In vitro Characterization of SLK-deficient Mouse Embryonic Fibroblasts (MEFs)

Clinton Royce Cunha

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Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa, Ontario, Canada

Abstract

The Ste20-like kinase (SLK) has been shown to be expressed in all cell lines and tissues. Previous studies using siRNA and dominant negative approaches have established that SLK plays important roles in cell growth, cytoskeletal dynamics and cell migration. However, the SLK-dependent signaling mechanisms have yet to be elucidated. To further investigate the role of SLK in those processes, we have assessed the effect of a genetic deletion of SLK on cell growth, apoptosis and cell motility. Conditional SLK-floxed fibroblasts were derived and the SLK gene was inactivated in established cell lines. Using cell counts and flow cytometry, we show that SLK deletion does not affect cell growth or progression through the cell cycle. Similarly, Boyden chamber migration assay showed that SLK deletion did not affect cell motility. Using a scratch wound assays and immunofluorescence, we assessed the localization of cytoskeletal protein during migration. Our data show that Paxillin and FAK are still recruited to the leading edge of migrating cells in the absence of SLK. However, nocodazole release studies show that SLK-deficient cells have a faster focal adhesion turnover rate. As SLK has been shown to play a role in apoptosis, we tested the effect of SLK deletion on cell death. Using multiple apoptotic triggers, we show that SLK deletion does not affect apoptosis in fibroblasts and that signaling downstream of those triggers is unchanged. Overall, in contrast to siRNA studies, our data show that the genetic deletion of SLK doesn't affect cell growth, apoptosis or cell migration. This is likely due to the activation of compensatory mechanisms, bypassing the requirements for SLK.

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Abbreviations

A375	Human Melanoma Line
AdCre	Adenovirus expressing Cre recombinase
AdGFP	Adenovirus expressing Green Fluorescent Protein
AKT	Protein Kinase B (PKB)
ASK1	Apoptosis signal-regulating kinase-1
ATH	ATI-46 Protein (motif) Domain
BRAF	Proto-oncogene (the v-raf murine sarcoma viral oncogene homolog B1)
BSA	Bovine Serum Albumin
C2C12	Immortalized Mouse Myoblast Line
CAD	Caspase-activated DNase
CDK	Cyclin Dependent Kinase
CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
cDNA	Complementary DNA
CK	Casein Kinase II
c-KIT	Proto-oncogene that encodes the KIT protein

CpG	Cytosine phosphodiester bonded to a Guanine
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	(4, 6-diamidino-2-phenylindole)
DIABLO	Direct IAP-Binding protein with Low PI
DISC	Death-Inducing Complex
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ELK-1	ETS transcription Factor
ERK	Extracellular Signal-Regulated Kinase
FADD	Fas Associated Via Death Domain
FAK	Focal Adhesion Kinase
FasL	Fas Ligand
FasR	Fas Receptor
FBS	Fetal Bovine Serum
G proteins	Guanine nucleotide-binding proteins
G2-M	Gap 2/Mitosis

GCK	Germinal Center Kinase
H&E	Hematoxylin and Eosin
Her2/Neu	ERBB2, Epidermal growth factor receptor 2
HSF1	Heat Shock Factor 1
HSP70	Heat Shock Protein 70
HtrA2	HtrA Serine Peptidase 2/Omi
ICAD	(CAD inhibitor) See CAD
JNK1	c-Jun N-terminal kinase-1
LacZ	Gene Reporter
LDB1/2	LIM domain-binding protein 1/2
LMO4	LIM Domain Only 4
LOK	Lymph Oriented Kinase
MAP4K	Mitogen-activated protein kinase kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MITF	Melanocyte inducing transcription factor
MST1	Macrophage Stimulating 1
$p14^{ARF}$	ARF tumor suppressor

$p16^{INK4a}$	Tumor suppressor that inhibits Cyclin D-dependent protein kinases
p38	Mitogen activated protein kinase p38
p53	Tumor protein 53
PAK	p21-activated kinase
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
PDGFR	Platelet derived growth factor receptor
PEI	Polyethylenimine
PFA	Paraformaldehyde
pFAK	Phospho-Focal Adhesion Kinase
Phospho-RB	Phospho-Retinoblastoma Protein
PI	Propidium Iodide
PI3K	phosphoinositide (PI) 3-Kinase
p-JNK	Phospo- c-Jun N-terminal kinase-1
PLK1	Polo-like Kinase 1
P-P38	Phospho-Mitogen activated protein kinase p38
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride membrane

RA	RAS association (protein domain)
RASSF1A	Ras association domain family 1 isoform A
RB	Retinoblastoma
RIPA	Radioimmunoprecipitation assay buffer
Rpm	Revolutions per minute
SARAH	Sav/Rassf/Hpo domain
SH2	SRC homology 2 domain
SH3	SRC homology 3 domain
SLK	Ste-20 like Kinase
Smac	Second Mitochondria-derived Activator of Caspases
SOX10	SRY-Box10
Src	Steroid receptor co-activator
Ste20p	Ste 20 protein
TBST	Tris-buffered Saline, 0.1% Tween
TNF- α	Tumor Necrosis Factor - α
TNF	Tumor Necrosis Factor
TNFR1	Tumor Necrosis Factor Receptor 1
TPR	Translocated Promoter Region

TRADD	TNFR Associated Via Death Domain
v-Src	Viral Src Proto-oncogene
xPlkk1	Xenopus polo-like kinase 1
Δ3-6	Delta 3-6, SLK region in this context

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1. INTRODUCTION

1.1 The Ste20-like Kinase SLK

The archetype Sterile 20 protein (Ste20p) plays a role in the yeast mitogen-activated protein kinase kinase kinase kinase (MAP4K) mating pathway (Dan I, Watanabe NM, Kusumi A., 2001). There are several homologs in different species and those are divided into the p21-activated kinase (PAK) and germinal center kinase (GCK) families. Their modular structure allows them to crosstalk with several regulatory proteins and signaling molecules that are involved in multiple biological processes (Dan I, Watanabe NM, Kusumi A., 2001). They play critical roles in morphogenesis, cytoskeletal dynamics and apoptosis. The Ste20-like kinase SLK was first identified and cloned from the guinea pig (Itoh et al, 1997). The mouse and human homologs were subsequently isolated and characterized (Sabourin et al, 1999, Yamada et al, 2000). A yeast two-hybrid screen was used to isolate murine SLK (Sabourin and Rudnicki 1999). Using a muscle specific transactivator as the bait protein, SLK was isolated as a false positive clone from a skeletal muscle cDNA library. Supporting previous studies, homology searches revealed that SLK was present in virtually all eukaryotic species, including flies. Northern Blot analysis of different mouse tissues showed three different isoforms of SLK (6, 7, 8 Kb) with the largest one being the most abundant (Sabourin, Seale, Wagner et.al, 2000). All tissues surveyed expressed similar levels of SLK mRNA with lower levels in the liver. The SLK protein has been shown to be expressed in most cell lines and tissues (Al-Zahrani et. al, 2013) and localizes to the cytoplasm.

SLK is a member of the group IV Germinal Center Kinases (GCK-IV). The amino-terminal kinase domain displays sequence similarity to the Xenopus polo-like kinase 1 (xPlkk1), MST1 and LOK serine/threonine kinases, playing a role in cell cycle control and apoptosis

(Graves JD, Gotoh Y, Draves KE, et al, 1998). The general structure of SLK consists of 1202 amino acids arranged in three distinct domains: a Ste20 catalytic kinase domain (aa 1-338) near the N-terminal, a central coiled-coil region (aa 339-788) and an AT1-46 homology domain (ATH: aa-789-1202) at the C-terminal end (Figure 1) . A putative SH3 binding site is present at amino acid position 735, suggesting that SLK could directly interact with SH3 domain-containing proteins (Sabourin, Tamai, Seale, et al., 2000).

SLK has been shown to be phosphorylated at serine 189, threonines 183 and 193 (T183 and T193) following auto-activation (Cybulsky et al. 2017). Experiments have shown a 60% reduction in kinase activity with a serine 189 to alanine 189 mutant (S189A) and a T183A/S189A double mutant showed an 80% reduction (Luhovy, Jaberi, Papillon et al., 2011). Both sites are never completely phosphorylated, suggesting that they become phosphorylated in a primary and secondary manner. A glutamine 185 mutation (Q185P) that prevents SLK from dimerizing, shows reduced auto-phosphorylation, suggesting that SLK forms a dimer in a trans-orientation prior to auto-phosphorylation (Pike, 2008). Based on deletion studies, it was reported that an important regulatory region lies between amino acids 373 to 592 (Sabourin, Tamai, Seale et al., 2000). The C-terminal ATH domain of SLK also plays a role in the regulation of its activity. SLK-binding proteins include the transcriptional co-activators LMO4, Ldb1, Ldb2, the cytoskeletal protein Actinin and Translocated Promoter Region (TPR) (Jaberi A, Hooker E, Guillemette J et.al, 2015, Storbeck CJ, Wagner S, O'Reilly P, et al., 2009). Binding to Ldb1/2 appears to downregulate kinase activity (Storbeck et al., 2009). Supporting this, deletion of the ATH region enhances SLK kinase activity. The various pathways regulating SLK, its regulators and downstream effectors are still being uncovered.

SLK has been shown to play a role in apoptosis, cytoskeletal dynamics and cell migration (Hao W, Takano T, Guillemette J et al., 2006; Wagner et al., 2002; Quizi JL, Baron K, Al-Zahrani KN, 2013). SLK has distinct functions and likely signals differently depending on the cellular context. In a renal ischemia-reperfusion injury model, SLK reduces the effect of the endoplasmic reticulum stress response and activates the apoptosis signal-regulating kinase-1 (ASK1) (Hao W, Takano T, Guillemette J et al., 2006). ASK1 activation through phosphorylation leads to direct phosphorylation of its downstream target, the p38 mitogen-activated protein kinase (G. Bunkoczi, E. Salah, P. Filippakopoulos et al., 2007). This leads to the release of cytochrome C from its mitochondrial complex that culminates in the activation of caspase 8 and 9. In glomerular epithelial cells, SLK over expression induces apoptosis through the c-Jun N-terminal kinase-1 (JNK1) JNK dependent pathway (Cybulsky AV, Takano T, Guillemette J et. al, 2009). SLK has also been shown to be required for cell cycle progression from G2 to Mitosis (G2/M) and activate the polo-like kinase homolog (PLK1) and cyclin dependent kinase (Ellinger-Ziegelbauer H, Karasuyama H, Yamada E et al., 2000; Reilly P., Wagner S, Franks D., et al. 2005). Both these kinases are important to regulate transit through the cell cycle. In addition to cell death and cell cycle progression, SLK has been demonstrated to regulate cytoskeletal dynamics and cell migration through actin destabilization and focal adhesion dynamics (S.Wagner, C. Storbeck, K. Roovers et al., 2008; Quizi JL, Baron K, Al-Zahrani KN, 2013).

SLK has been shown to play a critical role in development as the global knock out results in embryonic lethality (Pryce et al., 2017). Consistent with this, an SLK gene trap allele (SLK-LacZ) resulting in an SLK truncation at the ATH domain was found to be embryonic lethal in homozygote mice (Al-Zahrani, K. N., Sekhon, P., Tessier et al, 2014). At embryonic day E12.5, H&E staining showed severe developmental defects in about 40% of the embryos. At E14.5,

almost all of the tissue analysed had anomalies. Mouse embryonic fibroblasts (MEFs) were isolated from SLK-LacZ mice and cultured in vitro. Proliferation assays were performed and it was noted that SLK-LacZ cells grew poorly compared to wild type MEFS, supporting previous data on SLK and cell cycle progression (Ellinger-Ziegelbauer, H., Karasuyama, H., Yamada E., et al., 2000; Wagner, S., Flood T.A., O'Reilly P. et al, 2002).

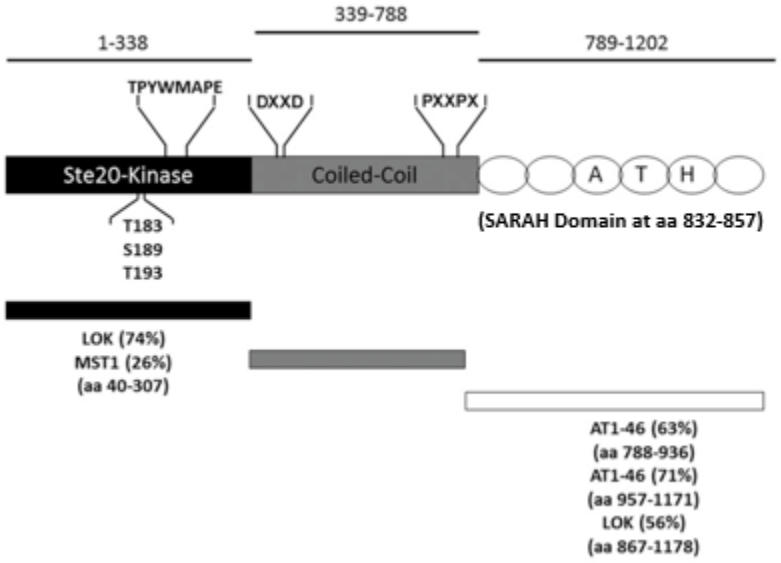


Figure 1. Schematic of the murine SLK structure. The Ste20-like kinase SLK bears a Ste20 signature kinase region (amino acids 1-338) located at the amino terminus (Al-Zahrani et. al, 2013). The kinase domain contains the Ste20 kinase sequence TPYWMAPE. The central coiled coil region extends from amino acids 339-788 and bears a putative SH3-binding domain consensus site (PXXPX) and a consensus caspase 3 cleavage site (DXXD) (Cybulsky et al. 2017, Conway et al. 2017, and Sabourin et al. 2000). The ATH domain is a disorganized region located at the C-terminus and contains a SARAH domain. Proteins that are similar in sequence to the kinase region of SLK include LOK and MST1. The AT1-46 and LOK proteins share similarities to the ATH region (Al-Zahrani et. al, 2013). SLK activity is dependent on three main sites (S189, T193, and T183) (Cybulsky et al. 2017, Conway 2017).

