The role of LMO4 in the regulation of SLK localization & activation within migrating cells and in murine mammary tumorigenesis

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Ancora imparo.

<I am still learning.>

~ Michelangelo, Aged 87 years

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Dedication:

I dedicate this PhD thesis to the memory of my late father John (Giovanni) Sergio Garbuio (1950 – 2013). ALS cruelly robbed him of being able to share in this, my greatest achievement and the trauma of his suffering almost robbed me of the ability to take advantage of this opportunity. He came from Italy with his family with nothing and made a wonderful life for my mother, brother and I. He was the quietest yet biggest cheerleader of my academic and personal goals. Dedicating this to him is the least I could do to repay him. I wish you were here, Poppo!

Abstract:

The Ste20-like kinase SLK plays a pivotal role in cell migration and focal adhesion turnover. SLK activity is regulated by the LIM domain-binding proteins Ldb1/2. In addition to playing role in tumor initiation and progression, these proteins have been demonstrated to interact with LMO4. Therefore, this project assessed the ability of LMO4 to interact and regulate SLK activity. Results show that LMO4 can directly bind to SLK and activate its kinase activity. LMO4 can be co-precipitated with SLK following the induction of cell migration by scratch wounding. Cre deletion of LMO4 inhibits cell migration and SLK activation, and impairs Ldb1 and SLK recruitment to the leading edge of migrating cells. Src/Yes/Fyn-deficient cells (SYF) express very low levels of LMO4 and do not recruit SLK to the leading edge. Src-family kinase inhibition impairs SLK recruitment to the leading edge, suggesting that both expression of LMO4 and the recruitment of SLK to the leading edge require c-Src activity. In conclusion, cell migration and activation of SLK requires its recruitment to the leading edge by LMO4 in a Src-dependent manner. This study also investigated whether LMO4 deletion through MMTV-Cre-driven excision would impair mammary tumorigenesis in a PyMT mouse model of breast cancer. No difference in Overall Survival was observed between animals with and without LMO4 expression. Western blot analysis and IHC showed that tumors expressed LMO4 protein in animals genotyped as Cre-positive. This result suggests that expression of LMO4 is required for tumor initiation in the PyMT model of murine mammary carcinoma. This project has established a novel cytosolic role for the transcriptional co-activator LMO4 and validated it's involvement in the regulation of SLK and cell migration. This pathway may provide a novel therapeutic strategy as LMO4 appears to be critical to the initiation and progression of breast cancer.

***Please note:** Permission has been obtained from the applicable parties for the inclusion of material and figures in this thesis from the following two peer-reviewed published articles (for copies of the License Agreements, please refer to the Appendices section of this thesis):

1. Baron, K. D., K. Al-Zahrani, et al. (2015). "Recruitment and activation of SLK at the leading edge of migrating cells requires Src family kinase activity and the LIM-only protein 4." Biochim Biophys Acta **1853**(7): 1683-1692. (Elsevier Limited)

2. Al-Zahrani, K. N.*, K. D. Baron*, and L. A. Sabourin. (2013). "Ste20-like kinase SLK, at the crossroads: a matter of life and death." Cell Adh Migr **7**(1): 1-10. (The Taylor & Francis Group) *These authors contributed equally to this work.

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List of Abbreviations:

1. aa: amino acid
2. AdCre: adenovirus expressing Cre recombinase
3. AdLacZ: adenovirus expressing LacZ
4. ASK1: apoptosis signal-regulating kinase-1
5. ATH: AT1-46 homology
6. AT_2R: angiotensin II type 2 receptor
7. BSA: bovine serum albumin
8. Ci: Curie
9. DAB: 3, 3'-diaminobenzidine
10. DAPI: 4, 6-diamidino-2-phenylindole
11. DMEM: Dulbecco's modified Eagle's medium
12. DTT: dithiothreitol
13. E: Glutamic acid
14. EDTA: ethylenediaminetetraacetic acid
15. EGFR: epidermal growth factor receptor
16. EMT: epithelial-to-mesenchymal transition
17. ER: endoplasmic reticulum
18. F: phenylalanine
19. FA: focal adhesion
20. FAK: focal adhesion kinase
21. FBS: fetal bovine serum
22. G: glycine
23. GAPDH: glyceraldehyde 3-phosphate dehydrogenase
24. GCK: germinal centre kinase

25. GST: glutathione-S-transferase
26. HEK: human epithelial kidney
27. IHC: immunohistochemistry
28. IP: immunopreciptated
29. JNK: c-Jun N-terminal kinase-1
30. K: lysine
31. K63R SLK: SLK mutant unable to autophosphorylate
32. kDa: kilo-Dalton
33. LBD: LIM binding domain
34. Ldb: LIM domain binding transcriptional cofactor protein
35. LMO4: LIM-only protein 4
36. $LMO4^{fl/fl}$: LMO4 homozygous floxed
37. LMO4 Δ1: LMO4 construct with the LIM1 domain deleted
38. LMO4 Δ2: LMO4 construct with the LIM2 domain deleted
39. LOK: lymphocyte oriented kinase
40. MAPK: mitogen-activated protein kinase
41. MEF: murine embryonic fibroblast
42. MKK: MAPK kinase
43. MMTV: mouse mammary tumor virus
44. MST1: mammalian sterile twenty kinase
45. NLS: nuclear localization signal
46. OS: overall survival
47. P: proline
48. PAGE: polyacrylamide gel electrophoresis
49. PFA: paraformaldehyde
50. PI3K: phosphatidylinositol 3-kinase

51. Plk1: polo-like kinase homologue
52. PTPα: protein-tyrosine phosphatase-α
53. pTyr: phosphor-tyrosine
54. PVDF: polyvinylidene difluoride
55. Pxn: paxillin
56. PyMT: polyoma virus middle T oncoprotein
57. Q: glutamine
58. QUAD LMO4: quadruple LIM1 domain mutant - R33G/F34Q/L35Y/Y37Q
59. R: arginine
60. S: serine
61. SDS: sodium dodecyl sulfate
62. SH3: Src homology 3
63. siRNA: short-interfering RNA
64. SLK: Ste20-like kinase
65. SYF: c-Src, Yes & Fyn – deficient cells
66. T: threonine
67. TGFβ: tumor growth factor-β
68. TNF-α: tumor necrosis factor-α
69. V: valine
70. VO3: sodium orthovanadate
71. WCL: whole cell lysate
72. xPlkk1: *Xenopus* polo-like kinase kinase
73. Y: tyrosine

Chapter 1:

General Introduction

1. General Introduction:

1.1 Cell Migration and Cancer

Cell migration is essential to the process of metastasis involving the spread of a primary tumor to distant sites and is the cause of 90% of cancer-related deaths (Horwitz and Parsons 1999; Christofori 2006). Briefly, the process of cell motility involves assembly and turnover of focal adhesions (FA) regulated by tyrosine phosphorylation events by the focal adhesion kinase (FAK), structural proteins and the c-Src proto-oncogene (Burridge and Chrzanowska-Wodnicka 1996; Giancotti and Ruoslahti 1999; Schlaepfer, Hauck et al. 1999). Adhesion turnover is required for intracellular cytoskeletal dynamics and protrusive forces necessary to propel the cell in a specific direction (Amano, Mukai et al. 1996; Leung, Chen et al. 1996; Amano, Chihara et al. 1997; Ishizaki, Naito et al. 1997; Manser, Huang et al. 1997; Van Aelst and D'Souza-Schorey 1997; Watanabe, Madaule et al. 1997; Leung, Chen et al. 1998; Nobes and Hall 1999). Overexpression, increased activity or activating mutations in c-Src and FAK have been found in various forms of cancer including breast (Wiener, Nakano et al. 1999), ovary (Verbeek, Vroom et al. 1996), and pancreas (Lutz, Esser et al. 1998). In addition, increased cell motility has been reported in various forms of cancer (Schlaepfer, Hauck et al. 1999; Frame 2004; Playford and Schaller 2004; Mitra and Schlaepfer 2006).

