C$ for 5 min and centrifuged briefly. The denatured DNA was then added to the tube supplied by the kit. To the tube, 5 µl ^{32}P-dCTP (Sigma) was added to the reaction mixture, pipetted up and down 12 times, and incubated at $37^{o}C$ for 15 min. The reaction was column purified (Quick Spin Column (TE), Roche). The flow-through was boiled for 15 min, centrifuged briefly, and mixed. Labeled probe (14 µ) was used in 5 ml hybridization buffer.

PreHyb Solution

To 1M $NaPO_4$, 20% filtered SDS, 0.5M EDTA, fresh Poly A/PolyC solution (Sigma, # P9403 and P4903) was added. Poly A/Poly C stock solution was maintained at 10 mg/ml. To 1ml of TE, 12.5 µl of each was added and boiled for 10 min, quenched on ice, and added to PreHyb solution.

Hybridization Buffer

1 M $NaPO_4$, 20% filtered SDS, and 0.5M EDTA

Erase Solution

5 ml 20X SSC, 5 ml 20% SDS, and 490 dH_2O

Wash Solution

For 1L: 5 ml 20X SSC, 2 ml 0.5M EDTA, 50 ml 20% SDS, and 943 ml of dH_2O

Probing of BAC Filters

PreHyb solution was added to the filter membranes (CHORI), and they were incubated at 65^oC for 2 h. PreHyb solution was replaced with prewarmed hybridization solution. The labeled probes were boiled and then added overnight at 65^oC. Filters were washed with 1X SSC with 0.1% SDS for 30 min for a total of six times. The labeled membranes were placed in a Kodak Biomax cassette with film for 24-48 h, and the film was then developed.

Labeling of Southern Probes to Screen ES Cells

The Prime-It II Random Primer Labeling Kit was obtained from Stratagene. With some modifications, PCR fragments were amplified and then purified using the QIAquick Gel Extraction Kit (Quigen). After purification, 2 µl of DNA (25 ng total) was added to a microcentrifuge tube. To the reaction tube, 17 µl dH_2O and 10 µl of random oligonucleotide primers were added, and the tubes

were heated at 95°C for 5 min and briefly centrifuged. In order, 10 µl of 5X buffer, 5X dCTP primer buffer, 5 µl of labeled ^{32}P-dCTP (Sigma), and 1 µl Exo(-) Klenow enzyme (5 U/µl) were added and then mixed and incubated at 37°C for 45 min. The reaction was stoped with 2 µl of stop mix.

Southern Blotting

DNA was digested with EcoRI or BsaWI for a minimum of 8 h during the day. The digested DNA was run on a 0.8% agarose gel overnight without EtBr at 25 V in 1X TBE. The gel was stained in 4 µg/ml EtBr for 15 min and briefly rinsed three times with H_2O and photographed. Dilute HCl (10 ml HCl+490 ml H_2O) was added for 15 min and rinsed with H_2O three times. The gel was washed with 0.5M NaOH for 40 min on shaker at room temperature. DNA was transferred overnight in 0.5M NaOH in transfer apparatus (Altec Labs) to a Hybond-N+ (Amersham) membrane. The next day, the membrane was washed with 2X SSC for 10 min and then Stratalinked (Stratagene) for 30 min. Erase solution (500ml) was added to the blot at room temperature with shaking for 20 min. PreHyb (10 ml) solution was added for 2 h at 65°C. The PreHyb solution was removed and the boiled probe was added to 10 ml hybridization solution preheated to 65°C. The probe and blot were incubated overnight at 65°C. The following day, the hybridization mixture was discarded. Preheated wash solution (100ml) was added to the container with blot for 30 min at 65°C, for a total of three times. The labeled blots and film were placed in a Biomax cassette (Kodak) for at least 24 h, depending on the affinity of the probe, and developed.

Modified BAC DNA Midiprep (Qiagen)

A single BAC clone was obtained from CHORI and one colony was isolated and used to inoculate a 5 ml starter culture with chlorophenicol. To inoculate 100 ml of selective LB medium, 0.5 ml of starter culture was added and then grown at 37°C overnight with vigorous shaking. The cells were harvested by centrifugation. Pellets were suspended in 10 ml of P1 buffer. Buffer 2 (10 ml) was added to the tubes. It was mixed thoroughly by inverting six times and then incubated at room temperature for 5 min. Chilled P3 buffer (10 ml) was added and immediately mixed by inverting six times and incubated on ice for 15 min. The slurry was centrifuged at >20,000 x g for 15 min at 4°C. The supernatant was removed containing the BAC DNA. It was re-centrifuged as before and the supernatant with the DNA was removed. A Qiagen-tip 100 was equilibrated by applying 4 ml of Buffer QBT and then allowed to empty by gravity flow. The tip was washed two times with 10 ml Buffer QC. The DNA was eluted with prewarmed (65°C) 5 x 1ml Buffer QF. The DNA was precipitated by addition of 3.5 ml room temperature isopropanol. It was mixed with gentle inversion and centrifuged immediately at >15,000 x g for 30 min at 4°C. The supernatant was removed from the DNA pellet, and the pellet was washed with 2 ml of room-temerature 70% EtOH. Centrifugation was then conduted at >15,000 x g for 10 min. The pellet was air-dried and redissolved in TE.

Primers Used to Construct Probe for BAC Membrane Screen

5': AGCTGGGCCCTGGAAGAATGG,

3': AGCGCAGCTTCGAGAGAGTC

Primers for PCR Screening of BAC Clones

3.1: 5' CGAGTTGTCCTTTGAGTCCCTG,

 3' GAACCTACCGTCTGAGAACCACTG;

3.2: 5' TGGAAGAATGGAAGGAAGGTCTC,

3' CACAGGCAGGCAGCAATAAAG;

3.3: 5' GGGTGAACTCTGTAACCATTGACC,

3' CGGCTTTCCTTTGTCTGTCTTG;

3.4: 5' GAATGCTTGCGTGTAGTTTGGG,

3' TGCTCTAAGACAATGGTTCCTTCC;

3.5: 5' TTGTATGTGGCGTTCAGTAGATGG,

3' TGACTTGCTGTAACTCCTGGGTAAG;

3.6: 5' AGCAGTGAGGTATGTGAGCAGGAG,

3' TTTTGGACCAGGGAGATGACTTAG;

4.1: 5' GCAGTTTGAATCGCTCGTGC,

3' TACGGAACAACCTGTCAGCCAC;

4.2: 5' CTCTGAGAAGGGAAGGTGTTTGTG,

3' AAGCAAGGGAATCCAGCAGG

Primers for Cloning *Lek1* into pFRT.loxP

SA: 5' ATCGATGGACTGGGATCACAGGCAGAAG,

3' ATCGATTTCTTTCTGTCTTGTTTTGTCAATTGTG;

5' DEL fragment: 5' TTAATTAACAGTTGGGTCTGAGTGACAGTGGCAG,

3' CTTTTGCTCCATCGACAGAAATTAC;

3' DEL fragment: 5' CGGTAACTGTCTGAGGAACAGTCCTGT,

3' TTAATTAAGCAACAGTGTAAACCTCTACTACT;

5' LA fragment: 5' TATAGGATCCTCTTCAGTGGAGACAGAGCT,

3' GCAATGTCAGCAAAACACTTAACAA;

3' LA fragment: 5' ATTCCTCCAGGTCCTTAAGATTTG,

3' ATATAGGATCCCATGCCCACCTCATAAAGA

Primers Used for Screening ES Cells by Southern

Probe 4.1-1: 5' GCAGTTTGAATCGCTCGTGC,

3' TACGGAACAACCTGTCAGCCAC;

Probe 11.1-5: 5' TTAGCTGCCTTCCTGGGGAA,

3' AAATCTGGATATGCGATTCGC;

Probe 5.7: 5' AATCACTAGGCCAGTATCACTGC,

3' CATTTCACAATGTGTGCATGG

Primers for PCR Screening of ES Cells and Mice

Across loxP site: 5' AATAATGAAGCTGACACCAAAAACT,

3' GAACCTACCGTCTGAGAACCACTG;

Across FRT site: 5' CCAAAGAACGGAGCCGGTT,

3' AATGTCTTACTCGCGTCTTCACGGA;

Neomycin cassette: 5' CGATCCCATATTGGCTGCAG,

3' AACCGGCTCCGTTCTTTGG;

LA recombination: 5' TTGGAGGCATGTCTGTGTGTGGCT,

3' CATTTCACAATGTGTGCATGG

<div align="center">

Results

</div>

Isolation of *Lek1* gene

Our laboratory had previously constructed the nucLEK1 and cytLEK1 constructs separately. As a result, the entire gene with exons and introns needed to be cloned. To inititially screen the high density mouse BAC library filter set, a 140 bp fragment from exon 1 of *Lek1* was amplified by PCR. This fragment of *Lek1* was then randomly labeled to create a radioactive probe. The probe was then utilized to screen the filter set with 11 potential clones being identified in the screen.