1.2 SLK and Cell Cycle Control

The cell cycle is tightly controlled as it is an important process that allows cells the ability to multiply and create new daughter cells (Satyanarayana, Kaldis. 2009). The cell cycle is characterized into different sections which include Mitosis (M phase) and Interphase (Alberts B, Johnson A, Lewis J, et al., 1983, Schafer, K. A., 1998). Interphase consists of S, G1, G2 and G0 sub phases. S phase is when DNA doubles, hence the cell goes from a 2N to a 4N DNA content. In G1, critical proteins are created in order to help the cell move into S phase and create DNA. G2 phase consists of DNA proofreading and repair to get ready for mitosis or M phase (prophase, metaphase, anaphase and telophase), where chromosomes align and divide into two new daughter cells. G0 consists of not actively dividing cells.

Progress in the cell cycle has many proteins that control and coordinate cell cycle events through different checkpoints. Cyclin-dependent kinases (cdks) and cyclin proteins are in charge of various stages in the cell cycle (Devault A, Cavadore JC, Fesquet D et al, 1991; Parker LL, Piwnica-Worms H, 1992; Van den Heuvel S, Harlow E: 1993). Serine/threonine protein kinases, known as cdks, help in mitotic progression. Phosphorylation regulates cdks along with different cyclin levels (Schafer, K.A, 1998) and cyclin interactions that activate cdks. Changes in the levels of varying phase-specific cyclins direct cells through the cell cycle. When one specific cyclin increases, another one is destroyed from a previous phase.

The cell cycle, as described by classic literature, has classified the following: D-type cyclins bind to Cdk4 and Cdk6 during G1, which helps prepare for DNA synthesis (Malumbres, M., Barbacid, M., 2001). These complexes can cause a partial inactivation of the retinoblastoma proteins RBL2, RB and RBL1. E-type cyclins are then expressed and bind to CDK2 (Harbour, J. W., Luo, R. X., Dei Santi, A., et al., 1999). The CDK2-cyclin E complex pushes G1/S transition

(Hochegger, H., Takeda, S., Hunt, T., 2008). When DNA replication is almost over, cyclin A activates CDK2, allowing for transit between S to the start of M phase. Nuclear envelope and cyclin A degradation occurs. CDK1-cyclin B complex forms as a result of the degradation which pushes the cells to pass into mitosis (Malumbres, M., Barbacid, M., 2005).

Interestingly, SLK has been shown to co-localize with the mitotic spindle involved in chromosome alignment and segregation at M phase. Consistent with this, SLK activity is increased during M phase (O'Reilly PG, Wagner S, Franks DJ et al., 2005) and SLK has been shown to be associated with the microtubule network and co-immunoprecipitate with α-tubulin (Wagner S, Flood TA, O'Reilly P et al., 2002). Expression of kinase-inactive SLK in MEF-3T3 fibroblasts inhibited proliferation and blocked progression through G2. Overexpression of active SLK induced aberrant mitotic entry and spindle formation in MEF-3T3 fibroblasts. Supporting a role for SLK in G2/M, SLK has been shown to phosphorylate and activate Plk1 during the G2 phase of the cell cycle (Ellinger-Ziegelbauer H, Karasuyama H, Yamada E et al, 2000).

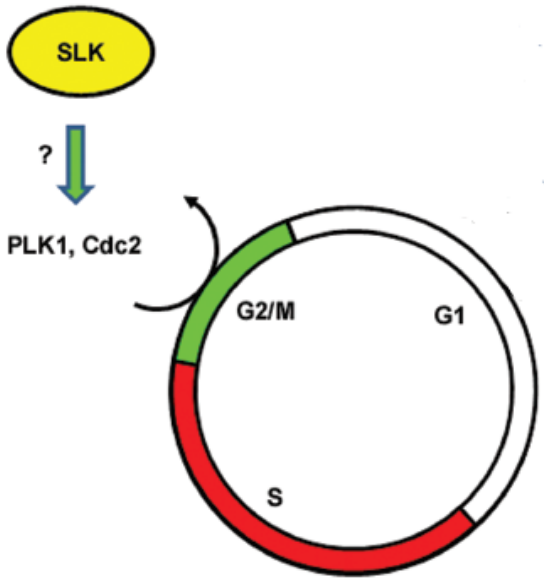

SLK
?
PLK1, Cdc2
G2/M
G1
S

Figure 2 – SLK and cell cycle control. SLK has been shown to act upstream of PLK1 and Cdc2 in cell cycle progression, during G2/M. Those mechanisms have yet to be clearly elucidated (Al-Zahrani, Khalid N., Kyla D. Baron, and Luc A. Sabourin, 2013).

1.3 SLK and Cell Migration

Cell migration is critical for several processes including morphogenesis, wound repair, maintaining homeostasis and development (Trepat et al. 2012). The cytoskeleton is required for integrin-dependent focal adhesion assembly and migration signaling (Bershadsky, 1996). During cell migration, integrins first attach to the extra-cellular matrix (ECM) (V Bolós, Gasent, J. M., Lopez-Tarruella S. et al., 2010; M. Nagano, D. Hoshino, N. Koshikawa et al. 2012), allowing signaling and scaffold/adaptor proteins to be recruited on the cytosolic side of the cell. These complex structures are referred to as focal adhesions and are made up of several proteins (Paxillin, Talin, Tensin, Zyxin, Vinculin) that provide a strong connection to the actin cytoskeleton (V Bolós, Gasent, J. M., Lopez-Tarruella S. et al., 2010; M. Nagano, D. Hoshino, N. Koshikawa et al. 2012). The assembly of focal adhesions involves the recruitment of focal adhesion kinase (FAK), a major regulator of adhesion signaling. The FAK-integrin complex interacts with the c-Src tyrosine kinase, triggering multiple downstream phosphorylation events critical for the signaling at focal adhesions (V Bolós, Gasent, J. M., Lopez-Tarruella S. et al., 2010; M. Nagano, D. Hoshino, N. Koshikawa et al. 2012). Through its SH2 domains, c-Src binds directly to phopsho-tyrosine 397 on FAK. The signaling that ensues induces focal adhesion disassembly. Disassembly is regulated by calpain and microtubules, however the full mechanism has not been fully elucidated. The successive cycles of assembly and disassembly propels the cell forward during migration (V Bolós, Gasent, J. M., Lopez-Tarruella S. et al., 2010; M. Nagano, D. Hoshino, N. Koshikawa et al. 2012).

Scratch wounding in mouse embryonic fibroblasts (MEFs) triggers cell migration and induces SLK activity (Wagner S., Storbeck C., Roovers K. et al, 2008). Interestingly, MEFs that were deficient for FAK showed a lack of SLK upregulation upon scratch wounding when

compared to wildtype FAK MEFs, suggesting that FAK is critical for SLK activation. Different inhibitors (U0126, SB203580) of the MAPK signaling system were used and showed a similar effect on SLK activation, suggesting that the upregulation of SLK activity during migration is dependent on MAPK signaling. It has been shown that SLK is associated with the microtubule network, a component of the cytoskeletal system (Wagner et al., 2002). Interestingly, SLK has also been shown to play a role in the microtubule-dependent focal adhesion turnover after scratch wound-induced migration (Wagner et al., 2008). SLK has been demonstrated to promote focal adhesion turnover by directly phosphorylating Paxillin on serine 250 (Quizi, 2013). It was observed that 293T cells that expressed a Paxillin mutant at the S250 SLK phosphorylation site (S250A), had a two-fold decrease in migration rates, suggesting that SLK is a critical regulator of cell migration. Interestingly, the same Paxillin mutant also delayed the phosphorylation of FAK-Y925, indicating that focal adhesion turnover was impaired.

Factors that have been found to regulate SLK activity and migration rates include the LIM domain binding co-factors Ldb1 and Ldb2 (Storbeck et al., 2009). When Ldb1 levels were reduced, SLK activity was upregulated and migration increased by two-fold while SLK knocked downs impaired cell migration. When both Ldb1 and SLK were knocked down, migration was unaffected, suggesting that the increased migration upon Ldb1 knock down is SLK dependent. In disease contexts, SLK activity has been shown to drive breast cancer cell invasion in the presence of Heregulin, an activator of the HER2/Neu oncogene (Roovers K., Wagner S., Storbeck CJ, et al., 2009). As for mouse fibroblasts, the FAK/Src complex was also required for invasion and migration of breast cancer cells and mammary epithelial cells (Roovers K., Wagner S., Storbeck CJ, et al., 2009). Recently, LMO4 was found to interact directly with the ATH domain of SLK (Baron et al., 2015). It has been shown that the activation and recruitment of

SLK at the leading edge of migrating cells requires the LIM-only protein 4 (LMO4) and Src family kinase activity (Baron et al., 2015). LMO4-deficient cells failed to recruit SLK at the leading edge and to induce SLK kinase activity upon scratch wounding. SLK also co-localizes with Rac1, a regulator of actin stress fiber dynamics, at the leading edge of migrating cells (Wagner et al., 2002). Supporting a role for SLK in focal adhesion breakdown, overexpression of SLK induces actin stress-fiber disassembly, focal adhesion disassembly and apoptosis in C2C12 myoblasts (Sabourin et al., 2000). The v-Src oncogene has been shown to inhibit SLK activity indirectly through CK2 activation. SLK has been shown to be directly phosphorylated by Casein Kinase II (CK2) downstream of v-Src, resulting in the downregulation of SLK kinase activity (Chaar et al., 2006). Supporting this, SLK catalytic activity could be restored following CK2 inhibition in v-Src transformed cells.

In adult mice, SLK-null skeletal muscles show Paxillin mis-localization while wildtype muscles have a normal Paxillin distribution at costameres. FAK also displayed an altered localization and patchy distribution throughout the myofibers. This impaired localization might be responsible for the observed defects in the myotendinous junctions and myofibers stability.

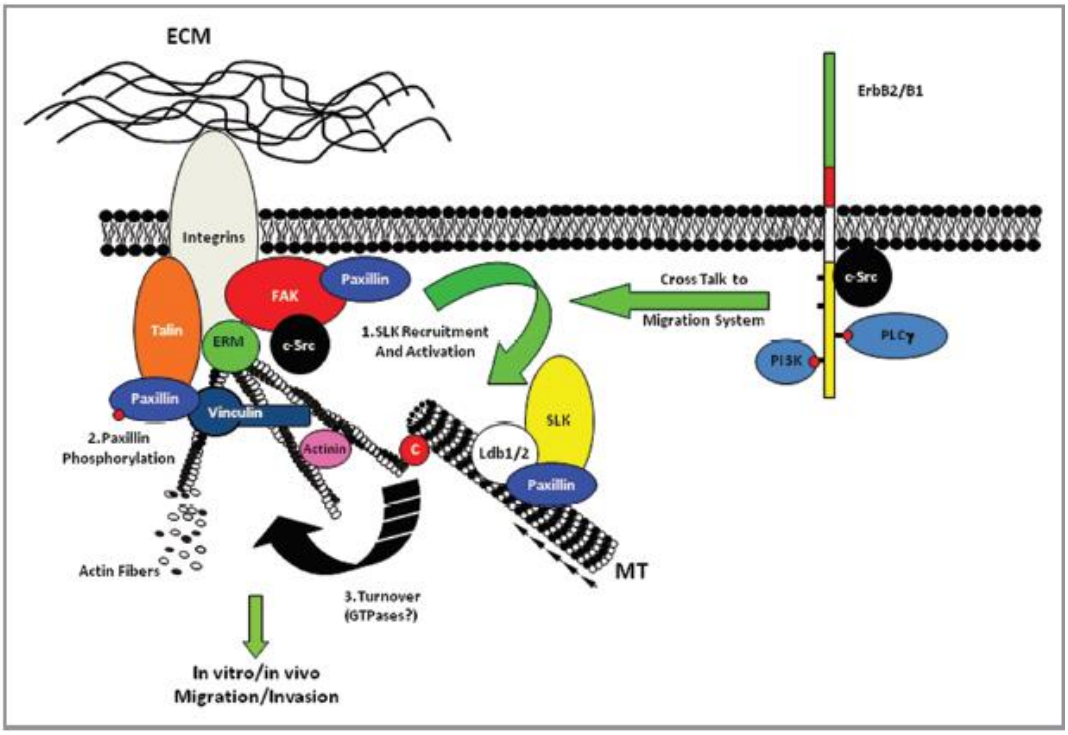

ECM
ErbB2/B1
Integrins
FAK
Paxillin
Talin
ERM
c-Src
1.SLK Recruitment
And Activation
Cross Talk to
Migration System
c-Src
PLCγ
PI3K
Paxillin
Vinculin
2.Paxillin
Phosphorylation
Actinin
C
SLK
Ldb1/2
Paxillin
MT
Actin Fibers
3.Turnover
(GTPases?)
In vitro/in vivo
Migration/Invasion

Figure 3. Mechanisms of SLK recruitment and activation during cell migration.

Downstream of growth factor receptor activation or direct integrin engagement with the ECM, the FAK/src complex is stimulated (Al Zahrani et. al, 2013), triggering signaling events that lead to SLK activation. SLK is recruited to the leading edge where it can phosphorylate Paxillin to induce focal adhesion destabilization. Focal adhesion turnover propels the cell forward in the direction of migration/invasion. The Ldb1/2 proteins can play a role in modulating SLK activity depending on the context (Storbeck, 2009).

1.4 SLK and Apoptosis

The word apoptosis was coined by Kerr and colleagues in 1972 to illustrate the physically unique appearance of a cell's "loss of life" (Kerr et al, 1972; Paweletz, 2001; Kerr, 2002; Susan E., 2007). Apoptosis is defined as programmed cell death (Susan E., 2007). In other words, cells lose their viability at distinct or regulated stops during certain cellular processes. Some features of apoptotic cells are that they have DNA breakdown, cleavage of proteins and cross-linking of proteins. Apoptosis was first studied in the nematode *Caenorhabditis elegans* (Horvitz, 1999; Susan E., 2007). Out of the 1090 somatic cells of the nematode, 131 underwent apoptosis between different stages highlighting the importance of this process for the control of cell number (Horvitz, 1999; Susan E., 2007). Other processes where apoptosis has been shown to occur include: aging, development and homeostatic cell population maintenance. For example, aging females have a higher rate of apoptosis for their oocytes than younger women possibly suggesting why older women have lower fertility (Wu J, Zhang I, Wang X, 2000; Muradian K, Schachtschabel DO., 2001). When cells have been damaged by toxins and/or pathogens or are under attack due to an immune response, apoptosis can occur to protect the organism (Norbury and Hickson, 2001; Susan E., 2007). Different stimuli cause apoptotic death through different signaling pathways. Current literature has divided apoptotic pathways into two categories: intrinsic (mitochondrial pathway) and extrinsic (death receptor pathway) (Igney FH, Krammer PH, 2002; Susan E., 2007). There is also evidence that connects both pathways to each other and studies that suggest the existence of a third system known as the perforin/granzyme pathway. These systems eventually lead to a final stage known as the execution pathway.