The regulation of cell migration has been implicated in breast cancer through the discovery that the ErbB2/HER2/Neu receptor tyrosine kinase signalling pathway is required for breast carcinoma cell motility (Marone, Hess et al. 2004). Advanced tumors have acquired the ability to invade surrounding tissues and migrate throughout the vasculature to colonize distant sites within the body (Hanahan and Weinberg 2000). The process of metastasis is highly dependent on an active migration system and cross-talk

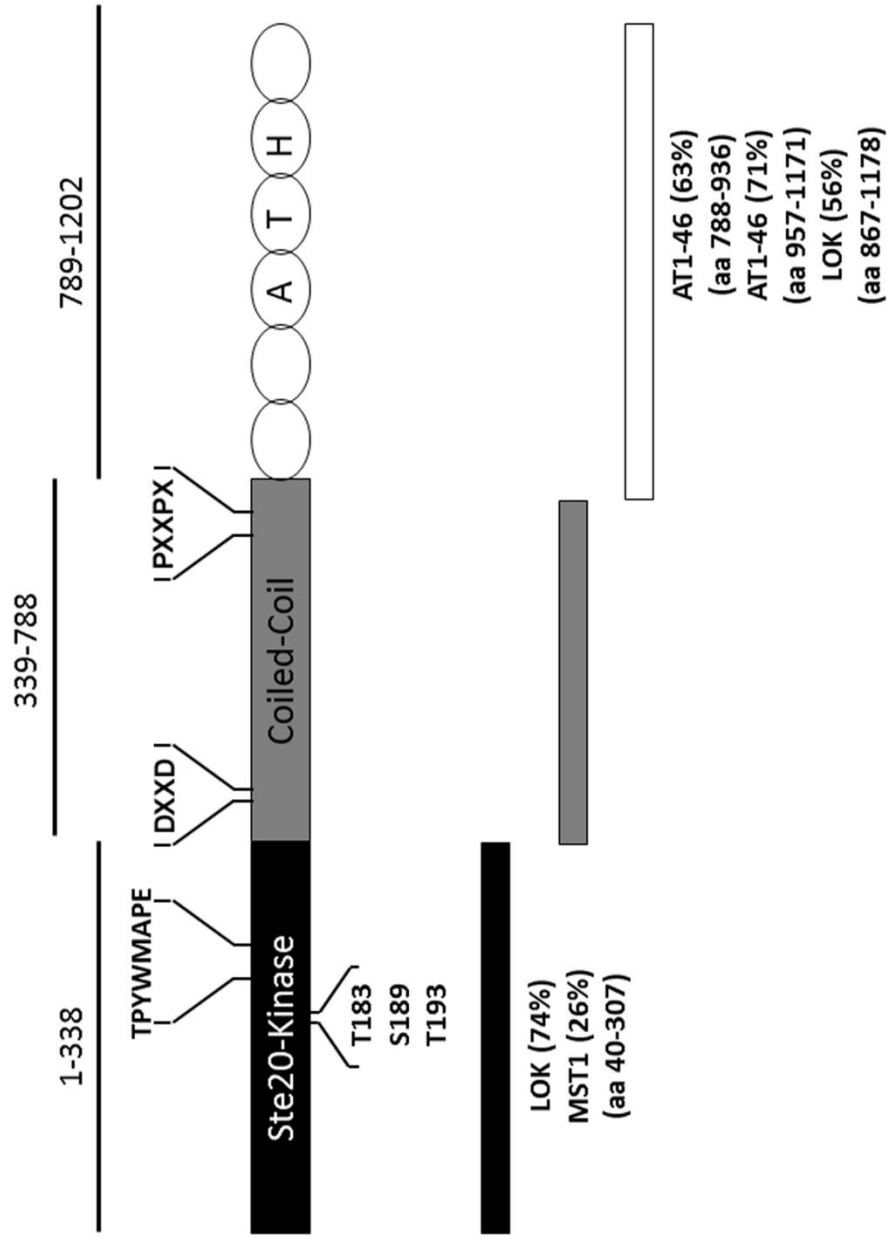
between growth factor receptors and focal adhesion signaling. HER2/Neu/ErbB2, a transmembrane receptor belonging to the epidermal growth factor receptor (EGFR) family, is an oncoprotein that is overexpressed in approximately 30% of human breast cancers and mediates anchorage-independent growth (Schechter, Stern et al. 1984; Mansour, Ravdin et al. 1994; Dankort and Muller 2000; Nanni, Pupa et al. 2000). Patients whose tumours overexpress HER2 receptors (categorized as HER2-positive cases) present with a more metastatic and invasive disease with poorer prognosis (Mansour, Ravdin et al. 1994). Specific docking proteins target trans-phosphorylated receptors and link activated HER2 to the Ras/mitogen-activated protein kinase (MAPK)-, phosphatidylinositol 3-kinase (PI3K)- and Src- dependant signalling cascades, initiating such cellular processes as migration, survival and apoptosis (Harari and Yarden 2000; Menard, Tagliabue et al. 2000; Dankort, Jeyabalan et al. 2001; Dankort, Maslikowski et al. 2001). The phosphorylation of tyrosine (Y) residues on the cytoplasmic tail of HER2 (Y1201 or Y1226/7) has been shown to mediate the epithelial-to-mesenchymal transition (EMT) induced by overexpression of HER2 in Madin-Darby canine kidney epithelial cells (Khoury, Dankort et al. 2001). Memo, a mediator of ErbB2-driven cell motility, has been found to interact with pY1227 through the Shc adapter protein, associating HER2 with the microtubule network (Marone, Hess et al. 2004). Activation of the MAPK, PI3K and Src cascades are critical for HER2-induced motility of breast carcinoma cells, but stimulation of these signals is not sufficient to induce migration in the absence of HER2 phosphorylation at Y1201 or Y1227 (Marone, Hess et al. 2004). Neu transformation activates FAK signalling which is required for cell motility and activation of MAPK, PI3K, and Src leads to ErbB2-dependent lamellipodia formation (Marone, Hess et al. 2004; Benlimame, He et al. 2005). These same pathways are responsible for inducing proliferation through the upregulation of cyclin D (Lenferink, Busse et al. 2001).

1.2 The Ste20-Like Kinase (SLK)

1.2.1. Kinase structure and activation

The mammalian Ste20-like kinase (SLK) was first isolated from guinea pig liver, followed by the cloning of the mouse homologue and then, the human (Itoh, Kameda et al. 1997; Sabourin and Rudnicki 1999; Yamada, Tsujikawa et al. 2000). SLK has been shown to be ubiquitously expressed in adult tissues and cell lines, as well as muscle and neuronal lineages in the developing embryo (Sabourin and Rudnicki 1999; Zhang, Hume et al. 2002). SLK was initially identified and characterized as a caspase3-activated kinase which played a role in the induction of apoptosis (Sabourin and Rudnicki 1999; Sabourin, Tamai et al. 2000; Hao, Takano et al. 2006; Cybulsky, Takano et al. 2009). The kinase is a 220 kDa germinal centre kinase (GCK)-related serine/threonine kinase containing three functional domains (Kuramochi, Moriguchi et al. 1997; Sabourin and Rudnicki 1999). The amino terminal Ste20 catalytic kinase domain (amino acids 1 – 338) shares homology with lymphocyte oriented kinase (LOK), mammalian sterile twenty kinase (MST1) and *Xenopus* polo-like kinase kinase (xPlkk1). The characteristic Ste20 kinase signature sequence (TPYWMAPE) is located in the kinase subdomain VIII at amino acid position 193 (Figure 1). SLK has also been shown to be phosphorylated at T183 and S189, resulting in the auto-activation of kinase activity. A number of protein kinases regulate themselves by autophosphorylation on at least one key residue within the activation segment, usually contained within the kinase lobe of the protein (Nolen, Taylor et al. 2004). Supporting this, T183 and S189 are located within the activation segment; subdomains VII and VIII, respectively (Pike, Rellos et al. 2008; Luhovy, Jaberi et al. 2012). For kinases like SLK that require phosphorylation in order to become activated, the phosphate group often confers a conformational change in the activation segment that promotes substrate binding, compared to the unphosphorylated activation

Figure 1. SLK structure and homologies (Al-Zahrani, Baron et al. 2013). Schematic representation of SLK structure showing the Ste20 kinase domain at the N-terminus and the AT1-46 homology region at the C-terminal end. Amino acid numbers are shown above the structure and homologies to related kinases are shown in percentage. The Ste20 signature sequence (TPYWMAPE), the consensus caspase cleavage site (DXXD) and a putative SH3-binding domain (PXXPX) are shown. Reprinted by permission from Taylor & Francis LLC.