Individual BAC clones were received and further analyzed. DNA was purified from each individual clone, and a PCR-based strategy was developed to screen for ones that contained *Lek1* (Figure 29). Initially, PCR was used to amplify a 140 bp product located in exon 1 (primer set 3.2). The screen identified two potential BAC clones that may have contained *Lek1*, clones 535-I18 and
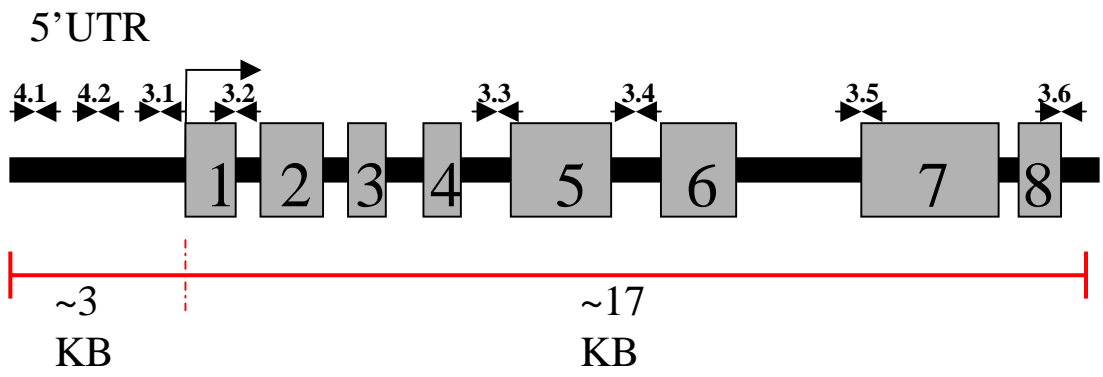
Figure 29. BAC screen. Eight unique primer sets were utilized to screen BAC clones that may have contained *Lek1*. In clone 335-N19, approximately 3 KB of 5'UTR and at least 17 KB of *Lek1* was identified in the PCR screen.
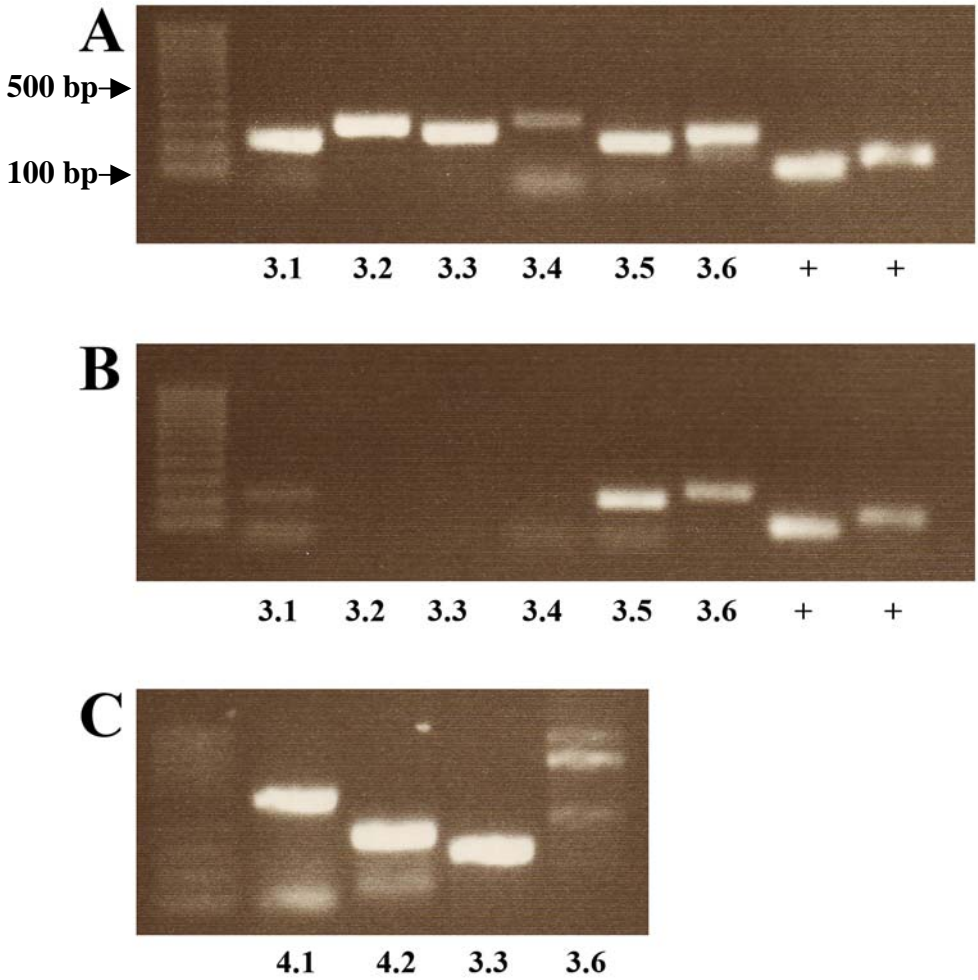
Figure 30. PCR screen with *Lek1* primer sets. Refer to Figure 27 to see where the unique *Lek1* primer sets amplify. Fragments from all the primer sets were amplified in clone 335-N19, while clone 535-I18 appears to have a region of *Lek1* missing from the BAC. **A** and **C** are PCR screens utilizing 335-N19 as template DNA and **B** uses 535-I18. From these screens, we determined that 335-N19 has at least 3 KB of 5'UTR and 17 KB of *Lek1* in the clone. Positive control reactions (+) utilized *Lek1* primer sets previously shown to amplify fragments of the gene.

335-N19. These two clones were then characterized to determine exactly which portion of *Lek1* they contained.

Again, a PCR-based analysis was utilized to further characterize how much of *Lek1* was present in each BAC clone. The clones were screened by eight unique primer sets that amplified different regions of *Lek1* (Figure 29). Clone 535-I18 was not found to have the entire gene and appeared to have a region missing between the first and fifth exons, while clone 335-N19 was found to have no such deletion (Figure 30). Further analysis showed that the 335-N19 BAC clone contained at least 3 KB of the 5'UTR and at least 17 KB after the translation start site (Figures 29 and 30). The BAC clone may contain more of *Lek1*; it has not been further characterized. In order to amplify the BAC clone, a modified Midiprep was used (see Materials and Methods).

Cloning of *Lek1* into knock-out vector

The knock-out vector, pFRT.loxP, was a gift of Dr. Mark Magnuson (Figure 31; Vanderbilt University). It contains the loxP sites, the neomycin resistance positive selection gene, the thymidine kinase negative selection marker utilized for random vector integration events, and FRT sites used to delete the neomycin resistance cassette. To construct the final knock-out Lek1 vector, three fragments of *Lek1* had to be ligated into different sites. The *Lek1* short arm (SA) and long arm (LA) were required for recombination in ES cells. Lastly, the fragment of *Lek1* to be floxed and excised (DEL) from the endogenous *Lek1* locus needed to be cloned (Figure 32).
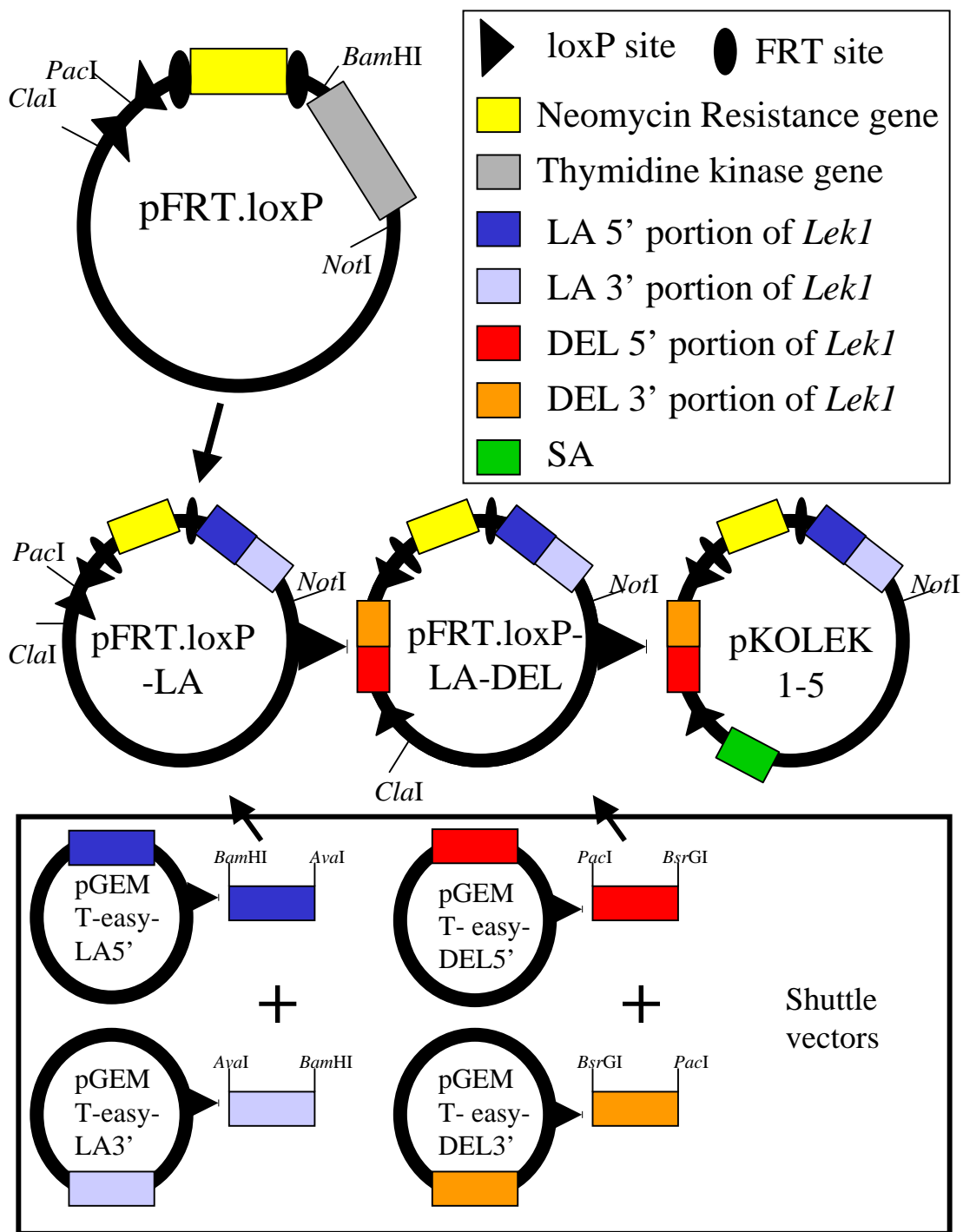
Figure 31. Cloning of *Lek1* targeting construct. The pFRT.loxP vector is depicted in the upper left corner of the figure. Shuttle vectors had to be constructed utilizing the pGEM T-easy system that were then used in intermediate steps to ligate the SA (1 KB), LA (6 KB), and DEL (1.4 KB)into pFRT.loxP. First, the *Bam*HI digested vector and *Ava*I and *Bam*HI digested LA5' and LA3' fragments were triple ligated to construct pFRT.loxP-LA. Then, *Bsr*GI and *Pac*I digested DEL5' and DEL3' fragments were triple ligated into *Pac*I digested pFRT.loxP-LA. This construct, pFRT.loxP-LA-DEL was then digested with *Cla*I and a shuttle vector containing the SA was also digested with *Cla*I. The cut vector and SA fragment were ligated together to form the final pKOLEK1-5 targeting construct.