The Extrinsic Signaling Pathway

This pathway is initiated by the activation of a transmembrane receptor-mediated interaction with a death signal or ligand (Hsu et al., 1995; Grimm et al., 1996; Wajant, 2002; Susan E., 2007). Death receptors, from the tumor necrosis factor (TNF) family, play an important role in this process. There are two critical death receptor pathways: the TNF-α/TNFR1 and the FasL/FasR. Ligand-mediated receptor clustering triggers the recruitment of adaptor proteins (FADD or TRADD) that interact with procaspase-8 (Hsu et al., 1995; Grimm et al., 1996; Wajant, 2002; Susan E., 2007). This death-inducing complex (DISC) activates Procaspase-8 through auto-catalysis, leading to the execution phase of apoptosis.

The Intrinsic Signaling Pathway

There are several stimuli that can cause apoptosis either through negative signals (lack of key components for cell survival) or positive signals (infections, radiation etc.). All of those stimuli disrupt mitochondrial function culminating in cell death (Saelens et al., 2004; Susan E., 2007). Two groups of pro-apoptotic proteins are key for intrinsic apoptosis. The first group is made up of the serine protease HtrA2/Omi, Cytochrome C and Smac/DIABLO (Cai et al., 1998; Du et al., 2000; Loo et al., 2002; Garrido et al., 2006; Susan E., 2007). The second is made up of endonuclease G, CAD and AIF (Joza N, Susin SA, Daugas E et al., 2001; Susan E., 2007). These factors all have pro-apoptotic activity in the intrinsic cascade.

Caspases are the ultimate effectors of the apoptotic response. They can be divided into initiator caspases (caspases 2, 8, 9, and 10), executioner (caspases 3, 6, and 7) and inflammatory (caspases 1, 4, and 5) (Cohen, 1997; Rai et al., 2005; Susan E., 2007). Other caspases include 11, 12, 13 and 14 with their own distinct roles. Caspase 11 helps cytokines mature during septic shock and regulates apoptosis (Kang et al., 2002; Susan E., 2007). Caspase 12 controls the cytotoxic effects of amyloid-β and apoptosis specific to the endoplasmic reticulum (Nakagawa et

al., 2000; Susan E., 2007). Caspase 13 was initially thought to be solely in humans, but later found to be in cattle (Koenig et al., 2001; Susan E., 2007). Caspase 14 is expressed in low amounts within adult tissues but at very high levels within embryonic tissues (Hu et al., 1998; Susan E., 2007). It has been shown to play a role in epidermal barrier formation (Denecker G., E. Hoste, B. Gilbert et al., 2007; Denecker G., Ovaere P., Vandenabeele P. et al, 2008). Most cells have inactive caspases. Once those inactive initiator caspases are activated, they cleave the pro-caspase forms to activate executioner caspases, resulting in a protease cascade (D. Mcllwain, T. Berger, T. Mak, 2013; Hengartner, 2000). This chain of events, known as the proteolytic cascade, accelerates the apoptotic signaling pathway and ultimately cell death. Both the intrinsic and extrinsic pathways eventually culminates in the activation of executioner caspases which then trigger several downstream processes including protein cross-linking, apoptotic body formation, degradation of nuclear and cytoskeletal proteins and DNA fragmentation (Igney FH, Krammer PH, 2002; Susan E., 2007).

Execution Pathway

Executioner caspases activate cytoplasmic endonucleases and proteases to breakdown nuclear material and proteins. They cleave multiple substrates such as poly (ADP-ribose) polymerase (PARP) and cytokeratins which causes the cell to change biochemically and morphologically. PARP helps in transcription, DNA repair and other processes (Morales J., Longshan L., Farjana F. et al., 2016). Caspase 3 cleaves ICAD (CAD inhibitor) to release the endonuclease CAD, causing degradation of chromosomal DNA (Sakahira H, Enari M, Nagata S, 1998; Susan E., 2007). Caspase-3 also cleaves the actin-binding protein Gelsolin and the cleaved portions negatively affects cell division, intracellular transport, the cytoskeleton and multiple signaling pathways (Kothakota S, Azuma T, Reinhard C, et al., 1997; Susan E., 2007). The

apoptotic cells also externalize phosphatidylserine residues on the cell surface (Susan E., 2007), allowing for non-inflammatory phagocytic recognition (Fadok VA, de Cathelineau A, Daleke DL, et al., 2001).

Interestingly, SLK was also shown to induce apoptosis in C2C12 myoblasts when overexpressed, by activating the c-Jun-N-terminal kinase 1 (JNK1)-dependent pathway (Sabourin LA, Rudnicki MA, 1999; Yan Y, Tulasne D, Browaeys E, 2007; Al-Zahrani, KN., Baron KD, and Sabourin LA, 2013). In another study, SLK overexpression lead to membrane blebbing, actin redistribution to the periphery of the cell, actin stress fiber dissolution and loss of substrate adhesion (Sabourin LA, Tamai K, Seale P, 2000; Al-Zahrani, KN., Baron KD, and Sabourin LA, 2013). Expression of active SLK caused rapid apoptosis while expression of the ATH domain caused a delay in apoptosis. A number of apoptotic triggers were shown to induce caspase 3-mediated cleavage of SLK, releasing the active kinase region, thereby accelerating apoptosis. In the context of renal ischemia-reperfusion injury, SLK expression increased the endoplasmic reticulum (ER) stress response through ASK-1 and p38. In glomerular epithelial cells, SLK overexpression induced p53 activity through the JNK pathway (Cybulsky AV, Takano T, Guillemette J et al., 2009; Al-Zahrani, KN., Baron KD, and Sabourin LA, 2013). The same effect was seen in a transgenic mouse line that overexpressed SLK in kidney glomerular podocytes (Cybulsky AV, Takano T, Papillon J et al., 2010; Al-Zahrani, KN., Baron KD, and Sabourin LA, 2013). This led to a loss of the podocytes, coupled with an elevation of p38 activity. In glomerular epithelial cells, SLK overexpression also induces the HSF1-HSP70 pathway (Cybulsky AV, Guillemette J, Papillon J. et al, 2016). HSF1 is involved in the transcription of various other heatshock proteins that help control protein folding, such as HSP70.

1.5 Rationale and Hypothesis

Numerous studies have shown that SLK plays a critical role in cell cycle transit and cell migration. It has also been demonstrated to be a substrate for Caspase 3, suggesting that it may have an active function in cell death (Ellinger-Ziegelbauer H, Karasuyama H, Yamada E et al., 2000; Reilly P., Wagner S, Franks D., et al. 2005; Hao W, Takano T, Guillemette J et al., 2006; Wagner et al., 2002; S. Wagner, C. Storbeck, K. Roovers et al., 2008; Quizi JL, Baron K, Al-Zahrani KN, 2013). The recent generation of a conditional SLK-floxed allele has allowed us to derive SLK-null fibroblasts to test the effect of an actual genetic deletion of SLK on those processes. Our hypothesis was that the deletion of SLK would markedly delay cell growth, migration and apoptosis. We tested this using the following approaches:

1.6 Objectives

1. Test the effect of SLK deletion on the proliferation of embryonic fibroblasts using cell counts, Flow Cytometry and Western blotting for cell cycle markers.

2. Test the effect of SLK deletion on the migration potential of fibroblasts using Boyden Chamber Migration Assays, scratch wounding and Nocodazole wash outs.

3. Test the effect of SLK ablation on cell death and apoptosis signaling using various triggers.

2. Materials and Methods

2.1. Cell Culture

Mouse Embryonic Fibroblasts (MEFs) were cultured in DMEM 1X (Dulbecco's Modified Eagle's Medium), 2mM L-glutamine (Invitrogen), with 200 U mL-1 penicillin/streptomycin (Invitrogen), and 10% fetal bovine serum (FBS; Invitrogen). All experiments were performed in complete medium as described above. Cells were grown in an incubator that was humidified and set at a temperature of 37ºC with 5% CO2. For Nocodazole (450 µM) arrest experiments, MEFs cells were always arrested for 24 hours prior to re-feeding with full media.

2.2 Western Blots and Protein Harvesting

Proteins were extracted with cell scraping from a 10 cm plate. 0.5 mL 1xPBS was the final volume, prior to washing with 1 mL of 1X PBS, which was collected to be spun in a centrifuge at 8000 rpm for 3 minutes (1xPBS was made from a 10x solution which consisted of: 2.1g Na_2HPO_4, 500mL H_2O, 45g NaCl, 0.72g KH_2PO_4) (Conway, J., Sabourin, L., 2016). Afterwards, the pellets were lysed with RIPA (1mg/mL leupeptin, 1M DTT, 150 mM NaCl, , 1M β- glycerophosphate, 0.05% SDS, 1M NaF, 1% Igepal CA-630, 0.1M PMSF, 1% Triton X-100, 1M Tris, 50 mM Tris-HCl, pH 7.5, 12 mM Na-Deoxycholate buffer, 1mg/mL pepstatin, 2 mM EDTA, pH 8.0, 0.2M NaVO3, 100µM benzamide, , 1mg/mL aprotinin)(Conway, J., Sabourin, L., 2016). Protein lysates were spun at 13500 rpm for 10 minutes to isolate the protein. Bradford Lowry Reagent was used to determine the protein concentration (Bio-Rad, Mississauga, Ontario, Canada) (Conway, J., Sabourin, L., 2016). For western blotting, 10-20 µg of total protein was electrophoresed on an 8, 10 or 12% polyacrylamide gel (depending on the experiment) and transferred onto a PVDF (polyvinylidene difluoride) membrane (Thermo Fisher Scientific, USA)

(Conway, J., Sabourin, L., 2016). BSA (5%) was used to block the membranes (Sigma-Aldrich, Oakville, Ontario, Canada). Membranes were mixed with primary antibodies in 1xTBST (150 mM NaCl, 0.05% Tween 20, 50 mM Tris, pH 7.4)(Conway, J., & Sabourin, L., 2016) for one hour at room temperature (or overnight). The membrane underwent 3 1xTBST washes, of 5 minutes. The primary antibody was then probed with a secondary antibody (Horseradish peroxidase-coupled from Bio-Rad, USA) and visualized with Western Lightning Plus chemiluminescence (Perkin-Elmer, USA). The following primary antibodies were used: anti-SLK (custom antibody, Japan, (Wagner, et al., 2008)), anti-α-tubulin (Sigma-Aldrich, Oakville, Ontario, Canada), Paxillin, FAK, phospho-FAK-Y397 (BD transduction laboratories, Mississauga, Ontario, Canada), Myc (Sigma- Aldrich; 9E10 mouse ascites), Beta-Actin (Sigma, A5316), PARP (Cell Signalling Technology, 9542), JNK1 (Cell Signalling, 9252), p-JNK (T183/Y185)(Cell Signalling, 9251), p38 (Cell Signalling, 9211), p-p38 (T180, Y182) (Cell Signalling 9211), ASK1 (Cell Signalling, 3762), p-ASK(966)(Cell Signalling Technologies), Caspase 3 (Cell Signalling, 9662), Cyclin D1 (Santa Cruz, sc-20044), Cyclin A (Santa Cruz, sc-271682).

2.3 Transfections

For transfections, MEFs were seeded at 50000 cells on a 6 cm plate and transfected the next day. The cells were transfected with Polyethylenimine (PEI - linear, 25,000 MW) in serum-free media, and changed for a 10 cm plate according to details specified by the manufacturer. The transfection mix was then added to the adherent cells and topped up with 10% FBS DMEM after 3 hours. The cells were harvested 48 hours following transfection.

2.4 Proliferation Assay

Cells were seeded in cell culture plates at an initial density of 50,000 cells in 6 cm plates in biological triplicates and counted every day for up to 8 days. Cells were removed through trypsinization and collected using a centrifuge at a speed of 1200 rpm for 3 minutes. The cells were then washed in PBS and collected in 2 mL of media. Technical triplicates, three individual counts from the same sample, were also taken using the Beckman Vi-Cell cell counter.

2.5 Flow Cytometry

Cells were seeded on 10 cm plates at around 70% confluency. On the following day, they were exposed to 450 µM Nocodazole for 24 hours. Cells were then released from arrest in complete media and collected at the indicated time points (0, 3, 6, 18, 20 hours). The cells were washed, trypsinized and spun at 1200 rpm for 5 minutes. They were then fixed in cold 70% ethanol and stained with Propidium Iodide Buffer (100 ug/mL RNaseA, 0.2% Triton-X, 25 ug/mL propidium Iodide, 0.5M EDTA, pH 8.0). The stained cells were then gated and counted using flow cytometry (Beckman Coulter MoFlo XDP).

2.6 Boyden Chamber Migration Assay

Boyden chambers were coated the night before with fibronectin (1% of total volume used, dissolved in PBS) and incubated at 4^{o}C. Cells were serum-starved 24h before the experiment. 50,000 cells were placed in the top portion of the Boyden chamber in a volume of 400 µL. The bottom chamber was also placed in 400 µl of media. SLK wildtype and SLK (-/-) cells were seeded into Boyden chambers in triplicate and allowed to migrate for 6 hours. Migration time and serum concentration were optimized in separate experiments and migration assays were performed for 6 hours at 1% FBS (bottom and top chamber), in DMEM to measure haptotaxis. The cells that remained on the bottom part of the chamber were fixed with 10%

buffered formalin phosphate and finally stained with 0.5% crystal violet (dissolved in 25% methanol) for quantification after imaging.