1-338
339-788
789-1202
TPYWMAPE
DXXD
PXXPX
Ste20-Kinase
Coiled-Coil
A
T
H
T183
S189
T193
LOK (74%)
MST1 (26%)
(aa 40-307)
AT1-46 (63%)
(aa 788-936)
AT1-46 (71%)
(aa 957-1171)
LOK (56%)
(aa 867-1178)

segment which would remain unstructured (Sicheri, Moarefi et al. 1997; Meng, Swenson et al. 2002). The mechanism of SLK activation is under ongoing investigation, however it has been demonstrated that SLK is able to self-associate in a *trans* orientation and autophosphorylate (Pike, Rellos et al. 2008). An SLK point-mutant Q185P exists as a monomer in solution and is unable to autophosphorylate, suggesting that dimerization of the kinase is critical for activity (Pike, Rellos et al. 2008).

A central coiled-coil domain (amino acids 339 – 788) contains a putative Src homology 3 (SH3) binding domain (PXXPX) and a caspase 3 cleavage site (DXXD) (Figure 1) (Pawson and Scott 1997; Sabourin, Tamai et al. 2000). However, the precise function of this central region has yet to be elucidated. Due to the presence of the SH3 domain, SLK may interact with proteins containing the proline-rich SH3 binding epitope, which have yet to be identified (Pawson and Scott 1997; Sabourin, Tamai et al. 2000). In addition, mutation of the caspase 3 cleavage site did not affect the ability of SLK to be cleaved by caspase3 *in vitro,* suggesting the presence of additional sites and more complex regulation (Sabourin, Tamai et al. 2000).

The C-terminal motif was termed the ATH domain, for AT1-46 Homology domain (Figure 1) (Schaar, Varia et al. 1996; Sabourin and Rudnicki 1999). The ATH domain is also homologous to the carboxy terminus of LOK (Sabourin and Rudnicki 1999; Sabourin, Tamai et al. 2000). The ATH domain consists of a coiled-coil region, which may facilitate dimerization allowing the catalytic site to come into proximity of the activation segment on the *trans* molecule and phosphorylate it (Delarosa, Guillemette et al. 2011). The GCK-related kinases have been shown to contain C-terminal auto-regulatory regions (Pombo, Bonventre et al. 1996; Graves, Gotoh et al. 1998). It was then hypothesized that the ATH domain could regulate SLK kinase activity. Indeed, a C-terminal truncation deleting the C-terminal two-thirds of the ATH domain ($SLK^{\Delta 950\text{-}1202}$)

dramatically increases SLK kinase activity (Sabourin, Tamai et al. 2000). These findings suggest that SLK is under complex regulation and that additional studies are required to fully understand the role of its various domains.

During the investigation of the auto-inhibitory role of the ATH domain, a yeast two-hybrid screen identified the LIM domain binding transcriptional cofactor proteins (Ldb1 and Ldb2) as SLK-binding factors. The Ldbs were found to bind preferentially within the central 86 amino acids (aa 981-1067) of the ATH domain ($SLK^{950-1202}$) (Storbeck, Wagner et al. 2009). Further study demonstrated that the dimerization and nuclear localization signal (NLS) within Ldb1/2 were required for the interaction with SLK (Storbeck, Wagner et al. 2009). *In vitro*, the ATH domain of SLK inhibited the kinase activity of the SLK^{1-373} fragment and the addition of either Ldb1 or -2 further reduced activity (Storbeck, Wagner et al. 2009). *In vivo* experiments demonstrated that the kinase and ATH domain fragments were more readily co-immunoprecipitated in the presence of Ldb1/2, suggesting that Ldb1/2 aid in the stabilization of the auto-inhibitory function of the kinase domain by the ATH domain (Storbeck, Wagner et al. 2009). It is currently unknown whether there is another scaffolding-type protein facilitating the recruitment of various components to this complex.

1.2.2. The multiple roles of SLK

1.2.2.1. Apoptosis

SLK was first characterized by Sabourin and Rudnicki in 1999 as a mediator of apoptosis. Stable over-expression of SLK in C2C12 myoblasts induced apoptosis via a c-Jun N-terminal kinase-1(JNK1)-dependent pathway, similar to the Ste20 kinase homologue recently characterized in the helminth parasite *Schistosoma mansoni*, SmSLK (Sabourin and Rudnicki 1999; Yan, Tulasne et al. 2007). It was found that

fibroblasts expressing a kinase inactive SLK^{1-373} ($SLK^{1-373\ K63R}$) were Annexin V negative, suggesting that SLK kinase activity was required for the induction of apoptosis (Sabourin and Rudnicki 1999). Interestingly, the expression of full length SLK^{K63R} also induced an apoptotic response albeit delayed, implying that perhaps SLK has the ability to induce programmed cell death independently of its kinase activity (Sabourin and Rudnicki 1999). In a follow-up study, the authors found that preceding apoptosis, SLK over-expression caused the dissolution of actin stress fibers, the redistribution of actin to the cell periphery, membrane blebbing and loss of substrate adhesion (Sabourin, Tamai et al. 2000). The stress fiber dissolution and actin reorganization observed was similar to that seen by over-expression of activated Rac1 and PAK3 (Sabourin, Tamai et al. 2000). It was established that expression of the kinase domain of SLK rapidly induced apoptosis due to its unregulated activity, whereas expression of the auto-regulatory ATH domain induced a delayed apoptotic response, preceded by actin stress fiber dissolution, cellular retraction and loss of adhesion (Sabourin, Tamai et al. 2000). Interestingly, incubation of SLK with recombinant caspase 3 or apoptotic lysates resulted in the release of the kinase and ATH domains and the induction of apoptosis. Supporting this, exposure of cell lines to various apoptotic stimuli, including myc expression, ultraviolet radiation and tumor necrosis factor (TNF)-α resulted in caspase 3-mediated SLK cleavage (Sabourin, Tamai et al. 2000).

In a study of renal ischemia-reperfusion injury, the mechanism of SLK-mediated apoptosis was established. SLK activity attenuated the endoplasmic reticulum (ER) stress response and induced p38 MAPK via the apoptosis signal-regulating kinase-1 (ASK1) (Hao, Takano et al. 2006). SLK activity released cytochrome C and activated caspases-8 and -9. The lack of JNK activation was attributed to cell line specificity as COS-1 only weakly express MAPK kinase (MKK) 4 and MKK7 (Hao, Takano et al.

2006). SLK over-expression in glomerular epithelial cells also stimulated p53 trans-activational activity via the JNK pathway (Cybulsky, Takano et al. 2009). SLK-induced apoptosis was attenuated with the use of the p53 inhibitor, pifithrin-α (Cybulsky, Takano et al. 2009). This was confirmed *in vivo* using a transgenic mouse line overexpressing SLK in the kidney glomerular podocytes (Cybulsky, Takano et al. 2010). Overexpression of the kinase in these cells resulted in injury and loss of these podocytes, accompanied by an increase in phosphorylation of p38 (Cybulsky, Takano et al. 2010). The multi-faceted role of SLK in mediating the apoptotic response suggests that it may play a significant role in apoptosis-dependent processes such as development and tumorigenesis.