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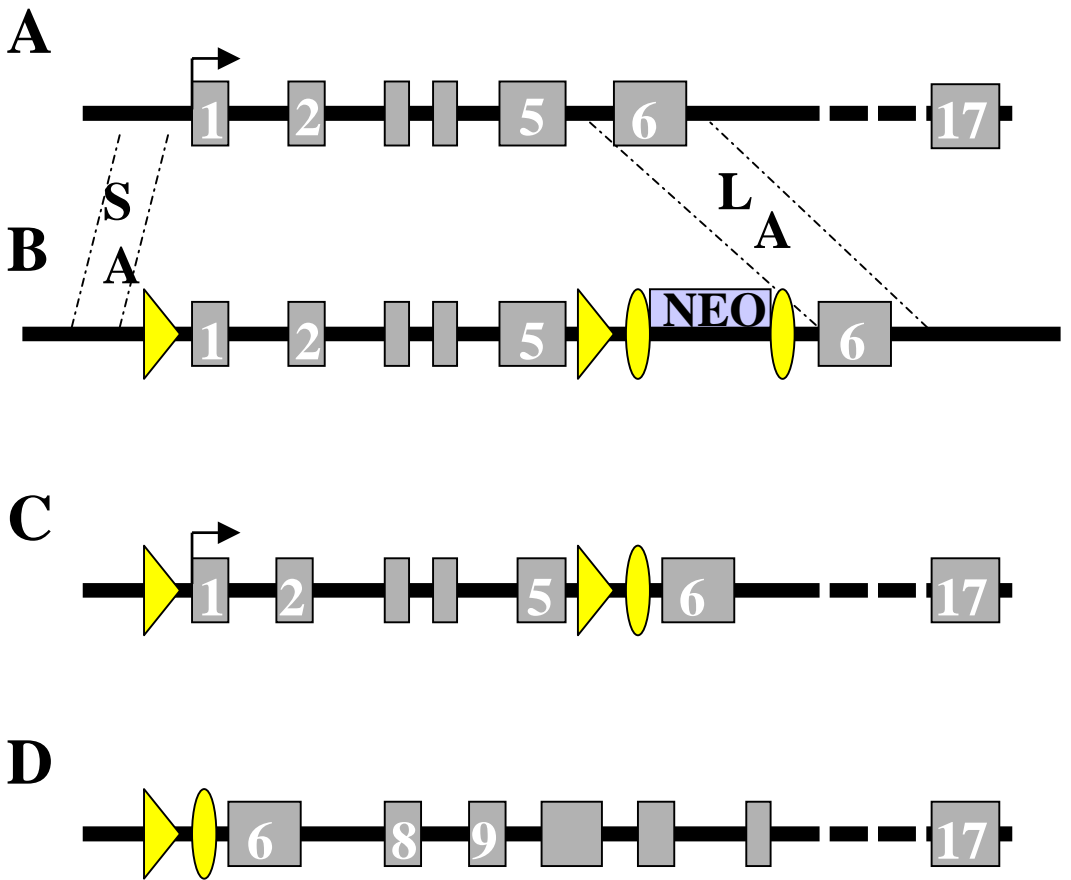
Figure 32. *Lek1* targeting. A) The 5'UTR was utilized for the SA, the first five exons, DEL, are to be excised, and a 3' region after DEL was used for the LA. **B)** After successful recombination, loxP sites will flank the first five exons of *Lek1*, and the positive selection cassette Neomycin resistance with corresponding FRT sites will also be integrated into the *Lek1* locus. **C)** Once the conditional *Lek1* mouse line carrying the Neo resistance cassette is identified, it will be crossed with a line expressing FlpE to delete the Neo resistance cassette. **D)** The line carrying the floxed *Lek1* allele without the Neo resistance cassette will be mated to Cre lines to delete the first five exons of *Lek1*, therefore ablating Lek1 function.

The *Lek1* gene is relatively large in size, 8994 bp, and contains 17 exons. Therefore, it was not feasible to flox the entire gene. We developed a strategy that floxed the first five exons of *Lek1* and included the translation start site. This corresponds to the first 788 bp Lek1 mRNA. By deleting the first five exons of *Lek1*, we postulated that since the translation start site is absent, Lek1 will not be translated. It must be noted that our laboratory has detected neither alternative start sites nor alterantive splice variants. However, there are several methionines located in frame throughout the remainder of the mRNA. If a truncated mRNA is transcribed, it is theoretically possible that a truncated Lek1 could be translated. Also, no consensus Kozac sequences would be present if a shortened mRNA is transcribed.

If a truncated Lek1 protein were to be translated, we have the necessary reagents to screen for the presence of the mutated protein. We have several antibodies that recognize different epitopes located throughout the protein. If a peptide was translated without the floxed region present (also termed 5'LSD), we have antibodies that recognize six epitopes after 5'LSD (see Figure 4). In the event that a truncated protein was produced, it was most likely that it would not function properly. Since a large region would be absent, it is quite possible that the protein would be misfolded or degraded, rendering it nonfunctional. The first five exons of *Lek1* correspond to the same region, 5'LSD, which was used in the Y2H screen (review chapters II and III). This is the region that is responsible for Lek1 interaction with SNAP-25 and syntaxin 4 in the plasma membrane recycling

pathway. In the scenario that a truncated Lek1 transcript is translated, we can test the consequences of Lek1 loss-of-function in these pathways.

In order for our laboratory to target ES cells and ablate Lek1 early in development, we constructed a *Lek1* knock-out vector. Unique restriction enzyme sites were required for each of the ligations, and each of the ligations had to be performed in a sequential manner. First, we utilized site directed mutagenesis to obtain a unique PacI restriction site in pFRT.loxP that was utilized for placement of DEL.

Second, a triple ligation strategy was used to ligate the LA into pFRT.loxP, since it is relatively large and difficult to PCR amplify. The LA contains a unique AvaI site that is found only once in the LA and not found in pGEM T-easy vector. Shuttle vectors were made that contained a 5' and 3' half of the LA (Figure 31). These shuttle vectors were then digested with BamHI and AvaI, purified, and ligated into the BamHI cut pFRT.loxP. After numerous attempts at the triple ligation, the LA was successfully ligated into pFRT.loxP, termed pFRT.loxP-LA.

Third, a similar triple ligation strategy was developed to place the DEL region into pFRT.loxP-LA. 5' and 3' halves were PCR amplified, placed into the shuttle vector, digested with PacI and BsrGI, and triple ligated into pFRT.loxP-LA. After several attempts, pFRT.loxP-LA-DEL was developed.

Lastly, the ClaI site of pFRT.loxP-LA-DEL was utilized to ligate the SA into the Lek1 knock-out targeting vector. The SA was PCR amplified, placed into the shuttle vector, digested, and ligated into the construct. This was relatively easier than the prior triple ligations. The targeting construct, pKOLEK1-5, was finished

after 11 months of ligation attempts. The vector was sequenced fully, and no mutations were present.

Development of conditional *Lek1* knock-out mouse line

No Lek1 family protein has been ablated *in vivo*. Figure 32 diagrams the wild type and targeted loci of *Lek1*. All ES cell electroporations were performed by the Vanderbilt Transgenic Mouse/ Embryonic Stem Cell Shared Core Resource Facility (Dr. Cathleen Pettepher, director) utilizing our pKOLEK1-5 targeting vector. Our laboratory also developed a Southern probe strategy to screen injected ES cell clones.

We obtained DNAs from 711 129/SvEv ES cell clones that survived positive and negative selections. Our initial screen used the radioactively labeled 4.1-1 probe of 545 bp (Figure 33). We identified four potential ES cell clones that had targeted recombination at the *Lek1* locus. In order to confirm these results, all clones were re-probed with 11.1-5, and these data supported the previous screen. We identified clones 1D-5, 5-C9-1, 6A7-1, and 8-D9 as potential clones containing the targeted *Lek1* locus. A third probe using the LA as a template, probe 5.7, confirmed our results.

Next, a PCR-based strategy was utilized to verify that the targeting construct recombined properly in the ES cell clones and that the entire construct was present in the floxed *Lek1* locus. As seen in Figure 33, four primer sets were used to identify a loxP site, a FRT site, the neomycin resistance cassette, and a primer set to identify proper LA recombination. Results from the PCR screen
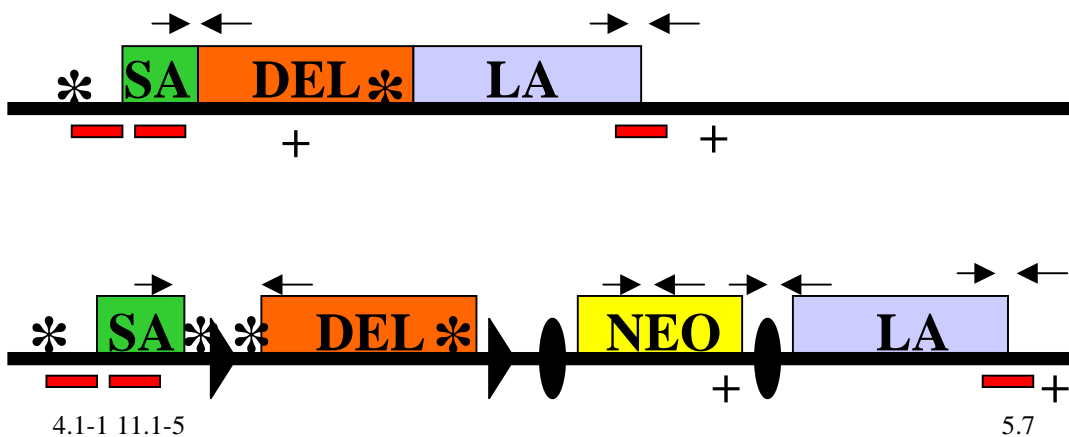
Figure 33. ES cell screening strategies. DNA was isolated from 711 ES cell clones and a Southern blot strategy was employed to screen the clones. To initially test for recombination at the SA, DNAs were digested at *Eco*RI sites (*) and the digested DNA was labeled with probe 4.1-1. The wild type *Lek1* band was 7.7 KB, while a targeted floxed allele would label a fragment of 3.6 KB. To confirm our initial results, probe 11.1-5 was also utilized on *Eco*RI digested DNA and gave the same results. DNA samples were then digested with *Bsa*WI (+) to test for recombination at the LA. Wild type fragments were 13 KB, while targeted alleles showed fragments of 8.8 KB. After Southern blot screening, clones demonstrating targeted recombination at the *Lek1* locus were also PCR screened. Primer sets (arrows) were designed to produce fragments that varied in size between wild type and floxed alleles. Primer sets were designed that showed the presence of a loxP site, the inclusion of the Neo resistance cassette, an FRT site, and recombination at LA locus.

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showed that clones 1-D5 and 8-D9 had the correctly targeted *Lek1* locus. By utilizing both Southern blotting and PCR-based strategies, we were able to identify two ES cell clones containing the correct targeted *Lek1* locus that floxed the first five exons of the *Lek1.*

Both ES cell clones 1-D5 and 8-D9 were injected into blastocysts and then placed into foster mothers by the Core. All litters had pups demonstrating >80% levels of chimerism (as seen by coat color), and therefore, germ line transmission was probable in one or more of these chimeras. One chimeric pup derived from the 1-D5 injected blastocyst demonstrated germline transmission and became the founder. Chimeras were crossed to the ICR background.

Preliminary studies characterizing conditional *Lek1* knock-out mice

Mice carrying floxed *Lek1* on both alleles, *Lek1* [loxP/loxP], have now been generated. We removed the neomycin resistance cassette gene by utilizing the FlpE deleter strain, and as a result, phenotypes characterized after *Lek1* excision can be based solely on Lek1 loss-of-function (Rodriguez et al., 2000). By utilizing the *cTnT-Cre* mouse line (a gift from Dr. H. Scott Baldwin; Jiao et al., 2003) and establishing *cTnT-Cre; Lek1* [loxP/+], we were able to ablate Lek1 function specifically in cardiomyocytes early in heart development, beginning at E7.5. To date, normal Mendelian distributions have been obtained, indicating that it is not an embryonic lethal phenotype. Lek1 is not required for cardiac specification or commitment and is not required for initial cardiac differentiation as we once speculated (Ashe et al., 2004). It must be noted that we would prefer to establish

a null *Lek* ^{flox/-} mouse line by utilizing the *E2A-Cre* (Lakso et al., 1996) line that expresses Cre ubiquitously, but the F1 generations have only demonstrated mosaic expression with no germ line Cre transmission. Attempts to develop a null *Lek1* allele are ongoing.