2.7 Alamar Blue Apoptosis Assay

SLK (fl/fl) and SLK (-/-) cells were seeded at 70-80% density (5000 cells) in a 96 well plate in quadruplicates for each time point. The following day, the cells were exposed to specific apoptotic triggers (1 µM Staurosporine/ 150 µM Etoposide/ 4 µM Chelerythrine; Cell Signaling and Sigma) for the indicated time (0, 3, 6, 12, and 24). Staurosporine is a protein kinase C inhibitor and also inhibits other kinases at higher concentrations (Ruegg, U. T. and Burgess, G. M., 1989). Etoposide is used for chemotherapy and it causes DNA damage by stopping Topoisomerase II (Jamil S, Lam I, Majd M et al., 2015). Chelerythrine acts as a strong inhibitor towards protein kinase C, causes DNA fragmentation and cell death (Barg, J. et al, 1992). Following treatment, the cells were mixed with Alamar blue (10x) solution and live/dead cells were quantitated, 4 hours after to allow the reagent to change color, using a Fluorescence Multiskan reader at 570 nm and 604 nm.

2.8 Immunofluorescence

For the Scratch Wound Assay, the cells were plated on glass coverslips in 6 well plates and grown to confluency. The monolayers were then scratched with a pipet tip and allowed to migrate into the wound for t=0 and 60 minutes. For the Nocodazole Arrest Assay, cells were plated at 50,000 cells on a 6 well plate. Subconfluent monolayers were re-fed with full media the following day and treated with 450 µM of Nocodazole for 24 hours, then fixed at t= 0 and 45 minutes with 4% PFA following nocodazole wash-out. From this point on, the procedure

remains the same for both assays. Media was removed and the cells were washed with 1X PBS. Cells were then fixed with cold 4% PFA (Paraformaldehyde) for 10 minutes. They were then washed with 1X PBS three times and permeabilized with 0.1% Triton (mixed with PBS) for 10 minutes. They were then washed again with PBS three times. The coverslips were blocked with 5% goat serum, diluted in PBS, (Original stock 45-75 mg/mL) for 1 hour. Primary antibody diluted in 5% goat serum was then added at room temperature. Fluorescent secondary antibodies were then added to bind to the primary antibodies with 5% goat serum. ProLong Gold antifade reagent with DAPI (4, 6-diamidino-2-phenylindole) was added to the coverslips and then sealed with nail polish. Images were captured using a Zeiss AxioCam Fluorescence Microscope.

2.9 Immunoprecipitation

Different Melanoma cell lines were seeded at an equal density on 10 cm cell culture plates. Cells were harvested and collected into an Eppendorf tube and lysed in RIPA lysis buffer. At least 300 µg of total protein were used in immunoprecipitations. 20 µL of protein A-Sepharose 4 Fast Flow beads were added to the tubes along with 1 µL of SLK primary antibody. The sample were incubated for 2 hours at 4 º C and washed three times with NETN (200 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.5% NP-40, 1 mM EDTA, pH 8.0) (Al-Zahrani, 2018) buffer to remove non-specific binding. SDS sample buffer was then added to the samples, heated at 100 º C for 5 minutes and fractionated by SDS-PAGE for western analysis.

2.10 In-vitro binding assay

Binding assays were performed to test the binding between the SLK and RASSF1A. For the binding assays, recombinant proteins were prepared using GST fusion constructs. GST and GST-fusion plasmids were transformed into DH5α E. coli and clones were isolated. Bacterial

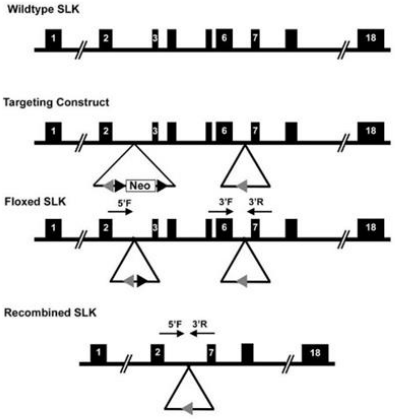
clones were inoculated the night before the experiment. One tenth of the culture was then transferred into a new flask and grown for an hour at 37 º C. Protein expression was induced using 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2 hours at 37ºC. The samples were then spun down and the cell pellets were lysed using 500 µL of RIPA lysis buffer. To prevent protein degradation, the tubes were constantly kept on ice. The cells were subjected to sonication and vortexed every 5 minutes for 15 minutes. The samples were cleared for 30 minutes at 13500 rpm at 4 º C and the supernatant was transferred into fresh chilled tubes and 20 µL of GST sepharose beads were added. The tube was rotated for 2h to isolate the GST-fusions. In vitro translation of HA-RASSF1A or Myc-SLK was performed using the TNT reticulocyte lysate system and purified plasmid DNA in the presence of ^{35}S-Methionine. The isolated GST and GST-fusion samples were washed with NETN to remove non-specific binding. The in-vitro translated products were then incubated with the GST-fusions for 45 minutes at room temperature. The samples were then washed with NETN, denatured and loaded onto SDS gels. The gels were stained with coomassie blue, washed with water and enlightening agent to increase the radioactive signal of ^{35}S. The gel was dried down and exposed to X-ray film at -80ºC.

3. Results

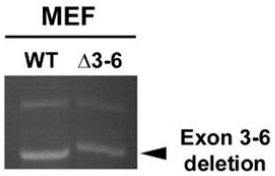
3.1 Generation of SLK-deficient Fibroblasts

Prior to the start of this project, Dr. Khalid Al-Zahrani from the Sabourin lab had generated SLK-deficient mouse embryonic fibroblasts (MEFs) from a conditional SLK floxed allele (**Figure 4A**). Briefly, 14.5 day old embryos were obtained from timed matings by caesarean section, decapitated and dissociated by trypsinization. The cell line was established following a 30 day period in culture. To delete SLK, cultures were exposed to AdCre virus or an AdGFP control (**Figure 4B)**. As shown in **Figure 4C**, AdCre-infected SLK floxed MEFs are devoid of SLK proteins, suggesting complete inactivation of the SLK gene.

A.


Wildtype SLK
1
2
3
6
7
18
Targeting Construct
Neo
Floxed SLK
5'F
3'F
3'R
Recombined SLK

B.


MEF
WT
Δ3-6
Exon 3-6
deletion

C.

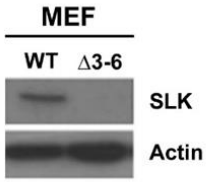

MEF
WT
Δ3-6
SLK
Actin

Figure 4. Generation of SLK-deficient Fibroblasts. (A) Diagram depicting the floxed SLK allele. Exons 3-6 were flanked by LoxP sites and a Neo cassette flanked by FRT sites was inserted in intro 2. Infection with AdCre deletes exons 3-6 (recombined locus) (Pryce, B. R., Al-Zahrani, K. N., Dufresne, S et al, 2017). **(B)** Validation of the genetic deletion of SLK illustrated by shift in the Δ3-6 PCR products encompassing the deleted region (done by John Abou-Hamad). **(C)** Western blot analysis of fibroblast extracts showing loss of SLK protein in Cre-infected cells.

3.2 SLK deletion in MEFs does not impair proliferation in vitro

Previous studies from our lab have shown that SLK kinase activity is induced during G2/M and that the expression of a kinase dead SLK in MEF-3T3 cells induces a G2/M cell cycle arrest (PG. O'Reilly, 2005). Supporting this, expression of active SLK induces ectopic spindle formation and mitotic catastrophe. Similarly, cells expressing a mutant SLK-LacZ gene trap fusion protein showed reduced proliferation (Al-Zahrani KN, Sekhon P, Tessier DR et al., 2014). Together these data suggest that SLK is critical for progression through the cell cycle. To assess the effect of an SLK genetic deletion on proliferation, we tested the growth potential of SLK-deficient MEFs. MEFs cells were plated at equal densities and growth was monitored over time. Surprisingly, the deletion of SLK had no effect on the proliferation of the MEFs in vitro in complete medium (**Figure 5**). This suggests that SLK is not required for the growth of MEFs in vitro or that compensatory pathways have been activated. To test the effect of SLK deletion on cell cycle distribution, Nocodazole-arrested cultures were released in complete medium and subjected to flow cytometry following Propidium iodide staining. As for the cell counts, no differences were observed in the cell content of the different cell cycle phases (**Figure 6A**). Supporting this, the expression pattern for cyclin A (G2/M phase) and cyclin E (S phase) were similar between the two cell lines, suggesting that SLK deletion does not affect cell cycle transit (**Figure 6B).** To further elucidate if there is a potential difference in growth, the time period of all the proliferation experiments could have been increased to 64 hours because 32 hours might have not been a long enough time frame to observe any changes.

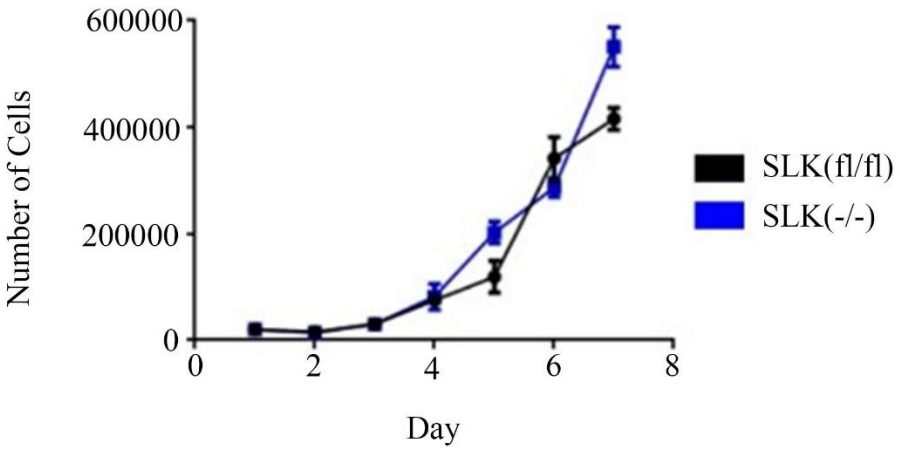
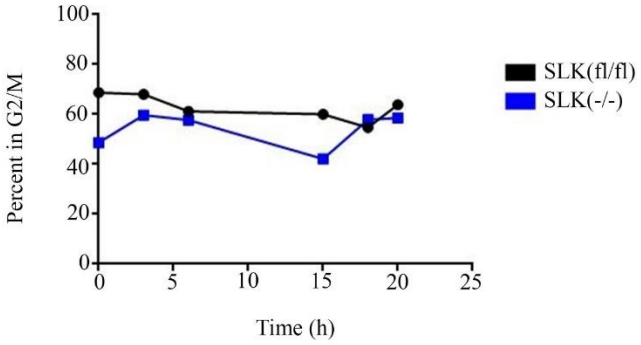

600000
400000
200000
0
Number of Cells
0
2
4
6
8
Day
SLK(fl/fl)
SLK(-/-)

Figure 5. SLK deletion in MEFs does not impair proliferation *in vitro*. Cells were plated at an equal density, in 6 cm plates, and counted daily for 7 days in biological and technical triplicates using the Beckman Vi-Cell cell counter (n=3). No difference in growth between SLK(fl/fl) and SLK(-/-) MEFs were observed.

A.


100
80
60
40
20
0
Percent in G2/M
0
5
10
15
20
25
Time (h)
SLK(fl/fl)
SLK(-/-)

B.

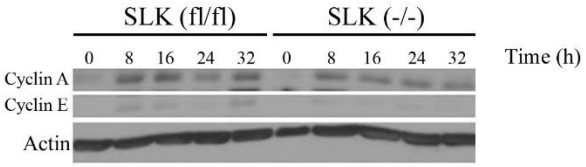
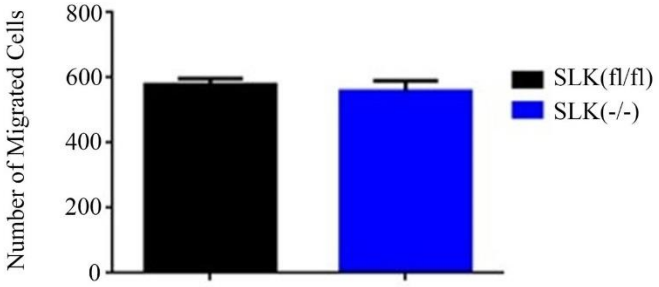

SLK (fl/fl)
SLK (-/-)
0
8
16
24
32
0
8
16
24
32
Time (h)
Cyclin A
Cyclin E
Actin

Figure 6. SLK deletion does not impair cell cycle progression. (A) Cells were arrested in 450 µM Nocodazole, refed in full media and fixed at specific time points for staining with Propidium Iodide and flow cytometry analysis. No difference in the percentage of cells in G2/M compartment was observed between SLK(fl/fl) and SLK(-/-) MEFs (n=3). **(B)** Cells were arrested in 450 µM Nocodazole and analysed by Western Blot for cyclin A and E. No change in cyclin levels were observed between SLK(fl/fl) and SLK(-/-) MEFs over a 32 hour time course.

3.3 SLK Deletion does not impair cell Migration

It has been shown that SLK is associated with the microtubule network (Wagner et al., 2002). In addition, SLK was shown to phosphorylate the signaling adapter Paxillin, critical for cell motility (Quizi, 2013). When a Paxillin mutant (S250A) at an SLK phosphorylation site was expressed in 293T, migration rates decreased two-fold. Similarly, when SLK was knocked down in cells that were activated by heregulin, migration was inhibited by 75% (K Roovers, S Wagner, CJ Storbeck et al., 2009), strongly supporting a role for SLK in cell migration. Therefore, motility was assessed in MEFs using a Boyden Chamber migration assay. Boyden chambers were coated with fibronectin the day before the experiment. Equal amounts of both wildtype and SLK-null cells were seeded in biological and technical triplicates and allowed to migrate to the fibronectin-coated side. Surprisingly, there were no detectable differences in motility rates between the two cells lines (**Figure 7A)**. Similarly, no differences were observed in the relative levels of migration signaling components such as Paxillin and FAK by Western blot (**Figure 7B**). These results suggest that SLK-deficient MEFs might have activated compensatory pathways to bypass the requirements for SLK during cell migration. There could also be compensation by a splice variant of SLK, as potentially predicted by past experiments, and they may not be detected by the regular SLK antibody due to possible post-transitional modifications ((Sabourin LA and Rudnicki MA, 1999) (Sierra Delarosa, 2009).

A.