1.2.2.2. The cell cycle

Initial characterization of SLK centered on the apoptotic response. However, the presence of active kinase in healthy exponentially growing cells suggested that the function of SLK was much more complex. Indeed, stable expression of kinase inactive SLK in fibroblasts cannot be achieved (O'Reilly, Wagner et al. 2005). The lack of an apoptotic response in cells transiently expressing kinase dead SLK lacking the ATH suggests that it interferes with proliferation, supporting a role for SLK in cell cycle progression (O'Reilly, Wagner et al. 2005). In the search for an upstream kinase to activate the polo-like kinase homologue (Plk1), SLK emerged as a candidate due to its high homology to the xPLKK1 (Ellinger-Ziegelbauer, Karasuyama et al. 2000). Plk1 was identified as a novel substrate of SLK, which phosphorylates and activates Plk1 during progression through the G2/M transition, at which point the kinase activity of SLK is highest (Ellinger-Ziegelbauer, Karasuyama et al. 2000). This phosphorylation by SLK is enhanced by the presence of the polo-box binding domain of Plk1 (Johnson, Antrobus et al. 2008). SLK was subsequently found to be required upstream of Cdc2 and depletion

of SLK caused cells to arrest in early G2. This arrest was accompanied by an inability to down-regulate cyclin A and progress through mitosis (O'Reilly, Wagner et al. 2005). Confirming a link between SLK and the microtubule network, the kinase co-localized with α-tubulin at the mitotic spindle and over-expression of the kinase caused ectopic spindle assembly (Wagner, Flood et al. 2002; O'Reilly, Wagner et al. 2005). Further study revealed that SLK was required for radial microtubule organization during interphase as depletion of the kinase resulted in the inability of centrosomes to anchor or cap microtubules (Burakov, Zhapparova et al. 2008). Interestingly, over-expression of SLK induced cell cycle re-entry of *Xenopus* oocytes (O'Reilly, Wagner et al. 2005). These observations offer a possible mechanism by which over-expression of SLK leads to apoptosis. Over-expression of the kinase may force cells to prematurely enter mitosis leading to their death through mitotic catastrophe.

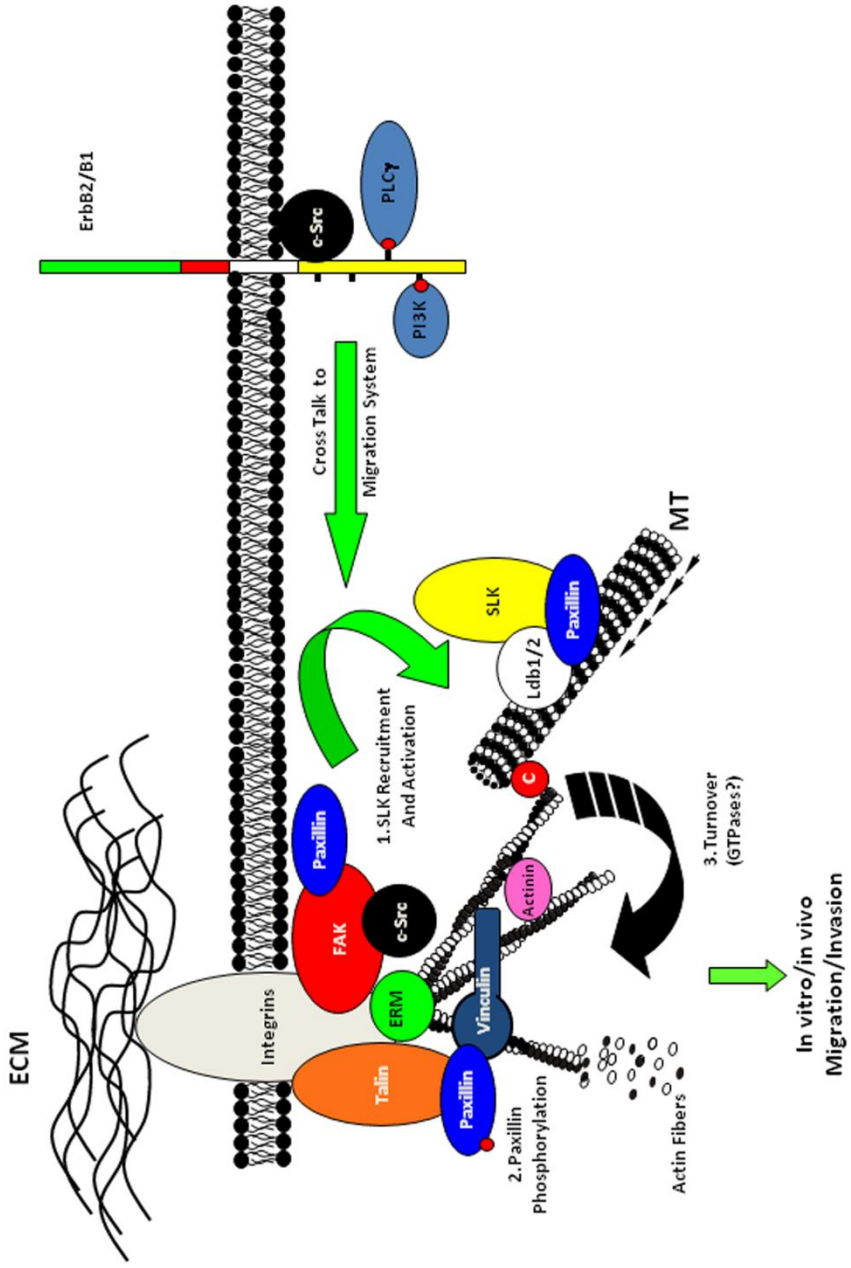
1.2.2.3. Cytoskeletal dynamics & cell migration

The recurrent theme in the characterization of SLK is its association with the cytoskeleton and the microtubule network. Initial work has shown that SLK localizes to the cell periphery. Furthermore, SLK overexpression induces the disassembly of actin stress fibers, cellular retraction and the re-localization of actin to the cell periphery (Sabourin and Rudnicki 1999; Sabourin, Tamai et al. 2000). Interestingly, disassembly of actin stress fibers induced by the over-expression of SLK can be attenuated by the expression of a dominant-negative mutant of Rac1 (Wagner, Flood et al. 2002). Accordingly, SLK co-localizes with Rac1 and the microtubule network in fibroblasts actively spreading on fibronectin (Wagner, Flood et al. 2002). RhoA activation is known to promote stress fiber and focal adhesion (FA) complex assembly, downstream of Rac1 (Nobes and Hall 1995; Van Aelst and D'Souza-Schorey 1997). In a study of vasodilation induced by angiotensin II type 2 receptor (AT_2R) activation, it was reported that AT_2R

signals through SLK which in turn phosphorylates Ser188 of RhoA, thus inhibiting its activity, preventing vascular smooth muscle cell contraction (Guilluy, Rolli-Derkinderen et al. 2008). This interpretation supports a mechanism by which SLK mediates cytoskeletal reorganization and stress fiber breakdown through a Rho/Rac pathway.

Actin fibers are anchored at FAs through protein complexes composed of α-actinin, vinculin, talin and zyxin (Figure 2) (Craig and Johnson 1996). The FAs control adhesion dynamics and contain FAK, paxillin and vinculin. In response to cell adhesion or migration stimuli, cells form integrin/FAK/Src complexes which recruit and activate numerous other adaptor molecules leading to FA turnover (Figure 2) (Brown and Turner 2004; Mitra and Schlaepfer 2006). The formation of a functional FAK/Src complex is critical for efficient cell migration by modulating FA turnover, while disruption of this complex results in reduced cell migration (Kaplan, Bibbins et al. 1994; Cary, Chang et al. 1996; Cary, Han et al. 1998; Fincham and Frame 1998; Kaverina, Krylyshkina et al. 1999; Owen, Ruest et al. 1999; Sieg, Hauck et al. 1999; Ren, Kiosses et al. 2000; Webb, Parsons et al. 2002; Brown and Turner 2004; Webb, Donais et al. 2004; Mitra and Schlaepfer 2006). Following fibronectin stimulation, it was observed that SLK co-localized with vinculin at large podosome-like adhesions (Wagner, Flood et al. 2002). SLK ablation resulted in an increase in the size and density of vinculin-positive adhesions, indicative of stable FAs in non-migrating cells (Wagner, Storbeck et al. 2008). These results suggest that SLK may play a role in adhesion dynamics. Supporting this, SLK kinase assays from cells induced to migrate by scratch-wounding show an upregulation in kinase activity (Etienne-Manneville and Hall 2001; Wagner, Storbeck et al. 2008). This activation was found to be dependent on c-Src and the MAPK kinase pathway as pre-treatment with the PP2 and U0126 inhibitors abrogated kinase activation (Wagner, Storbeck et al. 2008). Overexpression of v-Src leads to inhibition of

Figure 2. Upstream signals and SLK activation (Al-Zahrani, Baron et al. 2013). Upon integrin engagement, the FAK/src complex is activated and focal contacts are assembled while SLK is held inactive. Further signaling activates and recruits SLK through the microtubule network, along the actin fibers (Wagner, Storbeck et al. 2008). SLK can phosphorylate paxillin in the vicinity of adhesions to induce adhesion turnover (Quizi, Baron et al. 2012). Alternatively, microtubule-bound paxillin could be phosphorylated and recruited to newly formed adhesion and induce destabilization. Stimulation of growth factor receptor such as ErbB2 can also activate SLK through receptor-integrin cross talk (Roovers, Wagner et al. 2009). Reprinted by permission from Taylor & Francis LLC.