PCR analysis of mice carrying a floxed allele confirms the presence of a loxP site before the first exon, which contains the translation start site, and a site after the fifth exon. These mice also carry the cardiomyocyte specific cTnT-Cre, therefore ablation of the first five exons is expected. A truncated protein may or may not be present, as previously described.

In our pilot studies, we initially examined three time points in cardiac development to characterize *Lek1* loss-of function in cardiomyocytes. E12.5 is the time when the heart chambers become apparent and the heart wall cardiomyocytes are undergoing proliferation; trabeculae are becoming prominent. As seem in Figure 34, no outward structural defects were present when examining the entire organ at this timepoint. Histological analysis displayed distinct differences between mutant hearts and those from their heterozygous littermates. The ventricular walls, atrial walls, and epicardium appear highly thinned in the mutant hearts. Interestingly, epicardial thinning may result from signaling defects between the myocardium and the epicardium in the developing heart (Crispino et al., 2001). Some areas in the mutant hearts have only one myocyte thickness in the developing ventricles and the differences in nontrabeculated regions of the heart appear significant (Figure 34). Also, ventricular wall trabeculation is significantly reduced in the *cTnT-Cre; Lek1* ^{loxP/loxP}
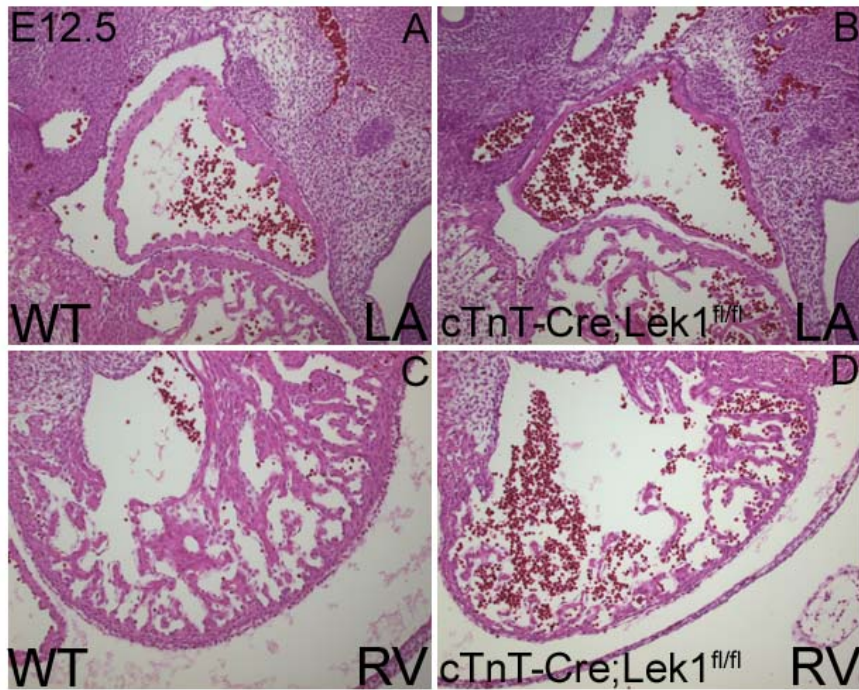
Figure 34. E12.5 mutant hearts. Wild-type (WT) and mutant *cTnT-Cre; Lek1^loxP/loxP* mice were compared at E12.5 by histological analysis by H and E staining. Hearts isolated from mutant animals demonstrate significant structural defects as compared to the littermates. Right ventricular (RV) and left atrial (LA) wall thickness are reduced in mutant hearts. Trabeculation in the developing chambers also appears to be significantly reduced and blunted in mutant hearts.
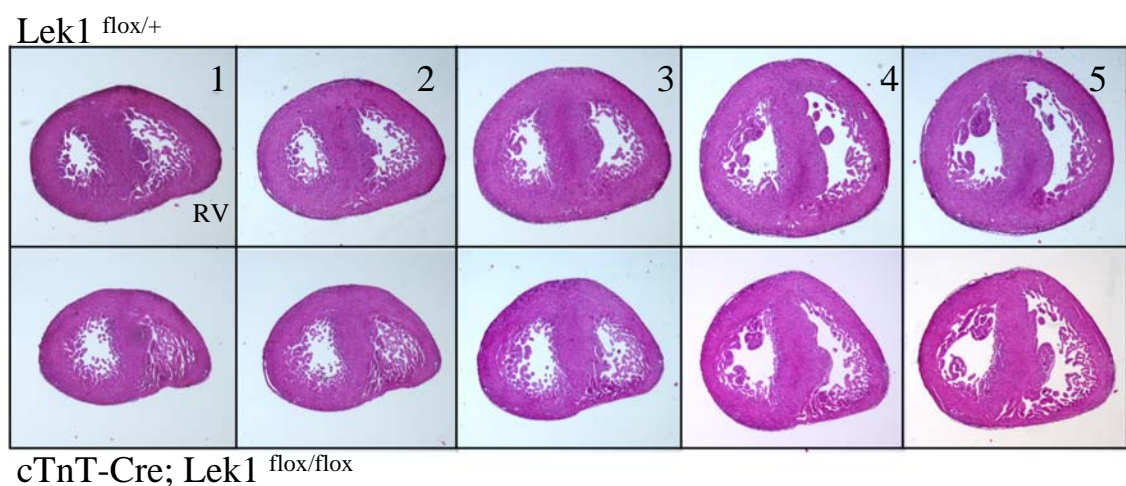
Lek1 ^{flox/+}

cTnT-Cre; Lek1 ^{flox/flox}

Figure 35. E17.5 mutant hearts. Control littermates and mutant *cTnT-Cre; Lek1loxP/loxP* mice were compared at E17.5 by histological analysis by H and E staining. Serial sections are depicted (1-5). As seen with E12.5 mutant hearts, a decrease in wall thickness is observed in ventricles. A 25-40% reduction in heart wall thickness is observed throughout. The decrease is most apparent in the right ventricle. Mutant hearts also show more blunted trabeculae, indicating that there may be a delay in heart wall compaction as compared to heterozygous littermates.

hearts as compared to heterozygous littermates. The developing atria also appear to have similar thinning and trabeculation defects.

We also compared E17.5 mutant and heterozygous hearts by immunohistochemical analyses. Heart walls should be thickened at this timpoint and trabeculae should be undergoing compaction. Hearts from the mutant mice have the same characteristics seen earlier at E12.5 with defects in myocardium thickness and blunted trabeculation (Figure 35). Mutant hearts demonstrate a 25-40% decrease in heart wall thickness compared to $Lek1^{flox/+}$ control littemates. The decrease is most apparent in the right ventricle. Interestingly, the walls are thinner in the mutant hearts, and it appears that trabeculae are more apparent in mutant hearts. This may indicated a delay in trabeculae compaction. Gross examination of the mutant E17.5 heart reveals that the mutant heart is significantly more angular and thinner than the control littermates, and they also appear to pool blood in both their atria and ventricles (Figure 36).

Lek1 loss-of-function results in a "small heart" phenotype in P4 hearts. Mutant $cTnT-Cre; Lek1^{loxP/loxP}$ hearts are consistently smaller than control littermate hearts, as there was a 30-40% reduction in heart size in mutant hearts (Figure 37), yet there was no significant body size difference between the animals at this timepoint. Mutant hearts have a more globular appearance and the ventricular apex is not as angular. Interestingly, studies examining valves, great vessels, epicardium, and endocardium show no distinct variations in these structures. The atrial and ventricular myocardium still appear to be significantly thinned in P4 hearts.
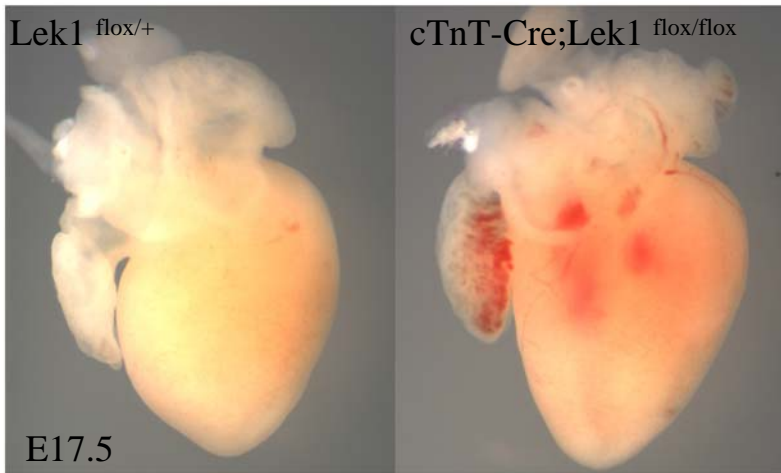
Figure 36. E17.5 mutant whole hearts. As compared to heterozygous littermates, mutant hearts are consistently thinner and more angular. Function also appears to be compromised in mutant hearts as blood is trapped in the atria and ventricles.
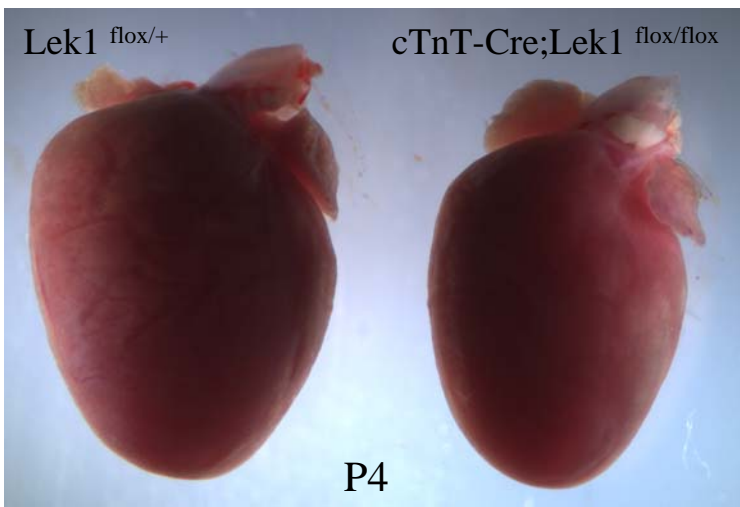
Figure 37. "Small Heart". Mutant hearts are 30-40% smaller than control littermates. Mutant hearts have a more globular shape and the ventricular apex is less angular than heterozygous littermates.