Number of Migrated Cells
800
600
400
200
0
SLK(fl/fl)
SLK(-/-)

B.

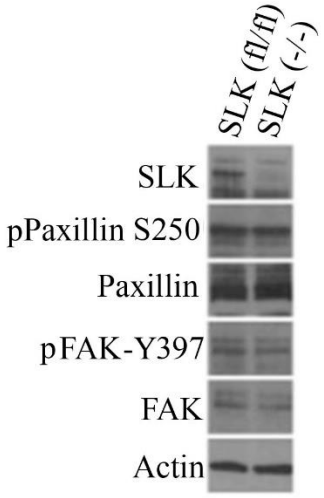
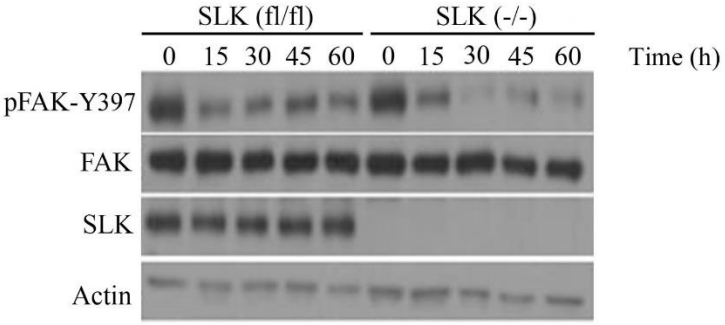

SLK (fl/fl)
SLK (-/-)
SLK
pPaxillin S250
Paxillin
pFAK-Y397
FAK
Actin

Figure 7. SLK deletion does not impair cell migration (A) An equal density of cells were placed on top of the Boyden chambers, left to migrate for 6 hours, fixed with 10% buffered formalin phosphate and finally stained with 0.5% crystal violet (dissolved in 25% methanol) for quantification after imaging. No difference in migration rates were seen between SLK (fl/fl) and SLK (-/-) MEFs. (**B)** Western blot analysis showing that the levels of critical migration signaling proteins are unaffected (n=3).

3.4 SLK deletion enhances Nocodazole washout-induced focal adhesion turnover

Microtubule polymerization and contact with focal adhesions have been shown to induce their destabilization (Kaverina et al., 1999). Similarly, Nocodazole treatment induces focal adhesion stabilization and accumulation of pFAK-Y397 (Kaverina et al., 1998), whereas Nocodazole release induces a loss of pFAK-Y397, indicative of focal adhesion turnover. We have previously shown that SLK is activated downstream of the FAK/Src complex, required for focal adhesion turnover and cell migration (S. Wagner, C. Storbeck, K. Roovers et al., 2008). To assess the effect of SLK deletion on focal adhesion turnover, we monitored the levels of pFAK-Y397 following Nocodazole treatment. Although no difference in motility was observed, the loss of pFAK-Y397 was more rapid in SLK-null MEFs, suggesting that microtubule-dependent focal adhesion turnover is faster (**Figure 8A**). An immunofluorescence experiment was done to validate this finding, however no difference was observed through immunofluorescence (**Figure 8B & 9)**. This is in stark contrast to what has been observed in SLK knockdown studies. Interestingly, scratch wounding of SLK-null MEFs still results in the recruitment of FAK and Paxillin at the leading edge of migrating cells, suggesting that SLK is not required for this process (**Figure 10**).

A.


SLK (fl/fl)
SLK (-/-)
0 15 30 45 60 0 15 30 45 60
Time (h)
pFAK-Y397
FAK
SLK
Actin

B.

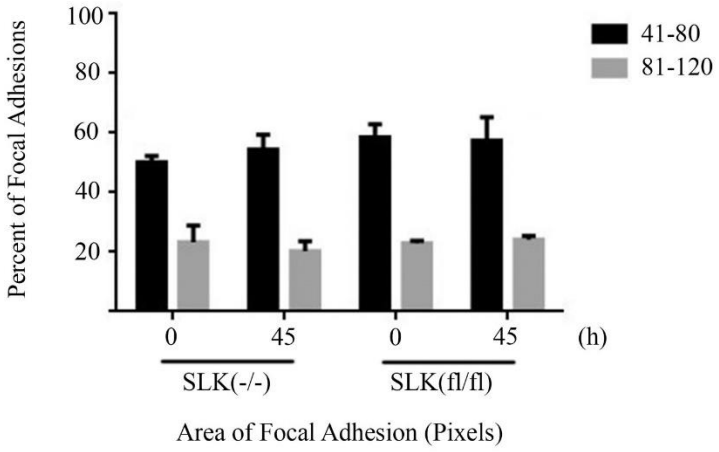
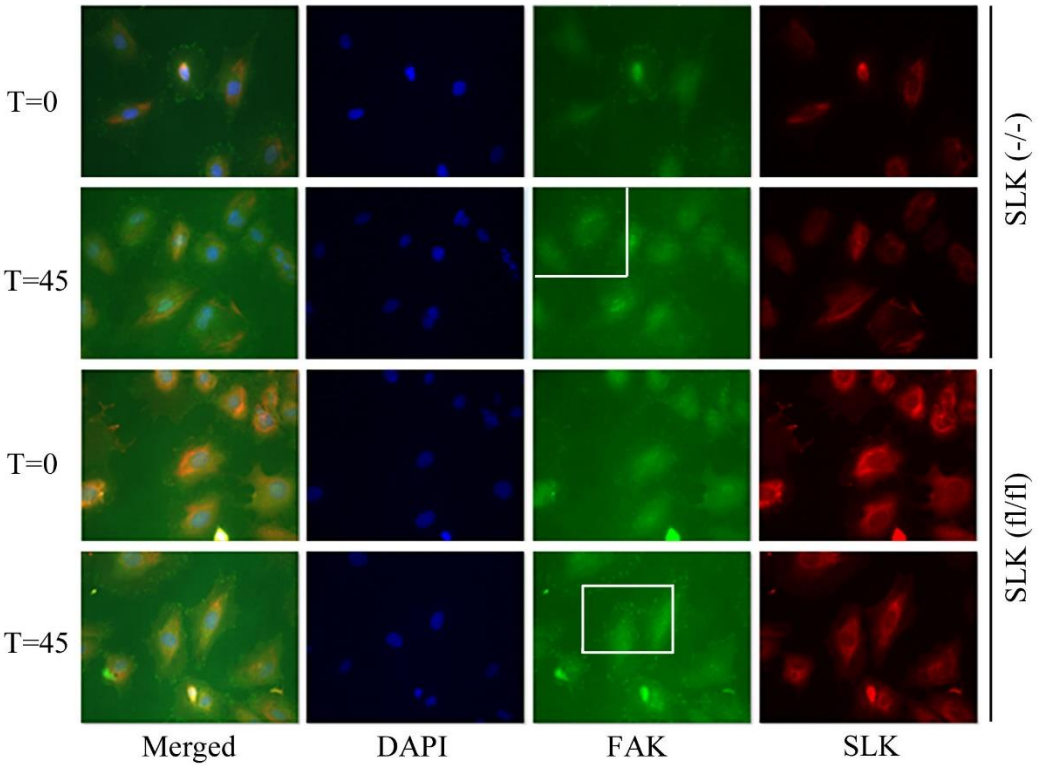

100
80
60
40
20
Percent of Focal Adhesions
41-80
81-120
0
45
0
45
(h)
SLK(-/-)
SLK(fl/fl)
Area of Focal Adhesion (Pixels)

Figure 8. SLK deletion enhances Nocodazole washout-induced focal adhesion turnover (A) Sub-confluent monolayers were arrested with 450 µM Nocodazole, refed with full media and finally harvested for western blot analysis at specific time points. A more rapid loss of pFAK-Y397 was observed in SLK (-/-) MEFs, suggesting altered kinetics. **(B)** Focal adhesion size was assessed by fluorescence microscopy following Nocodazole washout. No change in focal adhesion size was observed. Focal adhesions that did not fit the bin sizes were excluded from the graph due to them being false positives outside the average focal adhesion size. Quantification was done through ImageJ and is thoroughly explained by the following paper (Horzum U., Ozdil B., Pesen-Okvur D., 2014) (n=3).

A.



B.

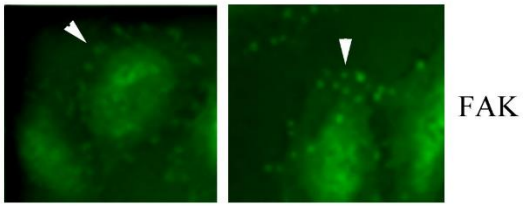
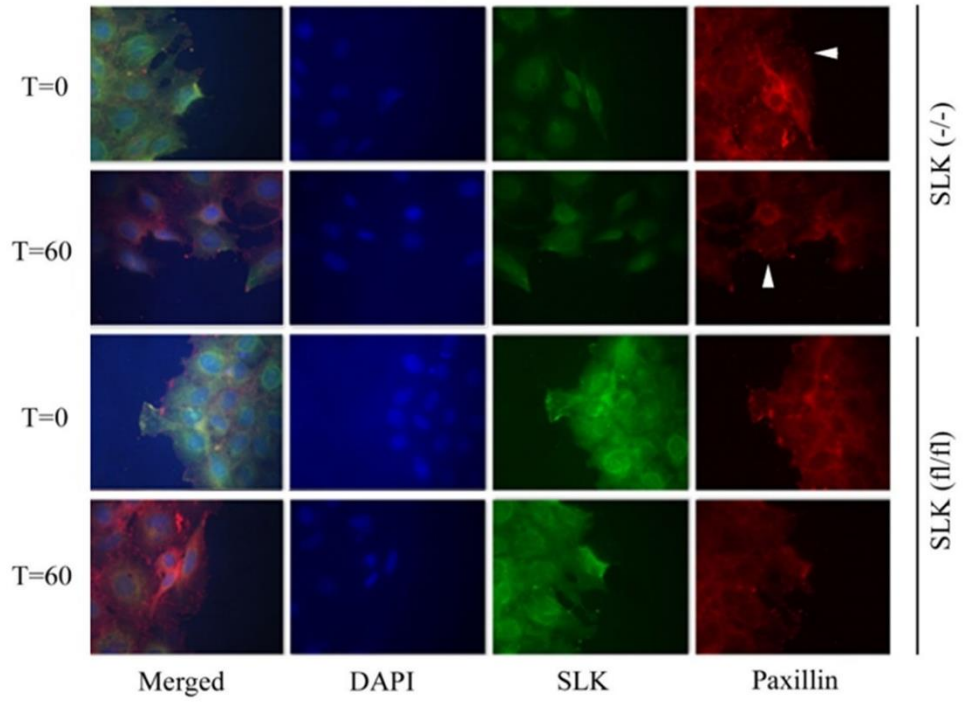


Figure 9. SLK deletion does not alter focal adhesion size (A) In Figure 8, cells were arrested with 450 µM Nocodazole, refed with full media and fixed with 4% PFA for total FAK immunofluorescence analysis. The size of the FAK-positive adhesions was quantitated. **(B)** The boxed regions in (A) are shown at higher magnification. Adhesions are indicated by the arrowheads (n=3).

A.



B.

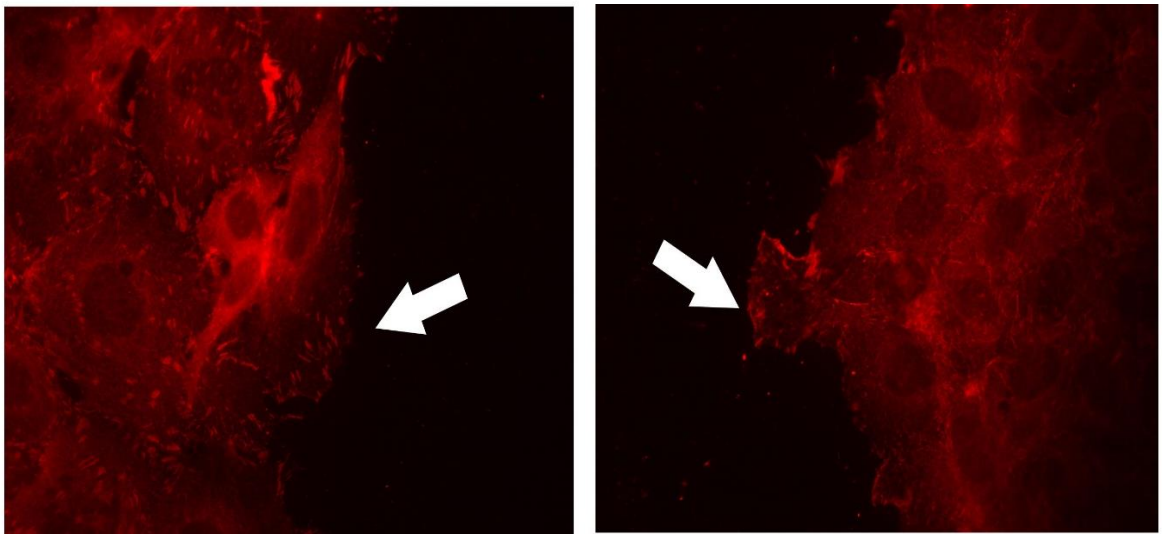


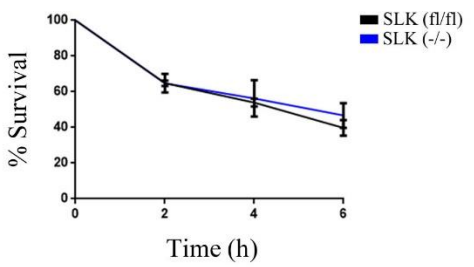
Figure 10 – SLK is not required for Paxillin localization at the leading edge. (A) Confluent monolayers were subjected to Scratch Wound Assays and Paxillin localization was assessed by immunofluorescence. Paxillin was still recruited to the leading edge in SLK-deficient cells. Arrowheads show Paxillin at the leading edge of cells migrating into the wound (n=3). **(B)** Close up immunofluorescent images of Paxillin (SLK (-/-) on the left, SLK (fl/fl) on the right).