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

SLK activity through hyper-phosphorylation of the kinase domain through casein kinase II, suggesting that the Src family kinases can also function to negatively regulate SLK activity during cell migration, perhaps during focal contact assembly (Chaar, O'Reilly et al. 2006). Confirming a role for SLK in cell migration, knockdown experiments and expression of dominant negative SLK show marked reductions in migration (Wagner, Storbeck et al. 2008).

Scratch wounding of confluent monolayers causes FAK activation as the cells migrate into the wound (Gates, King et al. 1994). FA turnover during cell migration induces the formation of a functional FAK/Src complex initiated by auto-phosphorylation of FAK at tyrosine residue 397 (pY397), stable focal adhesion assembly and FA turnover and migration (Figure 2) (Gates, King et al. 1994; Nobes and Hall 1995; Van Aelst and D'Souza-Schorey 1997; Etienne-Manneville and Hall 2001; Chaar, O'Reilly et al. 2006; Guilluy, Rolli-Derkinderen et al. 2008; Wagner, Storbeck et al. 2008). Similarly, nocodazole treatment of fibroblasts results in microtubule depolymerization and FA stabilization as evidenced by high levels of phospho-FAK-Y397 (Bershadsky, Chausovsky et al. 1996). Nocodazole wash-out and microtubule regrowth is accompanied by cyclical changes in the levels of FAK pY397 (Bershadsky, Chausovsky et al. 1996). Nocodazole wash-out in MEF-3T3 fibroblasts that express kinase inactive SLK or have been siRNA treated show impaired focal adhesion turnover as demonstrated by stabilization of FAK pY397 levels (Wagner, Storbeck et al. 2008). SLK-deficient cells also displayed enlarged adhesions following the wash-out, further supporting impaired FA turnover (Wagner, Storbeck et al. 2008). Supporting a role for SLK in turnover, scratch wounding of a confluent monolayer of FAK-null fibroblasts results in almost no upregulation in SLK kinase activity, suggesting that SLK activation is FAK-dependent (Wagner, Storbeck et al. 2008).

Further investigation into the mechanisms of SLK activation and activity during cell migration showed that the regulatory Ldb1/2 co-factors co-localized with SLK at the leading edge of migrating cells (Storbeck, Wagner et al. 2009). Both the knockdown and over-expression of Ldb1/2 caused an increase in the rate of cell migration, recapitulating the changes in SLK activity seen in the SLK-Ldb complex studies *in vitro* (Storbeck, Wagner et al. 2009). Changes in the expression level of Ldb1 resulted in the loss of either Ldb factor from the SLK complex, activating the kinase and increasing cell migration (Storbeck, Wagner et al. 2009). Adding complexity, as SLK kinase activity increased during cell migration, the amount of Ldb1 associated with the kinase increased but SLK-associated Ldb2 remained stable, suggesting that the stoichiometry of the SLK-Ldb complex is crucial for cell migration (Storbeck, Wagner et al. 2009). These observations also hint that a heretofore unidentified component of the SLK-Ldb complex initiates a shift in the stoichiometry of this complex thus allowing the kinase to activate and signal downstream to the FAs (Figure 2). Recent evidence from our lab shows that activated SLK regulates FAK-mediated FA turnover by phosphorylating paxillin on serine 250, an integral FA complex component (Figure 2) (Quizi, Baron et al. 2012). A paxillin serine 250 mutant (S250T) abolished phosphorylation of paxillin by SLK resulting in stable FAK pY397 levels, stabilized FAs and impaired cell migration, further supporting a role for SLK as a modulator of adhesion turnover and cell migration (Quizi, Baron et al. 2012). As proposed previously, SLK might represent part of a microtubule-associated complex that targets focal adhesions for disassembly (Figure 2) (Kaverina, Krylyshkina et al. 2002; Palazzo and Gundersen 2002).

1.2.3. The role of SLK in HER2/Neu/ErbB2 signaling

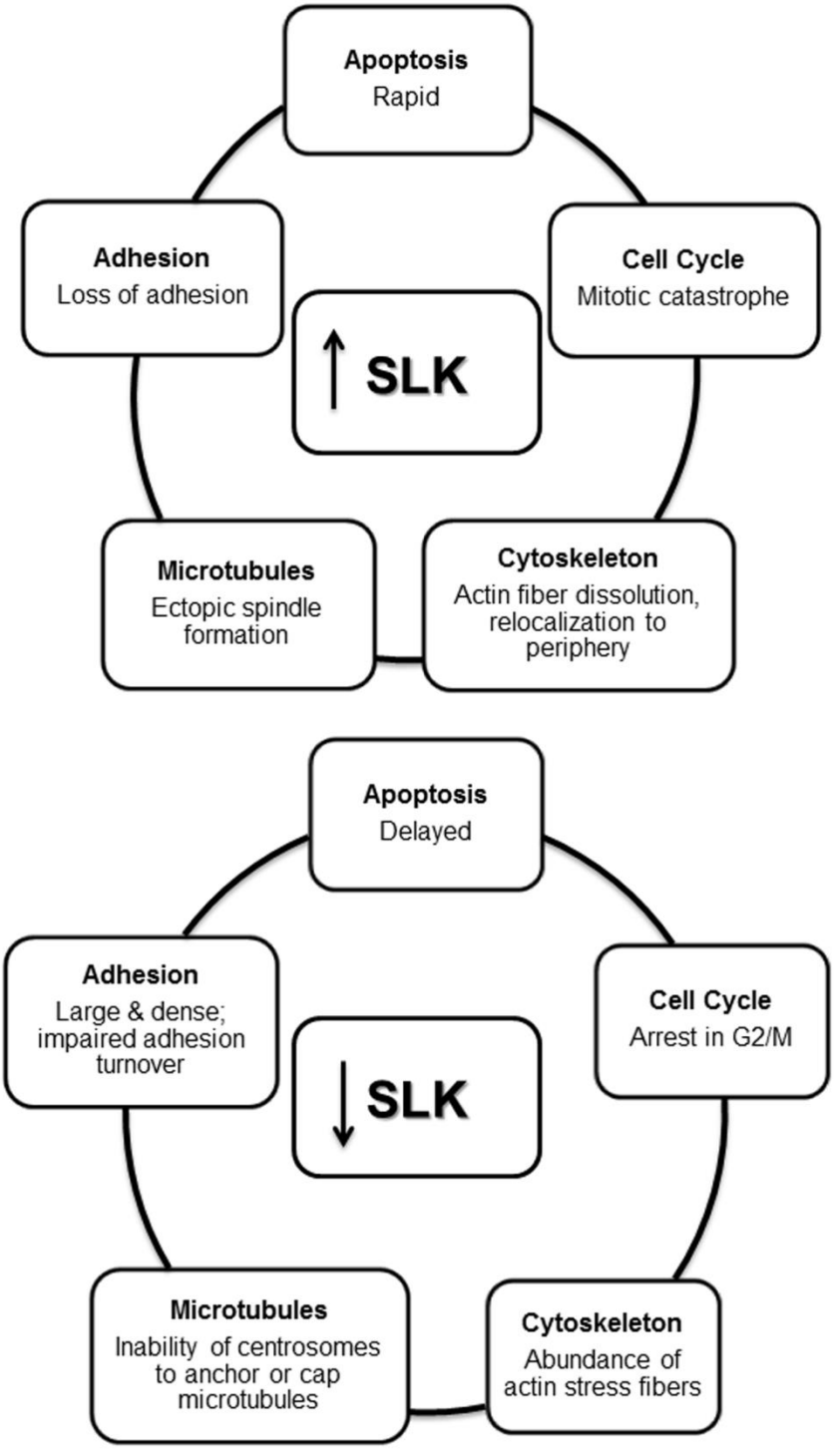
FAK signalling is required for HER2-induced transformation and invasion (Benlimame, He et al. 2005). Chemotaxis and invasion of breast cancer cells in the