The first litters were not sacrificed, but instead were analyzed for cardiac function beginning at four weeks of age with the help of the Vanderbilt University Murine Cardiovascular Core (Dr. Jeff Rothman, director). After four weeks of age, major differences in heart function became apparent. Control and mutant mice were analyzed by serial conscious transthoracic echocardiography until 26 weeks of age. At 12 weeks, control (n=11) and mutant (n=5) subjects did not differ in terms of body weight or heart rate. However, Lek1 mutant mice developed progressive dilated systolic cardiomyopathy with age (Figure 38 and 39), demonstrating increased diastolic and systolic left ventricular dimension and decreased systolic function reflected in diminished fractional shortening defect. At 26 weeks, left ventricular dimension was markedly increased in mutant mice (LVIDs 0.19+0.03 vs 0.13+0.01 cm, p<0.01, FS% 44+1.9 vs 54+1.9, p<0.001; *cTnT-Cre; Lek1* $^{loxP/loxP}$ vs control littermates). In other words, contractile performance was significantly diminished and the heart had become enlarged by 26 weeks of age. Also, some mice demonstrated alterations in ventricular contraction, thus suggesting conducting abnormalities. Analyses of long-term electrocardiographic (EKG) recordings show differences in mutant mice. Mutant mice demonstrated prolonged PR intervals, low QRS complex amplitude and prolonged duration, and evidence of sinus node dysfunction or sino-atrial exit block (Figure 40). Ventricular arrhythmias or higher grade block may progress rapidly in the near-terminal stages of cardiomyopathy (Kozlov et al., 2005). Our EKG results may indicate deficiencies in the electrical coupling and alterations at the intercalated disk. Interestingly, when mice were allowed to age to 9 months, mutant mice
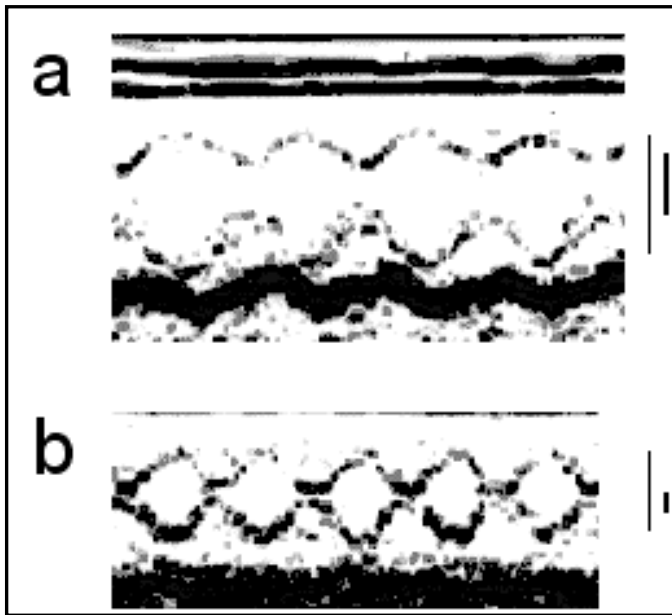
Figure 38. M-mode echocardiograph. Images of conscious *cTnT-Cre; Lek1* [flox/flox] (a) and wt (b) mice at the mid-papillary level. The diastolic (thin line) and systolic (thick line) chords are indicated next to the images. The *cTnT-Cre; Lek1* [flox/flox] mice show marked ventricular dilatation (~2x increase in left ventricular end-diastolic dimension) and moderate ventricular systolic dysfunction (decreased FS% at comparable afterload).
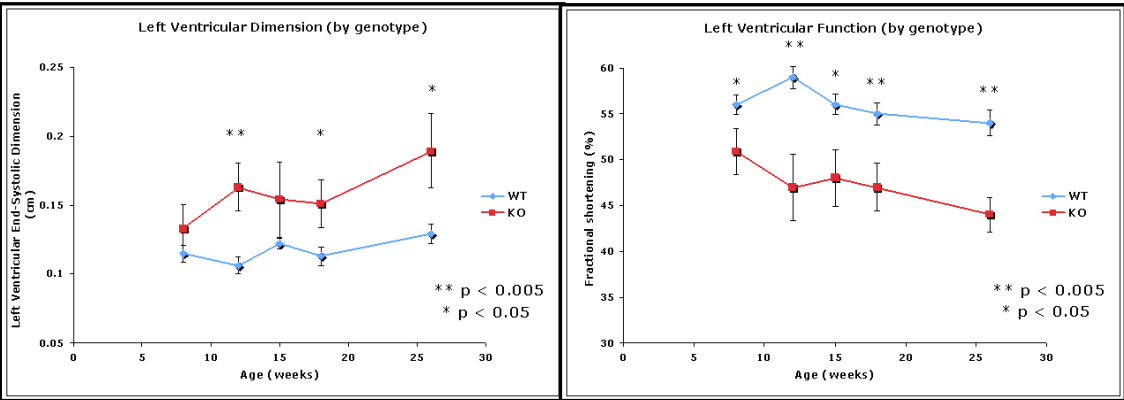
Figure 39. Longitudinal echocardiography of WT and *cTnT-Cre; Lek1 flox/flox* hearts.
Progressive dilation of *cTnT-Cre; Lek1 flox/flox* hearts is seen as compared to wt hearts (left)
while decreased fractional shortening is observed with age of knockout mice. Dysfunction is
observed at all time points after birth. Contractile performance was significantly diminished and
the size of the heart has become enlarged.

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Figure 40. Ambulatory mouse electrocardiogram. Ambulatory murine electrocardiogram from a *cTnT-Cre; Lek1 flox/flox* mouse (Mouse Holter) showing erratic sinus node function. P-P (and R-R) intervals range from 100ms (normal) to 410ms (markedly prolonged). QRS complex annotation is performed on arbitrarily long recordings, which can then be searched using a logical command structure.

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have a 20% mortality rate during this time period. All surviving mutant mice had irregular P-to-P intervals (the time between successive cardiac impulses), consistent with sino-atrial exit block (Figure 40), while control mice appear normal and no deaths occured. Lek1 mutant hearts show structural, but most importantly, functional defects.

Mutant mice have altered electroconducivity in their myocardium shown by their EKG. As a result, we utilized an *in vitro* technique developed by our collaborator, Cecilia Lo (NIH), to measure cell coupling between NIH3T3 fibroblasts. Her laboratory has demonstrated that NIH3T3 fibroblasts form gap junctions and therefore couple in culture. Microelectrode impalements of a cell with dye allow one to measure coupling between cells. A single cell is impaled and loaded with dye. Then, the numbers of cells containing dye are counted that surround the injected cell.

For our experiment, the results depicted in Figure 41, two different experimental cell populations were transfected with two different dominant negative complexes. One population was transfected with 5'LSD and GFP-syntaxin 4. In chapter III, we show that these proteins form a dominant negative complex. The second cell population was transfected with 5'LSD and GFP-SNAP-25. This complex was shown to retard endosomal recycling (Chapter II). These data shown in Figure 41 demonstrate that coupling is inhibited in dominant negative cells as compared to the control cell population that was transfected with EGFP only. In control cells that express EGFP, coupling is not inhibited and dye transfers to numerous second tier cells. Whereas in both experimental cell
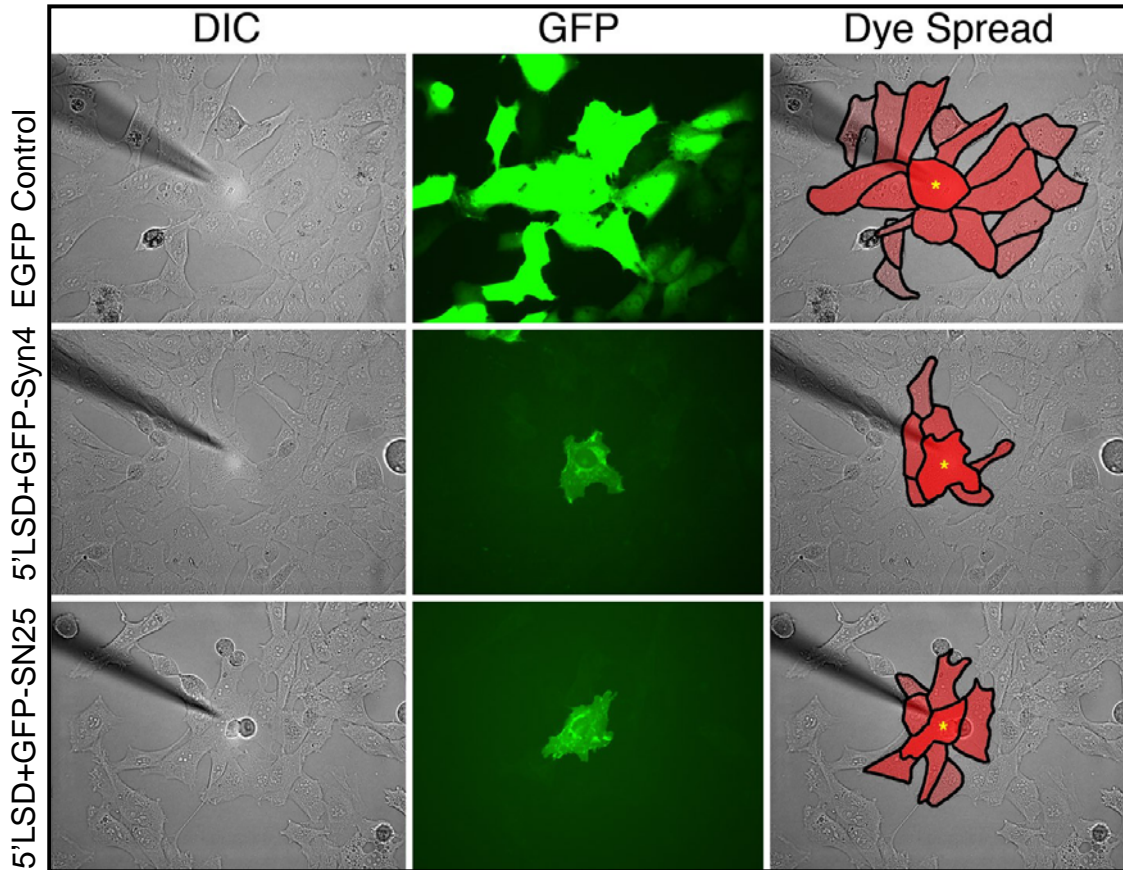
Figure 41. Coupling is inhibited. As a control, NIH3T3 fibroblasts were transfected with EGFP. One transfected cell was injected with dye and dye transfer was visualized. As seen, eight 1st and ten 2nd tier cells were observed containing dye. Then, a 5'LSD-GFP-syntaxin 4 cell was also loaded with dye. Only five 1st tier cells and one 2nd tier cell had dye transfer. Also, a 5'LSD-GFP-SNAP-25 cell was examined. Five 1st tier cells and two 2nd tier cells had dye transfer. The experimental cell populations have reduced dye transfer and therefore have significantly less coupling between the transfected cell and its surrounding cells.

populations, coupling is significantly inhibited. As demonstrated, one second tier cell contains dye when a cell expressing 5'LSD and GFP-syntaxin 4 is loaded with dye. Two second tier cells contain dye when a cell expressing 5'LSD and GFP-SNAP-25 is injected with dye. These results show that coupling is inhibited in a cell autonomous manner, and protein localization at gap junctions may be abnormal.