3.5 SLK is not required for programmed cell death

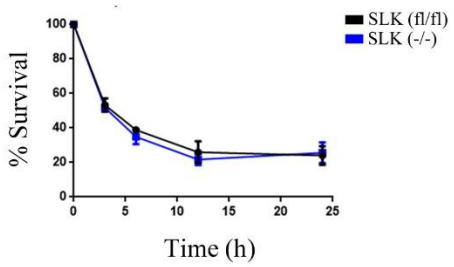
Previously SLK overexpression was shown to induce apoptosis and to activate JNK in various cell lines and podocytes (Sabourin LA, Rudnicki MA, 1999; Yan Y, Tulasne D, Browaeys E, 2007; Al-Zahrani, KN., Baron KD, and Sabourin LA, 2013; Cybulsky AV, Takano T, Guillemette J et. al, 2009). Furthermore, apoptotic triggers induced the caspase 3-mediated cleavage of SLK into its active kinase domain and the actin-regulating ATH region (Sabourin LA and Rudnicki MA, 1999). To test the effect of SLK deletion on cell death, wildtype and SLK-null MEFS were exposed to various apoptotic triggers. Both cell lines were treated with etoposide, Staurosporine or Chelerythrine and monitored for cell viability by Alamar blue over 24 hours. Surprisingly, SLK deletion had no effect of cell viability and did not delay cell death upon exposure to the apoptotic triggers (**Figure 11**). As previously reported, SLK was observed to breakdown during cell death (see **Figure 12-14;** Sabourin L.A., Tamai K, Seale P., et al., 2000). To further investigate the role of SLK in cell death signaling, both wildtype and knockout cells were treated with Staurosporine for a period of 6 hours and the activation or levels of apoptotic markers were assessed. Western Blot analysis showed no differences in PARP cleavage and caspase 3 activation between the two cell lines (**Figure 12A).** Other drugs also displayed the same phenotype (**Figure 12B, C).** Similarly, no changes were observed in the activation status of JNK and p38 (**Figure 13**). Interestingly, higher levels of total and inactive ASK-1 (pS966) were observed in SLK-deficient cells, suggesting that the loss of SLK delays ASK-1 activation and degradation during cell death (**Figure 13)**. However, this had no effect on p38 activity, an ASK-1 target (Watanabe T., Sekine S., Naguro I., et al, 2015). One possibility is that ASK-1 de-phosphorylation at serine 966 affects its stability and might be SLK-dependent. Due to the

availability of an SLK-deficient Neu-expressing breast cancer line (Al Zahrani K., Sabourin L., 2018), we tested the role of SLK in apoptosis in the context of transformation. Wildtype and SLK-null NDL cell lines (Neu Deleted Line; Al Zahrani K., Sabourin L., 2018) were treated with Staurosporine for 48 hours and assessed for apoptotic markers. As for MEFs, SLK was also shown to breakdown in the NDLs. Along with PARP cleavage and p38 activation, this was markedly delayed (**Figure 14**), consistent with an apoptosis-resistant transformed phenotype. Overall, our data show that SLK deletion does not significantly affect cell death in normal or transformed cells. In conclusion, the results show that SLK is not required for proliferation, migration or apoptosis in MEFs that were genetically deleted for SLK. However, pFAK(Y397) levels differ post Nocodazole arrest which suggests a possible change in focal adhesion turnover.

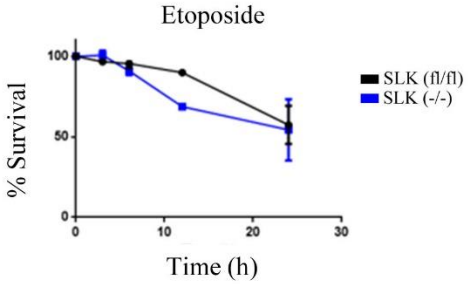
A.


Staurosporine (short)
% Survival
Time (h)
SLK (fl/fl)
SLK (-/-)

B.


Staurosporine (long)
% Survival
Time (h)
SLK (fl/fl)
SLK (-/-)

C.


Etoposide
% Survival
Time (h)
SLK (fl/fl)
SLK (-/-)

D.

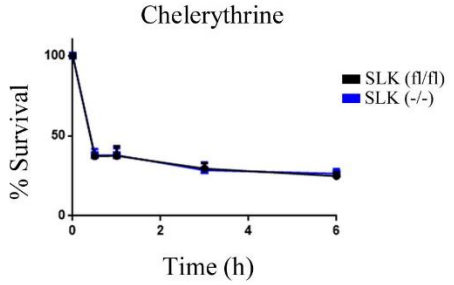

Chelerythrine
% Survival
Time (h)
SLK (fl/fl)
SLK (-/-)

Figure 11. SLK is not required for programmed cell death. Cultures of SLK-null and wildtype cells were exposed to 1 µM Staurosporine (A, B), 150 µM Etoposide (C) or 4 µM Chelerythrine (D) and cell viability was assessed using Alamar blue (5x). No difference in viability or Apoptosis rates were observed throughout the time courses between the two cell lines.

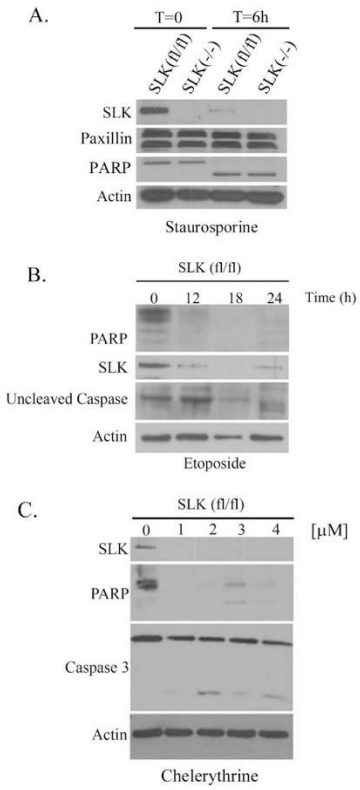

A.
T=0
T=6h
SLK(fl/fl)
SLK(-/-)
SLK(fl/fl)
SLK(-/-)
SLK
Paxillin
PARP
Actin
Staurosporine
B.
SLK (fl/fl)
0
12
18
24
Time (h)
PARP
SLK
Uncleaved Caspase
Actin
Etoposide
C.
SLK (fl/fl)
0
1
2
3
4
[μM]
SLK
PARP
Caspase 3
Actin
Chelerythrine

Figure 12. SLK deletion does not affect PARP breakdown or Caspase 3 activation. (A) MEFs were treated with 1 µM Staurosporine for 6 hours and assessed for SLK, PARP and Paxillin levels by Western blot analysis. **(B)** Western analysis for SLK, PARP and Caspase 3 following 150 µM Etoposide treatment **(C)** Western analysis for SLK, PARP and Caspase 3 following 4 µM Chelerythrine treatment. All treatments showed SLK and PARP breakdown as well as caspase 3 activation (cleaved caspase).

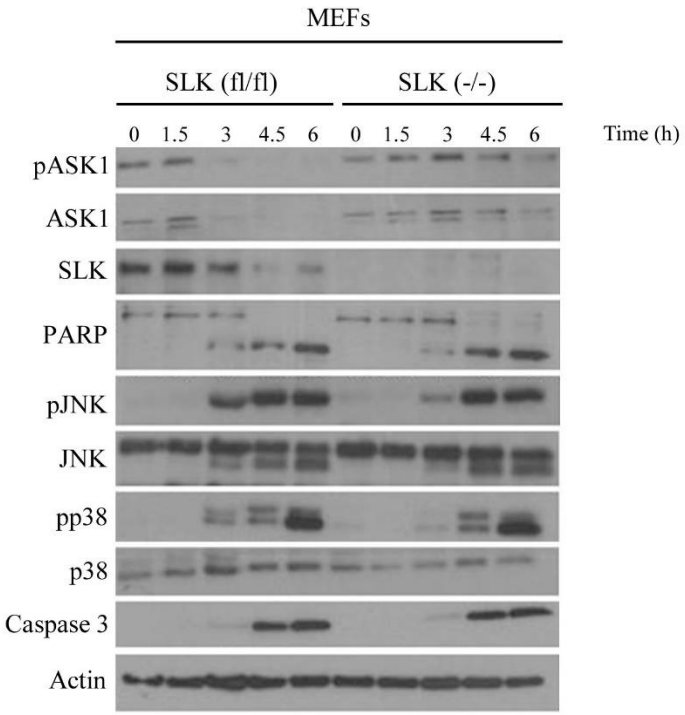

MEFs
SLK (fl/fl)
SLK (-/-)
0 1.5 3 4.5 6 0 1.5 3 4.5 6
Time (h)
pASK1
ASK1
SLK
PARP
pJNK
JNK
pp38
p38
Caspase 3
Actin

Figure 13. Apoptotic signaling in SLK-null cells. Staurosporine (1 μM) was used to induce apoptosis in MEFs over a time course of 6 hours. Protein extracts were analysed by Western blot for the activation status of various kinases implicated in cell death and stress response [pp38 (phospho-p38, Thr180 and Tyr182), ppJNK (phosphor-JNK, Thr183 Tyr185), pASK1 (phospho-ASK1 Ser966)]. Caspase 3 shows cleaved and active caspase 3.

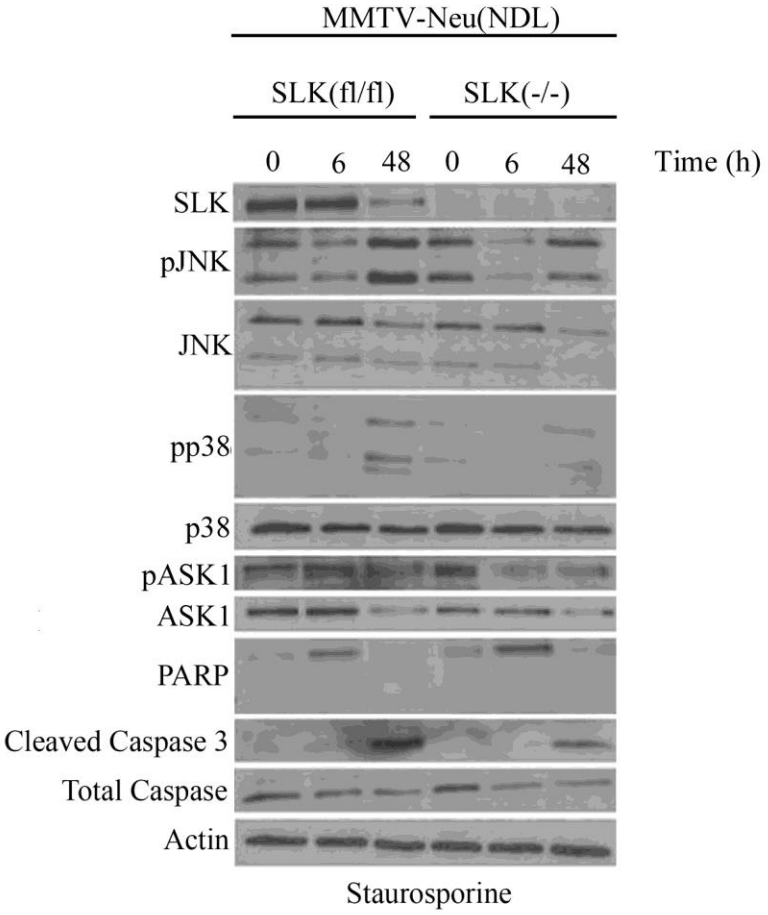

MMTV-Neu(NDL)
SLK(fl/fl)
SLK(-/-)
0 6 48 0 6 48
Time (h)
SLK
pJNK
JNK
pp38
p38
pASK1
ASK1
PARP
Cleaved Caspase 3
Total Caspase
Actin
Staurosporine

Figure 14. Apoptotic signaling in Neu-transformed SLK-null cells. Staurosporine (1 μM) was used to induce apoptosis in Neu-positive breast cancer cells (NDL; established by Dr. Khalid Al-Zahrani) over 48 hours. Protein extracts were analysed by Western blot for the activation status of various kinases implicated in cell death and stress response [pp38 (phospho-p38, Thr180 and Tyr182), ppJNK (phosphor-JNK, Thr183 and Tyr185), pASK1 (phospho-ASK1 Ser966)].

4. Discussion

To assess the role of SLK in various biological processes we have analyzed the growth potential, motility and apoptotic response of SLK-deficient MEFs. In contrast to previous observations using acute knock down, our data show that the genetic deletion of SLK in MEFs does not affect proliferation or susceptibility to apoptotic triggers. Although motility was unaffected, differences in focal adhesion turnover were observed, suggesting that compensatory mechanisms have been activated in SLK-null cells.

4.1 SLK is not required for the growth of Mouse Embryonic Fibroblasts in vitro

Our lab has previously shown that SLK co-localizes with the mitotic spindle during M phase and is required for progression through G2/M (O'Reilly PG, Wagner S, Franks DJ et al., 2005). SLK was also shown to phosphorylate and activate PLK1 during mitosis (Ellinger-Ziegelbauer H, Karasuyama H, Yamada E et al, 2000). Expression of a kinase dead SLK results in a G2/M arrest in fibroblasts and sustained expression of cyclin A, suggesting that SLK is required for cell cycle progression. Supporting this, MEFs expressing an SLK gene-trap (SLK truncation), which involved removing the ATH domain (known for regulating SLK kinase activity) (Sabourin, Tamai, Seale et al., 2000), displayed reduced growth potential when compared to wildtype MEFs (Al-Zahrani, K. N., Sekhon, P., Tessier et al, 2014). Using viable cell counts, we did not observe any differences in the growth rate of SLK-deficient cells (**Figure 5)**. Similarly, flow cytometry and Nocodazole release studies did not show any delay in the G2/M transition for the SLK-null cells (**Figure 6A)**. Supporting this, western blotting showed similar levels of cyclin A expression following release (**Figure 6B)**. Together, our data suggests that SLK is not required for proliferation in MEFs in vitro. For future experiments, it would be better to increase the time-course to see whether the cells require a longer period of time to exit

Nocodazole arrest. This is in stark contrast to previous data using kinase inactive SLK (O'Reilly PG, Wagner S, Franks DJ et al., 2005). One particular study could potentially explain these differences (Hall EA, Keighren M, Ford MJ et al. 2013). It focuses on a gene known as 5-azacytidine induced gene 1 (Azi1)/Cep131 that plays a role in ciliogenesis. Azi1/Cep131 was shown through siRNA knockdown and genetic deletion, in the context of MEFs, to have different phenotypes. In siRNA knockdown, cilia formation was reported to be reduced (Graser et al., 2007; Hall.E, Keighren M., Ford M. et al, 2013). Although male mice were shown to be infertile, a genetic deletion of Azi1 showed that it was not absolutely necessary since the phenotype of cilia formation was unaffected. This is likely due to the activation of compensatory mechanisms during development in the context of a genetic deletion (Graser et al., 2007; Hall.E, Keighren M., Ford M. et al, 2013). In a similar way, an SLK siRNA knockdown shows reduced cell proliferation, however the deletion of SLK had no effect. One possibility is that alternative pathways have been activated, rescuing the proliferation phenotype. An acute siRNA knockdown could also not give the cells enough time to adapt or compensate in this context. As shown previously, a kinase mutant version of SLK has been shown to be embryonic lethal (Al-Zahrani, K. N., Sekhon, P., Tessier et al, 2014). Therefore, the genetic deletion of SLK and expansion of those cells could have also selected for mutants that can grow without SLK.