HER2 tumour model has been shown to signal through SLK via the FAK complex (Figure 2) (Roovers, Wagner et al. 2009). SLK has been shown to be upregulated in cell lines that express high levels of the HER2 receptor, including the T47D and 4T1 lines (Roovers, Wagner et al. 2009). In cell lines where basal expression of the HER2 receptor is relatively low (HeLa and NIH3T3 cells) overexpression of an activated Neu (Neu V64E) was sufficient to upregulate SLK activity in the absence of heregulin stimulation (Roovers, Wagner et al. 2009). Expression of kinase inactive SLK (SLK^{K63R}) reduced heregulin-induced cell migration by up to 75% as measured in a Boyden chamber assay (Roovers, Wagner et al. 2009). Similarly, chemotaxis was inhibited in MCF-7, MDA-MB-231 and Sk-Br-3 cells when SLK^{K63R} was expressed (Roovers, Wagner et al. 2009). Autophosphorylation of Neu on tyrosine 1201 and tyrosine 1226/7 has been shown to couple the Neu/Neu receptor to its downstream signaling cascades (Marone, Hess et al. 2004). The presence of a tyrosine residue at either of the two auto-phosphorylation sites (Y1202 or Y1226/7), in an otherwise phosphorylation-deficient mutant (NYPD), is sufficient to activate SLK downstream of heregulin stimulation (Roovers, Wagner et al. 2009). As previously noted, both MEK1 and Src family kinase inhibitors are sufficient for the attenuation of SLK-mediated cell migration. Similarly, they were also shown to inhibit Neu-mediated SLK activation (Wagner, Storbeck et al. 2008; Roovers, Wagner et al. 2009). The use of inhibitors for both PI3K and PLCγ results in a partial inhibition of SLK activation in cells expressing activated Neu (Roovers, Wagner et al. 2009). In cells expressing Neu with only Y1201 available for phosphorylation, inhibition of PLCγ prevented SLK activation, but inhibition of PI3K had no effect on SLK activity (Roovers, Wagner et al. 2009). In cells expressing Neu with only Y1226/7 available for phosphorylation, the opposite effects are observed (Roovers, Wagner et al. 2009). These results suggest that multiple signaling pathways contribute to SLK activation downstream of HER2/Neu/ErbB2. Over-expression of activated Neu in a FAK-

null fibroblast cell line is not sufficient to induce SLK activity upon heregulin stimulation, suggesting that FAK is required for Neu-mediated SLK activation (Roovers, Wagner et al. 2009). Although it is unclear how SLK is directly activated downstream of the Neu-FAK cross-talk, it appears to be an important downstream mediator of the Neu receptor (Figure 2).

Albeit SLK regulation appears to be quite complex, it is likely to be dependent on the biological context, whether it is apoptosis, motility or proliferation (Figure 3). SLK responds to oncogenic signals such as activated HER2/Neu/ErbB2 to drive chemotaxis of breast cancer cells, suggesting that SLK inhibition might suppress the invasive and metastatic process. Whether SLK is required at any other levels during cancer progression remains to be elucidated. The kinase presents itself as a potential therapeutic target in HER2-positive breast cancer patients. Specific inhibition of SLK activity may abrogate the progression of early-stage HER2-positive tumors or stabilize disease in advanced-stage cases and may achieve favourable responses in a combinatorial therapeutic approach. However, as SLK is ubiquitously expressed in adult tissues, the use of SLK inhibitors might be detrimental to normal tissue function. Therefore, the identification of additional regulatory pathways or binding partners that are tissue specific will be beneficial, providing an opportunity to target upstream regulators in a tissue-specific manner. The global SLK knock out is embryonic lethal (Al-Zahrani, Sekhon et al. 2014), therefore the establishment of a conditional SLK allele in a cancer model will allow for the testing of its therapeutic potential.

Figure 3. Summary of SLK functions (Al-Zahrani, Baron et al. 2013). Studies have shown that high levels of SLK activity induce apoptosis, loss of actin fibers and adhesion, and ectopic spindle formation. Conversely, knock down experiments or expression of kinase inactive SLK results in delayed cell death, cell cycle arrest and inhibition of cell migration. Together, these data suggest a role for SLK in multiple processes. Whether the role of SLK is restricted to cytoskeletal remodeling in all cases remains to be verified. Reprinted by permission of Taylor & Francis LLC.


Apoptosis
Rapid
Cell Cycle
Mitotic catastrophe
Cytoskeleton
Actin fiber dissolution, relocalization to periphery
Microtubules
Ectopic spindle formation
Adhesion
Loss of adhesion
↑ SLK
Apoptosis
Delayed
Cell Cycle
Arrest in G2/M
Cytoskeleton
Abundance of actin stress fibers
Microtubules
Inability of centrosomes to anchor or cap microtubules
Adhesion
Large & dense; impaired adhesion turnover
↓ SLK

1.3 The LIM-Only Protein 4 (LMO4)

The LIM-only protein 4 (LMO4) is a member of the LIM-only (LMO) protein family of transcriptional transactivators characterized by two tandem LIM domains. The LIM motif itself consists of two cysteine-rich zinc fingers and mediates protein-protein interactions [reviewed by (Dawid, Breen et al. 1998; Bach 2000)]. Despite the direct DNA binding properties of most zinc fingers, those contained within LIM domains establish multi-protein complexes and do not directly bind DNA themselves (Matthews, Lester et al. 2013). The LMO protein family (LMO1 – 4) comprises one small group of primarily nuclear proteins within a larger family of nuclear and cytosolic LIM-containing proteins that contain between one (e.g. CRIP) and five (e.g. PINCH/Unc-98) LIM domains (Bach 2000; Matthews, Lester et al. 2013). The LMO family is distinguished from other LIM containing proteins by the simple fact that they contain no other protein domains apart from their two LIM motifs (Bach 2000). Each of the four LMO proteins have been confirmed to play a role in cancer progression [reviewed in (Matthews, Lester et al. 2013)]. LMO1 and LMO2 were initially identified through the mapping of chromosomal translocations in human T cell acute lymphoblastic leukaemia, causing their overexpression in 50% of cases of this disease (McGuire, Hockett et al. 1989; Matthews, Lester et al. 2013). LMO1 and LMO2 participate interchangeably in haematopoietic transcriptional complexes promoting the stemness and expansion of T cell progenitor populations, while inhibiting their differentiation (Warren, Colledge et al. 1994; Visvader, Mao et al. 1997; Wilson, Foster et al. 2010). LMO3 was discovered through sequence homology to LMO1 and further investigation found that it acts as a neuroblastoma oncogene through its interaction with the neuronal transcription factor HEN1 (Foroni, Boehm et al. 1992; Aoyama, Ozaki et al. 2005). All LMO family members have been

found to play a role in embryonic development through cell fate determination of various tissue progenitors (Bach 2000; Matthews, Lester et al. 2013).