Discussion

Lek1 is a 8994 bp gene that contains 17 exons and the intervening introns. Since it a relatively large gene, it was determined that deleting the first five exons, including the translation start site, would be the most feasible way to ablate Lek1 function. At least three KB of the 5' UTR and at least 17 KB of the gene were identified in a BAC clone. Utilizing this clone, we were able to construct a targeting vector that was successfully recombined into ES cells. Southern blot and PCR-based analyses allowed us to identify two unique ES cell lines that demonstrated proper recombination at the *Lek1* locus. ES cells from both clone lines were injected into blastocysts and placed into foster mothers, and only one founder was obtained that showed germ line transmission. Homozygous floxed *Lek1* mice without the neomycin resistance cassette are now available and initial studies characterizing mutant hearts are on-going. We have identified a "small heart" phenotype. Importantly, mutant hearts are altered in function, demonstrating a requirement for Lek1 in proper heart function.

Lek1 loss-of-function had never been demonstrated *in vivo*. We have not had any success with RNAi, but we have had much success with MO in vitro. Cell culture based techniques have obvious short-comings. MO technology is more of a protein knock-down approach rather than complete ablation, and MO entry into every cell can not be tested. For the first time, we are now able to study Lek1 ablation in a tissue-specific and temporal manner in the developing mouse. We have chosen to study Lek1 ablation in terms of heart development utilizing a transgenic cTnT-Cre mouse line. Lek1 has demonstrated associations with Rb family members and has been implicated in having a function in myocyte cell proliferation and differentiation (Ashe et al., 2004). Our most recent data show that Lek1 interacts with Nde1 and the MT network (Soukoulis et al., 2005). A unique interaction between SNAP-25 and syntaxin 4 with the plasma membrane recycling system has also now been identified (Chapters II and III).

Cardiac-TnT is expressed early in mouse heart development. Jiao et al. (2003) isolated the rat TnT promoter and used it to drive Cre expression in a transgenic line. By crossing these mice with the ROSA26 reporter mice (R26R), it was determined that the cTnT-Cre line induced recombination early in cardiomyocyte lineage. Expression was determined to initiate as early as E7.5 and was restricted to the hearts until E10.5. They demonstrated high levels of recombination at E10.5. Interestingly, skeletal muscles also express cTnT during fetal muscle development (Sabry and Dhoot, 1991). Lek1 has functions in muscle cell proliferation and differentiation, at least *in vitro*. As a result, *Lek1* loss-of-function may also demonstrate a muscle development defect. Creating the *cTnT-*

Cre;Lek1 ^{loxP/loxP} is an invaluable tool needed to study Lek1 function in the developing heart by ablating the protein early in heart development.

It is of interest to note that in the conditional Lek1 knock-out early in heart development, a functional heart still develops. We initially postulated that a mutant may not develop properly, since it functions with Rb family members and has roles in proliferation and differentiation. Therefore, we speculated it may result in early embryonic lethality. These initial hypotheses were based on the only known interacting partners of Lek1 at that time, Rb family proteins. Rb proteins function in regulating the cell cycle, differentiation, and apoptosis. Our data demonstrate that Lek1 may bind and inhibit the fuctions of Rb proteins in these processes. Since cardiomyocytes continually proliferate and differentiate in the developing heart, and Lek1 was central in these critical cellular processes, it was not incorrect to postulate that normal heart organogenesis would have been delayed or mutated in such a way that resulted in early lethality. However, this is not the case. A mutated, but functional heart develops, and pups are born at expected Mendelian ratios. *Lek1* is not required for cardiac specification or commitment, and it is therefore not required for initial cardiac differentiation.

Ablation of the first five exons of *Lek1* results in the "small heart" phenotype. It must be noted that we have yet to determine if a truncated protein is translated, but protein characterization is on-going with our available antibodies. Our pilot studies examined E12.5, E17.5, and P4 hearts. At all time points, there was a reduction in myocardium thickness and blunted trabeculae existed. As seen in hearts isolated at P4, there is a significant reduction in overall

heart size and variations in heart structure. Thinning of the myocardial layer may be the result in a block of Lek1 function in proliferation and differentiation. Mutant hearts may not be able to compensate for the loss, resulting in alterations of the cell cycle and a reduction in cell numbers, which are also defined roles of Lek1. We also speculate that since trabeculae are more apparent in the E17.5 mutant hearts, there may be a delay in trabeculae compaction, which results in the reduction of wall thickness in the adult heart.

Lek1 has functions with Rb proteins, the MT network, and in vesicle transport. Therefore, it appears that these Lek1 functions are critical for proper heart development. Mutant adult mice demonstrate progressive dilated systolic cardiomyopathy, altered conducting function, and deficiencies in electrical coupling. Loss of Lek1 function in vesicle transport and membrane trafficking is likely to be responsible, at least in part, for these results.

Again, we utilized the cTnT-Cre line to induce recombination early in heart development and excise the first five exons of *Lek1* in cardiomyocytes. Our most recent data show Lek1 to be important for heart function. Within the cardiac muscle, two types of myocytes exist: cells of the working myocardium, in both the atria and ventricles, and the conduction cells (Moorman et al., 1998). The conduction system has separate components that have distinct functions in the adult heart. They primarily function in the generation and conduction of electrical impulses in the heart. The myocytes of the working myocardium and conduction system perform four basic functions: contraction, autorythmicity, intracellular conduction, and electromechanical coupling. Coupling between myocytes occurs

through intercalated disks. Intercalated discs are formed at the junction between myocytes. It is through the gap junctions of the intercalated disks that electrical impulses pass through from cell to cell for myocyte contraction. Proteins that have been shown to be important at intercalated discs include connexin43, catenins, cadherins, vinculin, and ZO-1.

cytLEK1 has direct association with SNAP-25 and syntaxin 4. All three proteins have been localized to myocytes (Ashe et al., 2004; Bryant et al., 2002; Goodwin et al., 1999; Jagadish et al., 1996; Ma et al., 2005; Pessin et al., 1999; Pooley et al., 2006; Sevilla et al., 1997; Soukoulis et al., 2005). Therefore, studying Lek1 ablation in the heart is an excellent model to define their functions. The ability of cytLEK1 to bind SNAP-25 is important for plasma membrane recycling. We do not know if cytLEK1 has a more broad role in plasma membrane trafficking along the MT network, or if it is specific to Rab11a positive recycling endosomes. Needless to say, plasma membrane recycling is important for every cell type, including myocytes, in trafficking and placement of proper proteins and ion channels to the cell surface, such as Tf and its receptor. cytLEK1 has now been shown to function in membrane trafficking in two pathways. First, cytLEK1-SNAP-25 association functions in Tf uptake. Second, syntaxin 4 was also found to associate directly with cytLEK1. *In vitro* knock-down of Lek1 severley retards GLUT4 trafficking, a well-defined function of syntaxin 4. For increased glucose uptake, GLUT4-containing vesicle transport is critical after insulin stimulation. GLUT4 is trafficked through recycling endosomes. We

postulate that cytLEK1 is a critical link between recycling vesicles and the MT network, and specifically functions with Tf and GLUT4- containing endosomes.

When considering the preliminary data on Lek1 loss-of-function in the heart, it is very interesting to postulate that alterations to normal plasma membrane recycling are present in mutant cardiomyocytes. If further evidence support our pilot studies, vesicle and protein trafficking may be responsible, at least in part, for decrease function in mutant cells. Intercalated disks form between the junctions of myocytes, and proteins need to be properly trafficked to the cell membrane for electrical current to pass through cells and tissues of the heart. Our EKG data indicate that cells may not couple properly and electrical conduction in affected. Our *in vitro* data shows that altering cytLEK1 function alters coupling. Therefore, protein trafficking to the intercalated disks may be affected. It is logical to postulate that mutant cells have an inability to traffic proteins to the cell membrane properly, therefore heart function would be severely altered.

In conclusion, the conditional *Lek1* mouse line is now available. Our preliminary studies indicate that Lek1 function is critical for proper heart organogenesis, as mutant *Lek1* hearts have decreased function and contractile ability. Coronary heart disease is the leading cause of death in Americans; over 28% of Americans die from diseases of the heart every year (American Heart Association). Initially, it appears that Lek1 functions with Rb family proteins may be important in myocardial wall development, and/or Lek1 function with plasma membrane transport and vesicle associations with the MT network may be just

as critical. We are now able to test Lek1 loss-of function during development for the first time.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

Entering these studies, we identified nucLEK1 as being involved in cell proliferation and differentiation, while the initial cytLEK1 data show that the C domain interacts and functions with Nde1 and the microtubule network. In my studies, I describe novel functions for cytLEK1 in intracellular trafficking. Chapter II characterizes the interaction between cytLEK1 and SNAP-25. We show that the endogenous proteins share a strikingly similar cytoplasmic distribution pattern in cultured myocytes and fibroblasts with little, if any, localization at the cell membrane. This is important because in most cell types, neurons for example, SNAP-25 is mostly cell membrane bound. As a result, most studies focus on neurotransmitter release in neurons, but intracellular roles for SNAP-25 are emerging. Furthermore, CoIPs utilizing endogenous and transfected cytLEK1 and SNAP-25 show that these proteins interact directly. The most intriguing part of the study identifies this association as being critical for Rab11a and recycling endosome function. Rab11a is a marker for recycling endosomes and has a perinuclear distribution similar to cytLEK1 and SNAP-25. In contrast, cytLEK1 and SNAP-25 also have a wider distribution in cytoplasm further towards the cell membrane. This indicates that the association between cytLEK1 and SNAP-25 may regulate other intracellular pathways or other endosomal compartments.

In chapter III, a direct interaction between cytLEK1 and syntaxin 4 is characterized. As with SNAP-25, cytLEK1 and syntaxin 4 colocalize in the cytoplasm, especially in undifferentiated 3T3-L1 cells (preadipocytes). This cell line is a model for syntaxin 4 function and GLUT4 trafficking which are both important for glucose uptake. GLUT4 movement is dependent, at least in part, on Rab11a and the recycling endosome network. For the first time, we have identified a membrane localized cytLEK1 in differentiated 3T3-L1 adipocytes and in neuronal cell lines, another model cell line used to study syntaxin 4 function. Chapters II and III show that cytLEK1 can associate with both syntaxin 4 and SNAP-25 in the same complex. In order for the SNARE complex to form, a VAMP family member is also required, and in each study VAMP2 was identified.