4.2 SLK is not required for the migration of Mouse Embryonic Fibroblasts

Previous knockdown studies in mouse fibroblasts showed that SLK is an important regulator of cell migration (Quizi, 2013). SLK has been shown to promote focal adhesion turnover by directly phosphorylating Paxillin at serine 250. A Paxillin mutant at the S250 SLK phosphorylation site (S250A) showed a two-fold decrease in migration rates. Surprisingly, in

MEFs with a genetic deletion of SLK, no effect on cell migration was observed using scratch wounding assays or Boyden chambers **(Figure 7A, B)**. FAK and Paxillin were also recruited to the leading edge of the wound despite the absence of SLK expression **(Figure 9A, B, 10)**, suggesting that SLK is not required for the redistribution of other migration components. Knock out of the SLK-related kinase MST1 also showed no impairment of migration in plasma cells (Bagherzadeh Y.S, Witalis M, Meli AP, 2019). This similar phenotype could be due to a compensatory mechanism that is activated early during development, resulting in the absence of an observable phenotype. However, in an acute knock down model, overt phenotypes might be observed due to the lack of compensation, leading to impaired motility (Shmidt T, Hampich F, Ridders M, 2007; Barabási AL, Oltvai ZN, 2004). There are several ways that the cell could potentially compensate for the loss of SLK (El-Brolosy MA, Stainier DYR, 2017). One possibility is that other related kinases that perform a similar function to SLK may have been upregulated. This is illustrated when a genetic knockout of Rpl22, a ribosomal protein involved in translation, caused an enhanced expression of Rpl2211 with no obvious translation problems but subtle phenotypes (O'Leary MN, Schreiber KH, Zhang Y, 2013).

As shown previously, SLK is activated downstream of the activated FAK/Src complex in the context of scratch-wound induced migration (Wagner et al., 2008). SLK recruitment to the leading edge of migrating cells also requires FAK/Src activity and the SLK-binding protein LMO4 (Baron et al., 2015). SLK can then directly phosphorylate Paxillin and promote focal adhesion turnover (Quizi et al., 2013). Nocodazole treatment and release induces synchronized focal adhesion turnover (Kaverina I., Klemens R.J., J. Victor Small, 1998). This is accompanied by a loss of pFAK-Y397, a marker of focal adhesion breakdown (Wagner et al., 2008). Nocodazole release studies showed a faster disappearance of pFAK-Y397 in SLK-null cells,

suggesting a more rapid turnover (**Figure 8A).** Surprisingly the motility rates were similar. We have previously shown that SLK phosphorylates Paxillin on serine 250, stimulating FAK-dependent focal adhesion turnover (Quizi et al., 2013). Since SLK can directly phosphorylate Paxillin, the rapid reduction of pFAK-Y397 could be in response to the loss of Paxillin S250 phosphorylation, resulting in increased dynamics of the pFAK-Y397. This is counter-intuitive and different from what has been observed in siSLK-transfected cells. One possibility is that the knockdown MEFs could not rapidly compensate for the downregulation of SLK while the knockout MEFs have activated compensatory pathways (El-Brolosy MA, Stainier DYR, 2017). It is possible that those pathways can effectively induce turnover independently of Paxillin S250 phosphorylation. Alternatively, the SLK-related kinase LOK could compensate for Paxillin S250 phosphorylation. This has been observed for other GCK members (Miller, C. J., Lou, H. J., Simpson, C., 2019). Another possible explanation could be the heterogeneity of the MEFs isolated and immortalized for this project compared to one of the standard cells lines (MEF 3T3) used previously (Singhal P., Sassi S., Lan L. et al, 2015).

4.3 The Apoptotic Response is not affected in SLK-deficient MEFs

When SLK was overexpressed in C2C12 myoblasts, apoptosis was trigged and JNK1 was activated (Sabourin LA, Rudnicki MA, 1999; Yan Y, Tulasne D, Browaeys E, 2007; Al-Zahrani, KN., Baron KD, and Sabourin LA, 2013). SLK was also shown to be cleaved by capase-3 during apoptosis, releasing the active kinase domain and the ATH region. Other studies have shown that SLK could activate the p38 MAPK pathway through ASK1 (Cybulsky AV, Takano T, Guillemette J et al., 2009; Al-Zahrani, KN., Baron KD, and Sabourin LA, 2013). SLK's role was studied in ischemia-reperfusion within kidney cells and was shown to affect ASK1 pathway and

increase the ER-Stress response (Hao W, Takono T, Guillemette J et al., 2006). To test the effect of SLK deletion on the apoptotic response, Staurosporine, Chelerythrine and etoposide were used on both MEF lines to trigger cell death. In contrast to knockdown studies, there were no differences in the rates of cell death between the two cell lines, suggesting that SLK deletion does not impair apoptosis in MEFs **(Figure 11)**. Similar findings have been reported in several other models (El-Brolosy MA, Stainier DYR, 2017). As above, there could be an alternative pathway that compensates for the genetic loss of SLK. There could also be another regulator that needed to be deleted, along with SLK, to notice an observable phenotype. An example of this hypothesis is illustrated when HEK293A cells were CRISPR deleted either with MST1/2 or MAK4K4/6/7 but only showed partly reduction of LATS1/2 in the Hippo pathway (Meng Z., Moroishi T., Mottier-Pavie V. et al., 2015). The Hippo Pathway plays a role in regulating tissue state, the final role a cell plays and how big an organ will be (Meng Z., Moroishi T., Mottier-Pavie V. et al., 2015). When both MST1/2 and MAK4K4/6/7 were deleted, phosphorylation of LATS1/2 and YAP/TAZ were abolished (Meng Z., Moroishi T., Mottier-Pavie V. et al., 2015). Their results show that there was a redundant role between MST1/2 and MAK4K4/6/7 in the phosphorylation of LATS1/2 and YAP/TAZ. Therefore, one possibility is that the genetic deletion of SLK along with another unknown gene could result in a more obvious phenotype as there could be parallel processing in this context (Sabourin LA, Rudnicki MA, 1999; Yan Y, Tulasne D, Browaeys E, 2007; Cybulsky AV, Takano T, Guillemette J et al., 2009; Al-Zahrani, KN., Baron KD, and Sabourin LA, 2013; Meng Z., Moroishi T., Mottier-Pavie V. et al., 2015). Similarly, if SLK is negative regulator of specific pathways, their sustained activation in the absence of SLK could lead to cross-talk and the induction of compensatory mechanisms. Alternatively, SLK function is dispensable in MEFs but critical in other cell types. The

embryonic nature of MEFs might not make them dependent on SLK and differentiation or maturation is required for SLK to take over the functions of a more embryonic protein.

4.4 Future Directions

Here, we have shown that the deletion of SLK in conditional MEFs did not affect growth, motility or apoptosis. The knockdown of SLK through siRNA was previously shown to impair all those processes. It is then likely that a genetic deletion (and expansion in culture) rather than an acute downregulation through siRNA has led to the activation of compensatory pathways. It would then be of interest to screen our cell lines for differentially expressed genes using arrays or RNAseq. Mass spectrometry could also be used to identify global changes in phosphorylated peptides in an effort to identify compensatory pathways. Furthermore, it would be helpful to identify more interacting partners and substrates through immunoprecipitation, mass spectrometry and global kinase assays. Candidate proteins could be knocked down or re-expressed in a genetically deleted SLK context in an attempt to identify proteins or pathways that can compensate for the loss of SLK. Those tagged proteins could also be tested in MEFs that express the gene trap version of SLK (obtained by mating SLK heterozygous mice due to its lethality). A focus for candidates involved in growth, motility and cell death could unravel SLK compensatory pathways in different contexts.

4.5 Physiological Relevance

Due to its potential role in growth and development, understanding the many functions of SLK is of major interest (Yi-Hong Zhang, Karri Hume, Robert Cadonic, et al., 2002). Specifically, SLK has been shown to play a role in key processes such as proliferation, apoptosis and migration in several contexts (Al-Zahrani, KN., Baron KD, and Sabourin LA, 2013). Currently, several SLK-deficient cell lines have been generated in the Sabourin lab, providing an opportunity to test the role of SLK in several contexts such as neuronal and muscle development (Yi-Hong Zhang, Karri Hume, Robert Cadonic, et al., 2002). We have shown that a genetic deletion of SLK affects focal adhesion dynamics in MEFs. However, whether this is the case in other cellular contexts remains to be investigated. Movement, morphogenesis, ECM interaction and cell division are all important processes that require focal adhesion signaling (K. Chinthalapudi, E. S. Rangarajan, Tina Izard, 2018). Therefore, a better understanding of the SLK-dependent pathways may unravel new therapeutic targets for the modulation of those processes in disease contexts.

5. Conclusion

Current literature has mentioned potential compensation mechanisms that occur with genetic knockouts when compared to transient knockdowns as a way to explain the observed phenotypes (Liang H., Wen-Hsiung Li., 2009; El-Brolosy MA, Stainier DYR, 2017). In this project, in contrast to siRNA knockdowns, the genetic deletion of SLK did not affect cell growth, motility or cell death. This is likely due to the activation of alternate pathways in SLK-deficient cells. SLK has been identified more than 20 years ago and several studies have highlighted its

importance in many cell types. Due to recent advances in CRISPR technology, SLK can now be genetically deleted in several cellular contexts. The existence of multiple cell lines and in vitro models makes it possible to identify specific pathways that involve SLK. The discovery of those pathways might lead to the identification of disease genes linked to SLK signaling. The development of SLK inhibitors or modulators could then useful in the treatment of developmental defects and cancer.

6. Appendix I – Validate the putative interaction between SLK and RASSF1A

This project was pursued due to the possibility of RASSF1A being a binding or activating partner of SLK due to their interplay in similar processes such as proliferation, migration and apoptosis. Both RASSF1A and SLK have a domain known as the SARAH domain which plays a role in apoptosis. It was thought that these proteins might potentially bind through their SARAH domains, however there was only binding in the binding assay but not within the Melanoma Cell lines. There might be a specific event that could cause these proteins to interact however it is currently unknown.

6.1 Introduction to Melanoma

According to the Canadian Cancer Society's Advisory, approximately 7640 people will be diagnosed with melanoma in 2019 (Canadian Cancer Society, 2017). Melanomas generally occur through genetic mutations in pigment producing cells, the melanocytes (Sullivan, R. J., 2015). Melanocyte development is regulated by a multitude of transcription factors and receptors, of which SOX10, MITF and the receptor tyrosine kinase c-KIT are the most important (Tudrej K., Czepielewska E., Wojciechowska M.K., 2016). Melanoma initiation and progression can be affected by genetic and environmental factors (Sullivan, R. J., 2015). In hereditary melanoma, mutations in genes that modulate the cell cycle such as cyclin-dependent kinase inhibitor 2A (CDKN2A) and cyclin-dependent kinase 4 (CDK4) are quite frequent (Sullivan, R. J., 2015). Environmental factors play a role and mostly consists in excessive sun exposure. There are two particular pathways that are commonly affected in melanoma, which are the MAPK and PI3K pathways (Sullivan, R. J., 2015). In melanomas, mutations in the BRAF proto-oncogene (the v-raf murine sarcoma viral oncogene homolog B1) is observed in 50-60% of cases (Perna D., Karreth F., Rust A. et al., 2014). BRAF is a serine/threonine kinase that is highly expressed

in melanocytes (Sullivan, R. J., 2015). In a normal melanocyte, mitogen-activated protein kinase (MAPK) signaling only occurs when there is a ligand interaction with cell membrane receptors and signaling occurs following the activation of those receptors (e.g. epidermal growth factor receptor (EGFR), Platelet derived growth factor receptor (PDGFR)) (Sullivan, R. J., 2015). MAPK signaling activates the RAS family of G proteins that can then activate the BRAF kinase (Sullivan, R. J., 2015). BRAF can activate the MEK-ERK cascade through direct phosphorylation of MEK. ERK then phosphorylates several other downstream targets critical for cell growth and survival (Sullivan, R. J., 2015). For example, one target that ERK phosphorylates is ELK-1 which can then bind to DNA and drive transcription (Sullivan, R. J., 2015). In malignant melanomas bearing BRAF mutations, the MAPK signaling pathway is constitutively activated (Sullivan, R. J., 2015). About 90% of BRAF mutations consist in a valine to glutamic acid change at codon 600 within exon 15 (V600E). This causes the kinase domain to remain in an active state. Two other proteins are frequently mutated with BRAF in melanomas: phosphatase and tensin homolog (PTEN) and CDKN2A. In 50-60% of melanomas, PTEN either has point mutations, small or complete deletions which affects its ability to negatively regulate phosphoinositide (PI) 3-Kinase (PI3K) (Sullivan, R. J., 2015). This leads to increased AKT activity, promoting growth and survival (Hemmings. B, Restuccia D., 2012). CDKN2A encodes for two tumor suppressor proteins, $p16^{INK4a}$ and $p14^{ARF}$, that are involved in the retinoblastoma (Rb) and p53 pathways, respectively (Sullivan, R. J., 2015). CDKN2A is usually inactivated by point mutations in melanoma, leading to a loss of gatekeeper function (inducing cell cycle arrest) of the RB or p53 pathway (Sullivan, R. J., 2015).