LMO4 is the most recently discovered member of the LMO family and was reported by four groups almost simultaneously (Grutz, Forster et al. 1998; Kenny, Jurata et al. 1998; Sugihara, Bach et al. 1998; Racevskis, Dill et al. 1999). The first three groups all discovered LMO4 through its interaction with Ldb1, which is required to retain LMO4 in the nucleus as the small LMO4 protein does not contain a nuclear localization signal (Grutz, Forster et al. 1998; Kenny, Jurata et al. 1998; Sugihara, Bach et al. 1998). LMO4 interacts with Ldb1 via its first LIM domain (LIM1) and the LIM binding domain (LBD) of Ldb1 (Deane, Mackay et al. 2003; Deane, Ryan et al. 2004). In the mouse embryo, LMO4 is highly expressed in mesenchymal cells uncommitted to regional fates and especially, in the neutral crest (Kenny, Jurata et al. 1998). In addition, it is also highly expressed in developing cartilage and peripheral and central nervous systems (Sugihara, Bach et al. 1998). Global deletion causes neural tube defects, hematopoietic anomalies and causes embryonic lethality (Hahm, Sum et al. 2004; Meier, Krpic et al. 2006). In adult tissues, LMO4 is expressed ubiquitously in the epidermis, as well as the epithelial cells of the mammary gland, pituary gland and the genitourinary, respiratory and gastrointestinal tracts (Sugihara, Bach et al. 1998). Mammary gland-specific deletion impedes lobuloalveolar development during pregnancy (Sum, Shackleton et al. 2005). These effects may be caused by its transcriptional co-activator function however, there is evidence that LMO4 may have cytosolic protein adapter abilities, unlike its three other LMO family members (Bach 2000), in the context of migration and invasion (Schaffar, Taniguchi et al. 2008; Gomez-Smith, Qin et al. 2010). Despite being the only member of the LMO family with cytosolic functions, LMO4 is not the sole LIM-containing protein with a role within the cytoplasm (Bach 2000). Indeed, there are LIM-containing

proteins which function exclusively within the cytoplasm (e.g. paxillin, LIM kinases 1/2, CRIP, etc.) as well as a number of which perform functions in both the cytoplasm and the nucleus (e.g. zyxin, CRP3/MLP, etc.) (Bach 2000). This highlights the aforementioned fact that LIM domains primarily mediate protein-protein interactions and do not solely imply a role in transcriptional control within the nucleus (Bach 2000; Matthews, Lester et al. 2013).

Relevant to this study, Racevskis et al. isolated LMO4 from a human breast tumor cDNA library, as an autoantigenic cDNA clone (Racevskis, Dill et al. 1999). Further evidence shows that LMO4 may play a role in the progression of multiple cancer types, including pancreatic and myogenic (Yu, Ohuchida et al. 2008; Armeanu-Ebinger, Bonin et al. 2011). LMO4 was confirmed to be overexpressed in 56% of mammary tumors that are ErbB2 positive and is a predictor of poorer outcome in breast cancer patients (Visvader, Venter et al. 2001; Sum, Segara et al. 2005). The overexpression of LMO4 in murine mammary epithelium was found to induce hyperplasia and promote cell invasion (Sum, Segara et al. 2005). Subsequently, LMO4 was shown to be required for ErbB2/HER2/Neu-induced cancer cell cycle progression via the upregulation of cyclin D at the G2/M checkpoint, downstream of the PI3K signalling pathway (Montanez-Wiscovich, Seachrist et al. 2009; Montanez-Wiscovich, Shelton et al. 2010). It has also been discovered as a tumor growth factor beta (TGFβ) responsive factor in breast cancer cell lines (Lu, Lam et al. 2006). As mentioned above, recent evidence shows that LMO4 has cytosolic protein adapter abilities in the context of migration and invasion (Schaffar, Taniguchi et al. 2008; Gomez-Smith, Qin et al. 2010). Supporting this claim, advanced human breast tumor tissue shows increased cytosolic LMO4 staining (Visvader, Venter et al. 2001; Montanez-Wiscovich, Shelton et al. 2010).

1.4 Thesis Rationale, Hypothesis & Objectives

Our lab has demonstrated that SLK activity is stimulated by ErbB2/HER/Neu signalling and is required for processes such as cell migration and cell cycle progression (Figure 2). Mechanistically, we have shown that SLK activity is negatively regulated by Ldb1/2 through direct interactions (Storbeck, Wagner et al. 2009). We have also found that SLK co-localizes with tubulin, paxillin and Rac1 at the membrane ruffle of actively migrating cells (Wagner, Storbeck et al. 2008). However, the regulation of kinase activity and its intracellular localization along with associated complex components remains to be elucidated. As LMO4 interacts with Ldb1 and due to the parallels found between the roles of SLK and LMO4 in ErbB2-mediated cell proliferation and motility (Figure 4), we investigated the possibility of the interaction between SLK and LMO4, as well as the potential consequences of this interaction. The hypothesis for the following study is that **the up-regulation of LMO4 and its interaction with SLK regulates kinase signalling and localization, contributing to breast cancer cell invasion and metastasis.** To verify this hypothesis, I will (**1**) define the interaction between LMO4 and SLK as well as between LMO4 and Ldb1/2, and determine the role of LMO4 in the regulation of SLK activity; (**2**) investigate the mechanisms responsible for kinase complex assembly and its role in the regulation of SLK localization and cell migration; and (**3**) investigate the role of LMO4 in ErbB2-mediated breast tumorigenesis in a mouse model.

Table 1. The similarities between LMO4 and SLK suggest a possible functional relationship. When reviewing the literature, there appears to be a correlation between LMO4 expression levels and SLK activity as well as major binding partners (Ldb1/2) common to both proteins. This suggests a possible interaction or functional relationship between these proteins which requires investigation. LMO4 expression is upregulated through ErbB2 signalling, during cell migration as well as during the G2/M checkpoint. The SLK is activated in response to cell migration, ErbB2 signalling and during the G2/M checkpoint.

LMO4	SLK
ErbB2 ↑ expression	ErbB2 ↑ activity
↑ expression during G_2/M phase	↑ activity during G_2/M phase
↑ expression = cancer cell invasion	↑ activity = cell migration
Binds directly to Ldbs	Binds directly to Ldbs

Chapter 2:

Materials & Methods

2. Materials & Methods

2.1 *In vitro* binding assays

Bacteria expressing Glutathione-S-transferase (GST) fusion proteins were collected by centrifugation at 4°C and resuspended in 500 µL RIPA lysis buffer including protease inhibitors (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 2mM dithiothreitol (DTT), 10 µg/mL leupeptin, 10 µg/mL pepstatin, 10 µg/mL aprotinin, 1 mM phenylmethylsulphonylfluoride and 100 µM benzamidine) and sonicated on ice for 15 seconds. Supernatants were cleared by centrifugation, Glutathione-Sepharose beads (GE Healthcare, Mississauga, ON) were added and rotated for 2 h at 4°C. Bound GST fusion proteins and beads were washed three times with 200 mM NETN buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 200mM NaCl and 0.5% Nonidet P-40). *In vitro* translation products were obtained with the TNT quick-coupled *in vitro* transcription translation kit (Promega, Madison, WI) with ^{35}S-methionine (PerkinElmer, Waltham, MA) as per the manufacturer's instructions and incubated with either GST alone or GST-fusion proteins in 200 mM NETN buffer. Complexes were washed 3 times with 200 mM NETN, eluted from the beads by boiling in sample buffer and fractionated by SDS-PAGE. Proteins were visualized using Coomassie Brilliant Blue (Sigma-Aldrich, St. Louis, MO) staining. Gels were dried and subjected to autoradiography.

2.2 Cell culture, transfections & infections

Murine embryonic fibroblast (MEF) 3T3, murine mammary carcinoma 4T1, PTPα +/+ and -/- murine fibroblasts, human epithelial kidney (HEK) 293, SYF (src/fyn/yes triple mutant), SYF+c-Src, and primary MEFs were maintained in Dulbecco's modified Eagle's

medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine (Invitrogen), and penicillin G (200 U/mL; Invitrogen) and streptomycin sulfate (200 µg/mL; Invitrogen) in a humidified 37°C incubator at 5% CO_2. Primary MEFs from LMO4 floxed homozygous FVB/N female mice were isolated from E12.5 embryos via caesarian section of timed matings. Immortalization of LMO4 floxed MEFs was achieved using a standard 3T3 spontaneous immortalization protocol (Aaronson and Todaro 1968) and cells were maintained in a humidified 37°C incubator at 5% CO_2. All tissue culture experiments using primary MEFs were seeded at 20 or fewer passages following caesarian sections.

Transient DNA transfections into SYF cells were performed as per the manufacturer's instructions using Lipofectamine/PLUS reagent (Invitrogen) with a total of 4 µg of plasmid DNA per each 10 cm plate. MEF3T3 cells were transfected with 150 nM siRNA (Dharmacon RNA Technologies, Lafayette, CO) duplex for LMO4 (target sequence: 5' - GCAAGUGAGCUCGUCAUGA - 3') or Dharmacon's non-targeting duplex as control using the Lipofectamine 2000 (Invitrogen) transfection reagent. Cells were incubated for 48 h at 37°C and 5% CO2 and assayed for cell migration by a wound closure assay and protein expression by Western blot analysis.