From our initial studies characterizing cytLEK1 function in plasma membrane trafficking, we put foward the following model (Figure 42). We propose that cytLEK1 is a link between recycling endosomes and the MT network. Previous data show that the MTs are important to recycling endosome movement as nocodazole treated cells halt endosome recycling (Apodaca et al., 1994; Casanova et al., 1999). However, no proteins have yet been identified that function linking endosomes to the MT network. Actin has long been known to have a role in endosomes trafficking through myosin Vb, but we have now identified a possible link to the MT cytoskeleton.

As shown previously, the C-domain of cytLEK1 associates with the MT network (see Chapter I). This should not be of surprise, since this region of cytLEK1 has a spectrin repeat, a domain that has been shown to function with
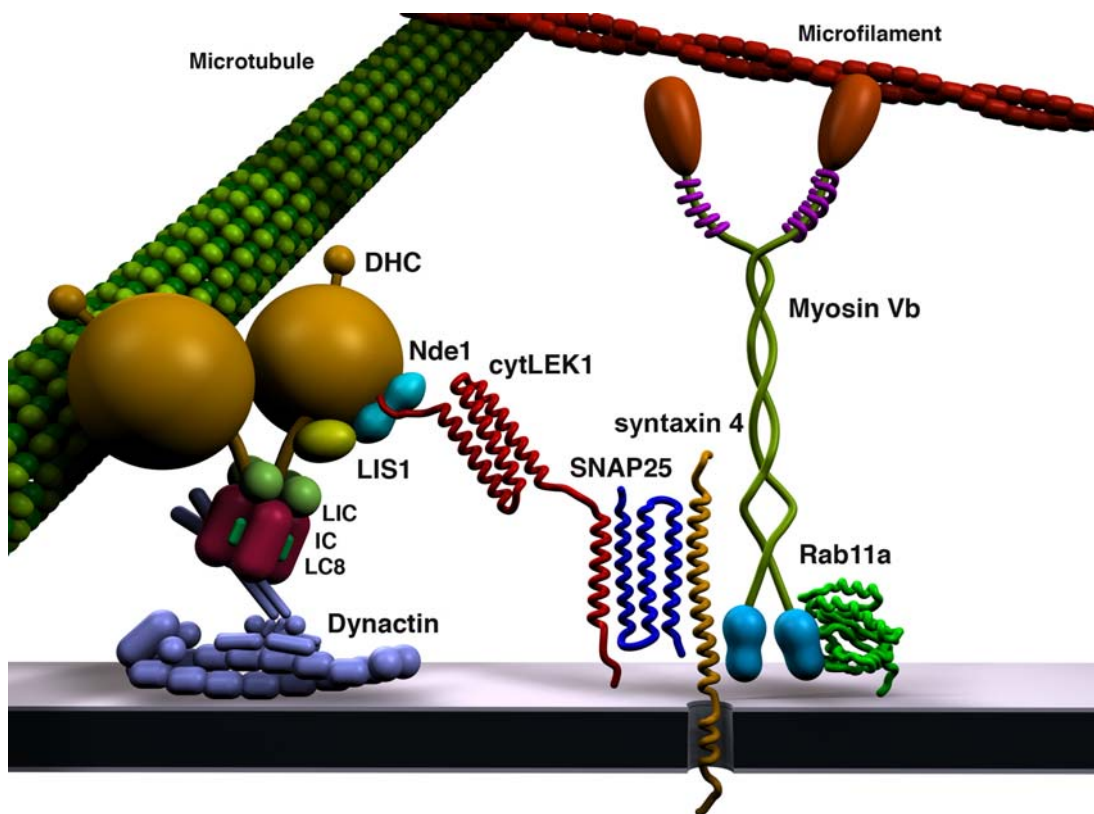
Figure 42. Our cytLEK1 model. We propose that cytLEK1 links recycling endosomes with the microtubule network. CytLEK1 has now been shown to associate with SNAP-25, and previously with Nde1 (Soukoulis et al., 2005), a protein of the Lis1/dynein complex that associates with the microtubule network. Rab11a, myosin Vb, and VAMP2 have been localized to recycling endosomes. CytLEK1 associates with SNAP-25 and syntaxin 4, and through this complex, recycling vesicles are linked to Nde1 and thus Lis1 and microtubules.

the cytoskeleton. Now through our most recent studies, we have demonstrated that cytLEK1 functions with the recycling endosome network through the 5'LSD domain. By combining these known functions of cytLEK1, we hypothesize that our protein is a key link between recycling endosomes and MTs. In order for organelles to move throughout the cytoplasm, one would postulate that interplay is required between the three networks that make up the cytoskeleton: MTs, actin filaments, and intermediate filaments. In our model, recycling endosomes can interact with two of the systems. First, they associate with the MT network by association with cytLEK1 and the Lis1 pathway. Second, they interact with the actin network by their association with Rab11a and myosin Vb. We further speculate that cytLEK1 is a key player in regulating trafficking between these two networks of the cytoskeleton.

Chapter V describes our initial characterization of the *Lek1* conditional knock-out. Since Lek1 is expressed at high levels in the developing heart, and most of our previous studies focused on its role in cardiomyocytes, we ablated the protein early in heart development. Deletion of *Lek1* leads to alterations in heart structure early in development and has severe effects to adult heart function. Interestingly, *Lek1* is not absolutely required for cardiac specification and commitment as we once postulated. This hypothesis was solely based on our understanding of nucLEK1 function in the key processes of proliferation and differentiation, a shared function with CMF1 (see Chapter I). Our initial characterizations show that *Lek1* has an important role in the regulation of heart development. At this point, it appears that any of the Lek1 functions may be

responsible for the small heart. Altered cardiomyocyte proliferation and differentiation may result in the thinning of the myocardium and changes in normal trabeculation and compaction during heart development. Retarding endosomal recycling could also be a factor responsible for the mutant phenotype.

Structuaral analyses and physiological tests conducted on mutant mice are quite revealing. Immunohistochemical anlalyses of heart sections from different stages of embryonic heart development demonstrate that the maturing heart wall is thinner and the trabeculae are blunted and not as pronounced in mutant mice. We initially speculate that this is the result of *Lek1* loss-of-function in the regulation of proliferation and differentiation. This would also help explain the "small heart" phenotype.

Interestingly, we observed that mutant adult hearts have dilated cardiomyopathy, which results in a dilated heart wall and diminished heart wall function. So as mutant hearts gets older, they actually increase in size. Additionally, EKG recordings demonstrate that the periodicity of heart contraction is significantly altered in mutant hearts. This allows us to postulate that not only are proliferation and differentiation compromised in mutant hearts, but coupling of cardiomyocytes is also affected. Therefore, electroconductivity through the heart wall is altered. Our initial *in vitro* data support these results and show that inhibiting Lek1 function retards coupling.

Recently, it has become evident that dilated cardiomyopathy is mainly due to mutations in cytoskeletal proteins (reviewed in Perriard et al., 2003). It is characterized by the thinning of the ventricular wall and its impared contraction,

both of which are altered in our mutant mice. It is unknown what causes the thinning of the wall, but our data indicate that altering Lek1 function may result in reduced myocyte numbers in the myocardial wall.

Relevant to our studies on cytLEK1 function in membrane trafficking and its association with the microtubule network, architecture and composition at intercalated disks may be a major contributing factor the dilated cardiomyopathy. Changes at contact sites between myocytes may diminish electroconductivity between cells (Ehler et al., 2001). Intercalated discs are comprised of three different types of cell-cell connections: gap, adheren, and desmosomal junctions. EKG recordings show that mutant hearts have altered electroconductivity, a function of gap junctions. As a result, it would be most informative to examine protein trafficking, such as connexin 43, to gap junctions. But, trafficking of any, or all, proteins to the intercalated disks may be inhibited or upregulated, as indicated by both studies in mice and humans (Perriard et al., 2003). Throughout development, it is of most interest to compare intercalated disk formation at the cell membrane. Mice suffering from dilated cardiomyopathy have shown significant changes in cardiomyocyte shape and intercalated disk localization. Cells isolated from diseased hearts have shown a more rounded shape and a broad intercalated disk localization (reviewed in Perriard et al., 2003). Cardiomyocytes are not able to make the normal bipolar connections with its surrounding cells.

Thus far, our studies have not addressed the compaction of the myocardium (see Chapter I). It is quite logical to hypothesize that Lek1 may also

function during this event in heart wall maturation. Compaction events have not been well-studied, but it is easy to postuate that protein and membrane trafficking are critical for compaction. In order for myocytes to make proper contacts and to function appropriately, protein localization to the cell surface must be unrestrained. It appears that Lek1 is a critical factor that functions in the basic pathways that are the foundation for proper heart development and function.

This leads us to the following question: What do all the known functions mean in terms of the global role of Lek1 in development? Even though we have chosen to characterize Lek1 function in terms of heart development, the protein is expressed throughout the developing embryo. I speculate that cytLEK1 has a critical function regulating organelle positioning and movement at the perinuclear region of cells. Examining the localization and distribution of endogenous proteins juxtapose to the nucleus allows me to hypothesize that it has a key function in this area of the cell. Studies looking at the C-domain demonstrate its localization at centrosomes. 5'LSD localizes to the recycling endosome network, a function primarily located in the perinuclear region. If the hook2-cytLEK1 association is validated, this is yet another line of evidence suggesting that cytLEK1 has an important role regulating organelles in the perinuclear region.

We also need to define whether or not Lek1 only has a role early in development, or if it does have some capacity to function in adult cells. The first possible explanation is that we have simply not examined adult tissues closely enough. It may be found at low levels in adult cells, just as SNAP-25, and we have not utilized assays sensitive enough to detect the protein. Also, now that we

have a better understanding of cytLEK1 function, it may be worthwhile to reexamine adult expression of the protein in certain cell types. SNAP-25 has well-documented functions in neurons. Syntaxin 4 has functions in adipocytes and neurons (see Chapter I). Recent reports show that SNAP-25 and syntaxin 4 function in insulin release in β-cells of the pancreas (Spurlin and Thurmond, 2006). These are tissues that may demonstrate cytLEK1 function in the adult. Lastly, stem cell proliferation and differentiation is a critical area of research today. It is imperative that we examine Lek1 function in embryonic and adult stem cell populations since it has roles in proliferation and differentiation.

Another explanation for cytLEK1 function in development is that another protein replaces Lek1 functions in the adult. This point appears to be the weaker of the two at this time. *In vitro* protein knock-down has a detrimental effect on cell proliferation and differentiation, as a result it appears that no proteins are able to compensate in cell culture with the loss of Lek1 function. Creation of the conditional knock-out may help alleviate this debate. With the inducible systems now available, cytLEK1 function in the adult can be further characterized.