6.1.1 The RASSF1A tumor suppressor

Before the year 2000, the 3p21.3 gene region was hypothesized to have potential tumor suppressor activity as many lung cancer mutations mapped to this region. Those cancers also displayed loss of heterozygosity, a hallmark of tumor suppressor mutations (Donninger H., Vos M., Clark G., 2007). The RASSF1A gene was later mapped to 3p21.3 region and shown to contain a domain known as the RAS association (RA) domain (Donninger H., Vos M., Clark G., 2007). Two isoforms are encoded by the RASSF1: RASSF1A and RASSF1C (Donninger H., Vos M., Clark G., 2007). RASSF1A was shown to be inactivated in many tumors due to its promoter region being methylated (Donninger H., Vos M., Clark G., 2007). It human cancer, RASSF1A is inactivated at a high frequency (Donninger H., Vos M., Clark G., 2007).

Hypermethylation of the RASSF1A promoter CpG islands causes reduced expression of RASSF1A mRNA and protein levels in melanoma cells and tumors (Gordon, M., Baksh, S., 2011; Yi M., Yang J., Chen X., et al., 2012). As expected, the RASSF1A isoform of the Rassf1 gene was shown to have tumor suppressor activity (Oh, H. J., 2006). RASSF1A is 340 amino acids long and has several different domains, including an SH3 binding domain, a zinc Finger Domain, a RAS Association Domain and a SARAH (Sav/Rassf/Hpo) domain (Oh, H. J., 2006).

RASSF1A has been shown to also play a role in apoptosis and cell cycle control in melanoma (Gordon, M., Baksh, S., 2011). During programmed cell death of A375 melanoma cells, RASSF1A can activate ASK1 which then targets JNK and p38 MAP kinase (Gordon, M., Baksh, S., 2011). This will eventually lead to the activation of caspase 3 and apoptosis (Gordon, M., Baksh, S., 2011). In the context of melanoma, RASSF1A overexpression has been show to block cell cycle transition from G1-S (Oh, H. J., 2006). RASSF1A prevents phospho-RB and cyclin D1 proteins from building up within the cell, preventing S phase entry (Oh, H. J., 2006).

RASSF1A has been shown to also modulate MST1 in apoptosis (Bitra A., Sistla S., Mariam J., 2017) through interactions between its SARAH domain and MST1 (Canadian Cancer Society, 2017). This is required for the apoptotic functions of RASSF1A. This supports numerous studies highlighting the disruption apoptosis in melanoma (Broussard L., Howland A., Ryu S. et al., 2018).

6.2 Purpose of Research

6.2.1 Rationale and Hypothesis

A putative interaction between SLK and RASSF1A was identified by immunoprecipitation and mass spectrometry. Extensive literature has highlighted the role of RASSF1A in tumor progression, metastasis and cell death in the context of melanoma. (Gordon, M., Baksh, S., 2011, Oh, H. J., Lee, K. K., Song, S. J. et al, 2006). As SLK plays a potential role in migration and tumor cell signaling (Al-Zahrani, PhD Thesis, 2018), we tested the potential of a direct interaction between RASSF1A and SLK. As SLK knockdown impairs growth and migration, our initial hypothesis was that RASSF1A could regulate SLK kinase activity through direct interaction.

6.2.2 Objective(s)

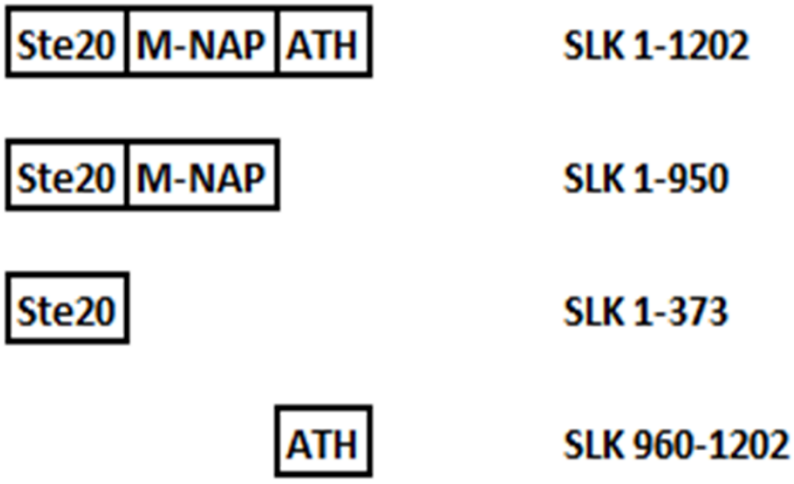
- To determine if RASSF1A binds directly to SLK *in vitro*.
- To assess complex formation between SLK and RASSF1A in melanoma cells.

6.3 Results

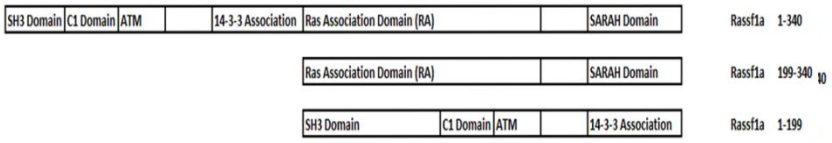
6.3.1 SLK does not interact with RASSF1A in a specific manner

RASSF1A has been shown to also play a role in apoptosis and cell cycle control in melanoma (Gordon, M., Baksh, S., 2011). In this context, RASSF1A overexpression has been show to block cell cycle transition from G1 to S (Oh, H. J., 2006). The identification of RASSF1A in SLK immunoprecipitates suggests that it binds SLK or that they are part of a complex. To test a potential direct interaction, both proteins were in vitro translated in the presence of 35S-methionine and subjected to in vitro binding assays with GST fusion proteins of the other partner. As seen in **Figure 15A**, all the domains of RASSF1A were capable of interacting with in vitro translated full length Myc-SLK. No binding was observed to a GST control, suggesting that the binding to RASSF1A fragments is not to the GST moiety but occurs through non-specific binding. Similarly, HA-RASSF1A bound to all SLK GST fusions, again supporting that the interactions are non-specific (Figure 15B). To further investigate this, SLK was immunoprecipitated from melanoma cell lines that were wildtype, deficient or re-expressed RASSF1A (**Figure 16)**. Immunoprecipitation for SLK and blot back for RASSF1A showed no RASSF1A-specific signal when compared to a control lysate, suggesting that they do not form a complex in vivo (**Figure 16A)**. Together these results suggest that the in vitro interactions are non-specific and that SLK and RASSF1A are unlikely to interact in vivo. One possibility is that the "sticky" and disorganized nature of the RASSF1A protein induces a high degree of non-specificity in vitro. As these proteins failed to interact, the project was abandoned.

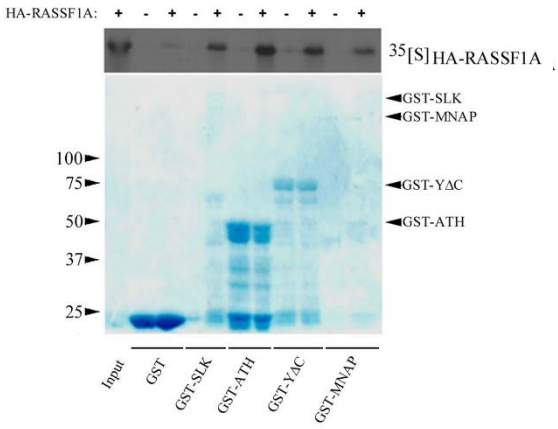
A.


Ste20
M-NAP
ATH
SLK 1-1202
Ste20
M-NAP
SLK 1-950
Ste20
SLK 1-373
ATH
SLK 960-1202

B.


SH3 Domain
C1 Domain
ATM
14-3-3 Association
Ras Association Domain (RA)
SARAH Domain
Rassf1a 1-340
Ras Association Domain (RA)
SARAH Domain
Rassf1a 199-340
SH3 Domain
C1 Domain
ATM
14-3-3 Association
Rassf1a 1-199

C.


HA-RASSF1A: + - + - + - + - + - +
35[S] HA-RASSF1A
GST-SLK
GST-MNAP
GST-YΔC
GST-ATH
100
75
50
37
25
Input
GST
GST-SLK
GST-ATH
GST-YΔC
GST-MNAP

D.

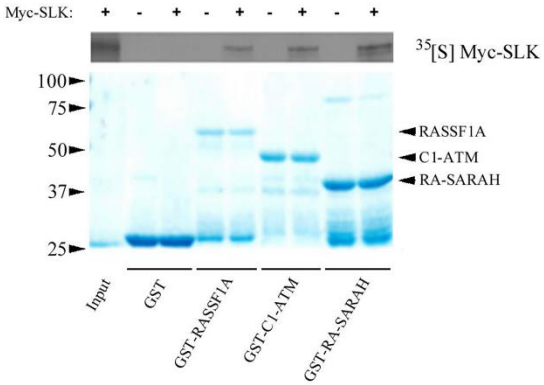
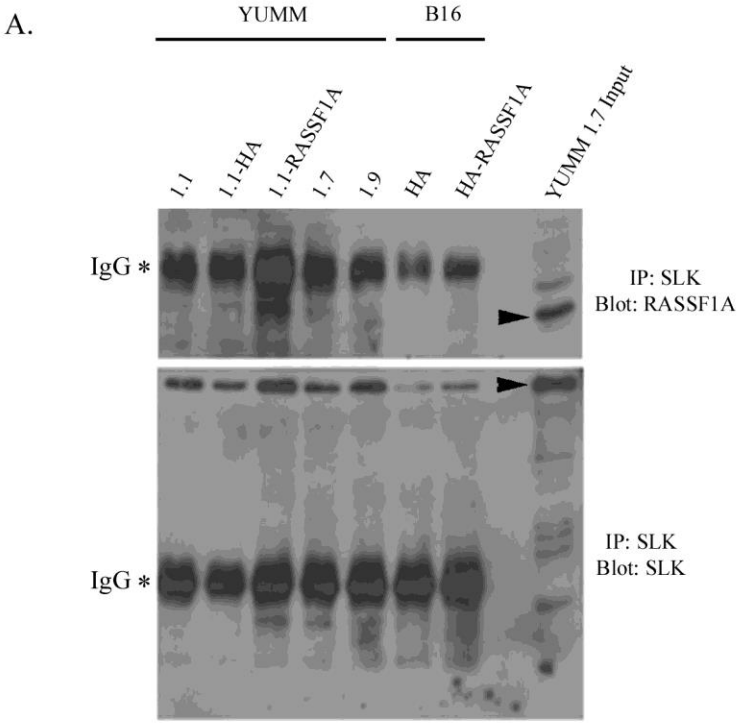

Myc-SLK: + - + - + - + - +
35[S] Myc-SLK
RASSF1A
C1-ATM
RA-SARAH
100
75
50
37
25
Input
GST
GST-RASSF1A
GST-C1-ATM
GST-RA-SARAH

Figure 15. The SLK-RASSF1A interaction is non-specific. (A) GST-SLK constructs used for In vitro binding assays (Quizi JL, Baron K, Al-Zahrani KN, 2013). (B) GST-RASSF1A constructs used for In-vitro binding assays (Gordon, M., Baksh, S., 2011). Numbers represent amino acid positions. In vitro binding assays were conducted with in vitro translated Myc-SLK (C) or HA-RASSF1A (D). The indicated immobilized GST-fusions were prepared in bacteria and incubated with the input amount of 35S-labelled protein at room temperature. Interaction was visualized by running the binding assays on SDS gels and exposure to X-ray film (n=3).


A.
YUMM
B16
1.1
1.1-HA
1.1-RASSF1A
1.7
1.9
HA
HA-RASSF1A
YUMM 1.7 Input
IgG *
IP: SLK
Blot: RASSF1A
IgG *
IP: SLK
Blot: SLK

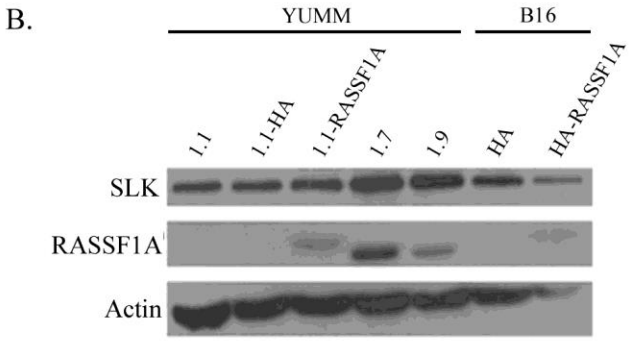

B.
YUMM
B16
1.1
1.1-HA
1.1-RASSF1A
1.7
1.9
HA
HA-RASSF1A
SLK
RASSF1A
Actin

Figure 16. RASSF1A and SLK do not interact in vivo. (A) SLK was immunoprecipitated from various melanoma cell lines (YUMM and B16) expressing different levels of RASSF1A or re-expressing RASSF1A (HA-RASSF1A). Immune complexes were subjected to Western blot for SLK. No co-immunoprecipitation was observed. (B) Western blot showing the levels of RASSF1A and SLK in the lines used in panel (A) (n=3).

6.4 Discussion

RASSF1A is frequently deleted in melanoma, suggesting that it is a critical tumor suppressor (Gordon, M., Baksh, S., 2011). Its overexpression also suppresses melanoma growth by blocking the G1 to S transition (Oh, H. J., 2006). SLK has a variety of roles different cellular processes including cell migration, changes of the cytoskeleton and cell death (Wagner et al., 2002; Hao W, Takano T, Guillemette J et al., 2006; Quizi JL, Baron K, Al-Zahrani KN, 2013). My data show that SLK and RASSF1A interact in vitro in a non-specific manner. Supporting this, no interaction was observed in vivo. The non-specific interactions could be due to the isolated system in which the binding assay is conducted. Those conditions do not contain exogenous proteins that could be required for specific interactions or to mediate structural changes in SLK or RASSF1A. Alternatively, their association only occurs under specific conditions or in distinct cell types. One possibility is that this interaction occurs during cell migration or cell death. This could be tested under conditions that stimulate those processes. In addition, the interactions could be transient and difficult to detect. It is also possible that this interaction is lost in melanomas. This could perhaps lead to increased SLK activity and enhanced migration and metastasis. Further studies will be required to test these various possibilities.

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