In summary, we have now shown that cytLEK1 functions in plasma membrane recycling. Along with what was previously known about cytLEK1, we have developed a model that depicts cytLEK1 being a key regulator that bridges recycling endosomes with the MT network. This would be the first time such a protein has been identified. Also, the conditional *Lek1* mouse line has been developed and pilot studies characterizing Lek1 function are ongoing. Lek1 appears to have a significant role in heart organogenesis. Even though we have

made great strides in defining the function of Lek1, additional studies are needed to fully understand its role in such key cellular processes.

Future Directions

Define cytLEK1, SNAP-25, and syntaxin 4 complexes

We have now shown that cytLEK1 binds both SNAP-25 and syntaxin 4 independently by Y2H. SNAP-25 function is best characterized in the brain, while syntaxin 4 is best defined in adipocytes, even though both proteins have a broader expression pattern and are found in numerous cell types. In fact, we have demonstrated that cytLEK1, SNAP-25, and syntaxin 4 form an intracellular complex in NIH3T3 fibroblasts and in C2C12 myoblasts. It remains to be determined whether or not a complex exists that contains all three proteins.

The SNARE complex is composed of a SNAP, syntaxin, and VAMP family member. SNAP-25 is mostly characterized with syntaxin 1 and 2 in the brain, while syntaxin 4 is best characterized with SNAP-23. We have strong evidence that cytLEK1 interacts with both SNAP-25 and syntaxin 4 in a complex. In all transfection experiments conducted thus far (see Chapters II and III), all three proteins are found at the perinuclear foci, and CoIPs demonstrate the presence of both SNAP-25 and syntaxin 4 with cytLEK1. To date, these data are only indirect evidence for their association. It is possible in such experiments that there are populations of cytLEK1-SNAP-25 complexes interspersed with cytLEK-1-syntaxin 4 populations. To address this question, biochemical competition

binding assays are necessary and would give the most insight. Further use of CoIP assays would also be a valuable tool. A series of pull-downs and immunoblots could be performed to identify which proteins are present in a complex.

It is of interest to examine the binding domains of the proteins. cytLEK1 binds the N-terminal region in SNAP-25. This is the same region in which syntaxins also bind SNAP-25. cytLEK1 binds syntaxin 4 at its C-terminus, again the same region that binds SNAP-25. It may be that cytLEK1 is a regulator of SNAP-25-syntaxin 4 binding. If all proteins do not form a complex, cytLEK1 may be a negative regulator of membrane fusion and deter complex formation, similar to Munc-18 binding of syntaxin 4 (Yang et al., 2000). Defining cytLEK1 binding in the SNARE complex is critical in understanding the role of cytLEK1 in intracellular transport.

We have identified cytLEK1 as being important in Tf and GLUT4 trafficking, which are both plasma membrane recycling processes. Future studies also can be conducted to determine the importance of cytLEK1 for other protein recycling pathways. To date, numerous proteins have been identified that traffic through Rab11 and recycling endosomes. These proteins include EGFR (Lapierre and Goldenring, 2005), p120 (Ducharme et al., 2006), apoE (Braun et al., 2006), NK1R (Roosterman et al., 2004), CXCR2 (Fan et al., 2004), and PAR2 (Roosterman et al., 2003), among many others. The recycling system has critical functions in protein localization to the cell surface in a wide variety of cell types. It is worthwhile to define the function of cytLEK1 beyond that of Tf and GLUT4

trafficking and to identify the roles of cytLEK1 in these protein trafficking pathways.

Further define Lek1 expression pattern and localization

To date, we have examined Lek1 function *in vitro* by utilizing fibroblast and myoblast cell lines. We now have a mouse line that conditionally ablates *Lek1* in the developing heart. Interestingly, SNAP-25 and Lis1 pathway proteins are most highly expressed in the brain, while syntaxin 4 is also highly expressed in adipocytes. To gain further insight into cytLEK1 function, we need to examine its function in these cell types.

Even though our initial expression studies show cytLEK1 localization in the brain, further detailed expression studies need to be conducted. It would be of great interest to determine whether cytLEK1 is highly expressed in proliferating cells of the brain and whether or not it is localized to the membrane, since this is where SNAP-25 functions in neurotransmitter release. We have begun to define cytLEK1 function in myocyte proliferation and differentiation. Since we have shown that Lek1 function is critical for heart development and function, it is also of interest to examine expression in postnatal hearts and determine if there is a proliferating population that expresses Lek1. Because syntaxin 4 is also highly expressed in adipocytes, studies need to commence on cytLEK1 localization and function in these cells. Additionally, further analysis in tumors would be of great interest to examine Lek1 function. One family member, CENP-F/Mitosin, is highly expressed in human tumors (de la Guardia et al., 2001), and since Lek1 has a

role in proliferation, characterizing Lek1 function in mouse tumor cells is of interest. Interestingly, one of the only cell populations to express both SNAP-25 and syntaxin 4 is β-cells in the regulation of insulin release (Spurlin and Thurmond, 2006). cytLEK1 expression and function should also be examined in these cells. Finally, stem cell research is an emerging field of research today. Lek1 may be expressed and function in various stem cell populations, since they are populations that retain their ability to proliferate and differentiate. Detailed studies on proliferating cells in the adult need further characterization to determine if cytLEK1 is definitively expressed and functions only during development.

To address these issues, we have undertaken several projects. We have produced several monoclonal antibodies that recognize unique epitopes in cytLEK1 and nucLEK1. Monoclonal antibodies have not previously been available. Once they are available, we will be able to look at nucLEK1 and cytLEK1 peptides concurrently. This will help to determine if a cleavage event exists that produces two peptides or whether two populations of full-length proteins reside in different compartments. Also, the conditional *Lek1* mouse will help define Lek1 function in different cell types. We now have the ability to specifically ablate Lek1 function in specific organs or at various developmental time points. This will allow us to define the broader role of Lek1 in proliferation, differentiation, and membrane trafficking with the MT network.

Lastly, we propose that Lek1 undergoes a cleavage event by an unknown enzyme at an unknown site. Mass spectrometry analysis and protein sequencing

techniques have been unsuccessful in determing the site of Lek1 cleavage if it exists (Ashe, Price, and Bader, unpublished data). To fully understand the function of Lek1 in such basic cellular processes, the full-length clone needs to be generated. However, our efforts in creation of the clone remain elusive, as no ligation strategy has been feasible. Development of the full-length clone by RT-PCR is required. Not only would it be beneficial in defining the cleavage event, but it would also allow us to perform overexpresion and rescue experiments. In summary, these experiments will allow us to define Lek1 expression in key cell types that have not been examined in detail thus far.

Characterize novel Lek1 binding partners

Thus far, we have identified and begun to characterize three novel protein associations initiated by Y2H screens. These screens were conducted with regions that account for less than 20% of Lek1. This technique has been invaluable in initiating studies in Lek1 function and should not cease. Structural analysis provides evidence that several leucine zippers are present throughout Lek1, which indicates regions for other potential protein-protein associations.

First, several interacting proteins have already been found through our Y2H analyses that have not been characterized. Besides Nde1, the Y2H screen conducted with the C domain also identified dysbindin as a cytLEK1 interacting protein (Soukoulis and Bader, unpublished data). This association is of interest because of its high expression in cardiac muscle, skeletal muscle, and the brain. One of its functions appears to be involved in lysosome-related processes (Li et

al., 2003). As previously discussed in Chapter V, proteins identified as interacting with 5'LSD need to be further addressed, especially p50 and Hook2. Studies examining these interactions previously identified by Y2H would be of great benefit in determining the function of Lek1 in intracellular processes with the MT network. Secondly, we have used the Y2H system with great success. Additional screens with other Lek1 bait constructs should be undertaken to identify Lek1 binding partners.

Once binding partners are identified, biochemical analyses will be of interest to support interaction and can also be used to define the binding domains within the proteins. Candidate approaches can then be used to identify the protein members that reside in the complex. From these data, hypotheses can be made on their functions in biological processes. *In vitro* MO protein knock-down can then be utilized to determine the ramifications to such complexes when Lek1 is no longer able to function in the cell. Lek1 may enhance or decrease complex formation. Once new associations are discovered, colocalization and dominant-negative studies should be undertaken.

Examination of Lek1 function in the mouse

First, analysis of cell proliferation in the heart needs to be conducted in our conditional Lek1 knock-out mouse by BrdU incorporation studies. Our most recent data suggest that Lek1 ablation results in a hypoplastic heart and the "small heart" phenotype. The phenotype may be due to decreases in myocyte number that would result in a decrease in wall mass. This phenotype is

supported by the ability of nucLEK1 to function in cell proliferation and differentiation. If there is a reduction in cell numbers, standard BrdU and PCNA analyses can demonstrate if a premature withdrawal from the cell cycle is present or if mutant cells undergo earlier differentiaton. By conducting these studies, it can be determined if the phenotype observed in the mutant heart is a result of altered cell proliferation and/or differentiation.

Second, further analyses of differentiation and protein trafficking are required. We have demonstrated that cytLEK1 associates with recycling endosomes and the microtubule network. We will apply the findings from our pilot studies on the conditional Lek1 knock-out to help define the "small heart" phenotype at the molecular level. Myocyte protein markers can be analyzed for both proper expression and localization in knock-out cells. To demonstrate consequences of altered protein trafficking, primary cardiac myocyte cultures can be purified and electrophysiological analysis can be conducted to measure gap junction mediated coupling in mutant cells. SNAP-25 and syntaxin 4 localization needs to be examined in these cells. This will demonstrate whether or not altered Lek1 function in protein trafficking is responsible for the "small heart" phenotype, at least in part.

Standard marker gene expression should be analyzed by immunofluorescence, PCR, and Northern blot assays to determine whether the overall pattern of myocyte differentiation is altered when Lek1 expression is disrupted. Analysis of contractile protein expression levels of myosin heavy and light chain, actinin, and cardiac troponin can be conducted (Ashe et al., 2004;

Gonzalez-Sanchez and Bader, 1984; Gonzalez-Sanchez and Bader, 1990). Changes in myocyte reshaping, movement, and adhesion during trabeculation and compaction should be visualized by immunohistological labeling with antibodies for N-cadherin, connexin 43, and other intercalated disc-specific proteins (Mikawa et al., 1992; Radice et al., 1997). Cardiac myocytes can be isolated to evaluate adhesive and migratory characteristics, and PCNA/BrdU will demonstrate mitotic capability *in vitro*.

Relatively little is known about the molecular regulation of cardiomyocyte proliferation and differentiation during trabeculation and maturation of the heart. Examining protein trafficking during heart organogenesis is a novel concept. The demonstrated roles for Lek1 in the regulation of cell division and movement, its high level of expression in the early heart, and its consequences to heart development in the conditional *Lek1* knock-out indicate that this gene is a key regulator of cardiac organogenesis. In the future, this conditional *Lek1* mouse line in combination with other Cre-expressing mice, such as α-MHC to ablate Lek1 function later in heart development (Sohal et al., 2001), will allow us to further define the role of *Lek1* in heart organogenesis.

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