For Cre-mediated deletion of LMO4, LMO4 floxed MEFs were plated at a density of 7.5 x 10^5 cells on 10 cm plates or 1.875 x 105 cells in 60 mm 6-well dishes in 10% FBS DMEM and incubated overnight at 37°C and 5% CO_2. The cells were then infected with an adenovirus expressing LacZ as a control (AdLacZ) or Cre recombinase (AdCre; both a generous gift of Dr. Robin Parks, OHRI, Ottawa, ON) at an MOI of 20 in serum-free medium for 90 minutes at 37°C and 5% CO2. The infection was followed by the addition of 10% FBS DMEM and the cells were incubated at 37°C and 5% CO2 for 48 hours prior to analysis via Western blot, cell migration or immunofluorescence.

2.3 Plasmid constructs

DNA plasmids were constructed using standard molecular cloning techniques. The GST-tagged SLK constructs were generated as described previously (Storbeck, Wagner et al. 2009). The murine LMO4 cDNA was PCR amplified (N-ter: 5′-ATGGTGAATCCGGGC and C-ter: 5′-TCAGCAGACCTTCTG) and sub-cloned in frame into the pCAN–Myc expression plasmid. The point mutations in pCAN–Myc–LMO4 for the quadruple LIM1 domain mutant (R33G/F34Q/L35Y/Y37Q; Myc–LMO4 QUAD (Deane, Ryan et al. 2004)) were generated according to Stratagene's (Agilent Technologies, Santa Clara, CA) 'QuikChange II XL site-directed mutagenesis kit' using the Myc-tagged LMO4 cDNA as the template. Clones were verified using DNA sequencing. The LMO4 deletion constructs LMO4 ΔLIM1 (aa 1–87) and LMO4 ΔLIM2 (aa 87–166), were constructed using PCR amplification with complimentary oligonucleotides (LMO4Δ1: 5′-ATGGTGAATCCGGGC and 5′-AGCACCGCTATTCCC; LMO4Δ2: 5′-TGCAGGGCCTGTGGA and 5′-TCAGCAGACCTTCTG) and sub-cloned into pCAN–Myc or pGEX 4T1. The pCAN-Myc-SLK ATH Y1182F mutants were generated using Stratagene's 'QuikChange II XL Site-Directed Mutagenesis kit' (Agilent Technologies) using the Myc-tagged SLK ATH cDNA as the template and the following primers: forward 3'-GCCGGATCTCTAAATTCTTCCCTATTCCCACCTTAC-5' and reverse 3'-GTAAGGTGGGAATAGGGAAGAATTTAGAGATCCGGC-5'. Clones were verified using DNA sequencing. The mutated ATH fragment was subcloned into the pGEX 4T2 expression vector.

2.4 Genotyping

Ear clippings were obtained from all 3-week-old pups at weaning and digested using proteinase K (QIAGEN, Mississauga, ON). Genomic DNA was used for LMO4 flox,

PyMT and MMTV-Cre genotyping by polymerase chain reaction (LMO4 flox: forward 5'-CGAGCTGAAATTGTCAGCAGCAAG-3' and reverse 5'-CGAGCTGCTGCCCGGATTCAC-3'; PyMT: forward 5'-GGAAGCAAGTACTTCACAAGG-3' and reverse 5'-GGAAAGTCACTAGGAGCAGGG-3'; MMTV-Cre: forward 5'-GGGATTGCTTATAACACCCTGTTACG-3' and reverse 5'-TATTCGGATCATCAGCTACACCAGAG-3').

2.5 Generation of mouse lines and tissue isolation

Male C57/B6 mice expressing the floxed *lmo4* gene (LMO4 flox) were generously provided by Dr. H. Chen (The Ottawa Hospital Research Institute, Ottawa, ON). Male C57/B6 LMO4 flox mice were crossed and back-crossed with female wild-type FVB/N from Charles River Laboratories (Sherbrooke, QC) for three successive generations to establish LMO4 flox in the FVB/N background. Background confirmation was provided by MAX-BAX congenic screening (Charles River Laboratories). Female LMO4 flox FVB/N mice were then crossed with male MMTV-Cre-expressing FVB/N mice (generously provided by Dr. W. Muller, McGill University, Montréal, QC) to establish MMTV-Cre LMO4 flox mice. Virgin mammary tissue samples were obtained from 11-week-old female mice, using glands #4 and 5 and frozen for protein analyses. Cre-negative LMO4 flox females and MMTV-Cre LMO4 flox females were crossed with male individuals expressing the polyoma virus middle T oncoprotein (PyMT), generously provided by Dr. W. Muller (McGill University, Montréal, QC). PyMT-expressing female individuals were monitored for tumor appearance by palpitation starting at 3-weeks-of-age. Tumor bearing animals were monitored daily until euthanasia at humane endpoint. Individuals were dissected immediately following euthanasia. All mammary glands, tumors and lungs were removed and either frozen for protein analysis or fixed in 10%

formalin for immunohistochemical (IHC) analysis. Endpoint data is represented as Overall Survival using Kaplan-Meier modelling.

2.6 Antibodies & immunofluorescence

The primary antibodies used in this study are as follows: FAK (BD Transduction Laboratories), FAK pY577 (Invitrogen), GAPDH (Cell Signaling Technology, Beverly, MA), Ldb1 (Santa Cruz Biotechnology, Dallas, TX), Myc (9E10 mouse ascites, Sigma-Aldrich), Paxillin (BD Transduction Laboratories), total phospho-Tyrosine (Cell Signaling), Src (Cell Signaling Technology), and α-tubulin (Sigma-Aldrich). The anti-LMO4 polyclonal antibodies were custom made using full length GST–LMO4 as the immunogen (Medical and Biological Laboratories Co. Ltd., Nagoya, Japan). The anti-LMO4 monoclonal antibody was generously provided by Dr. J. Visvader (Walter and Eliza Hall Institute Biotechnology Centre, Bundoora, Victoria, Australia) has been previously described (Sum, Segara et al. 2005). The anti-SLK polyclonal antibodies were custom made using full length GST–SLK as the immunogen (Medical and Biological Laboratories Co. Ltd., Nagoya, Japan) and have been previously described (Sabourin and Rudnicki 1999).

For immunofluorescence studies, cells were seeded in 10% FBS DMEM on coverslips coated with fibronectin (10 µg/mL,Invitrogen) and incubated overnight at 37°C and 5% CO_2 or until confluent for migration studies. For coverslips intended for migration studies, a micropipette tip was used to create two parallel wounds in untreated or inhibitor-treated (1 h pre-treatment prior to wounding with 10 µM PP2 or PP3; EMD-Millipore [Calbiochem], Darmstadt, Germany) cell monolayers. Growth media were replaced with fresh 10% FBS DMEM, including any small molecule inhibitors required for the experiment, and cells were incubated for 1 h at 37°C and 5% CO_2 prior to fixation

with 4% paraformaldehyde. Following permeabilization with 0.3% Triton X-100 in STO-PBS, cells were washed and blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 20 min. Cells were washed and a fresh blocking serum containing primary antibody was added directly to coverslips, which were then incubated for 1 h at room temperature. Primary antibodies were detected using anti-mouse, anti-goat or anti-rabbit secondary antibodies, conjugated to either fluorescein isothiocyanate (Alexa Fluor 488, Sigma-Aldrich) or tetramethyl rhodamine isothiocyanate (Alexa Fluor 594, Sigma-Aldrich). Cellular nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI, Invitrogen). Slides were visualized with a Zeiss Axioscope 100 epifluorescence microscope equipped with the appropriate filters and photographed using Axiovision software (Carl Zeiss Canada Ltd., Toronto, ON). Cells that showed reactivity at the leading edge of the wound were scored as a positive event. Counts were obtained from five images along the wound edge for two coverslips per transfection, infection or treatment.

2.7 Western blot analysis, immunoprecipitation & *in vitro* kinase assays

Cell pellets, mammary gland or tumor tissues were collected and lysed for protein analysis using a RIPA buffer including protease inhibitors as described above. Lysates were sonicated on ice for 15 seconds and cleared by centrifugation at 14,000 rpm at 4°C. Protein concentrations in the cleared supernatants were quantified using the Bradford protein assay reagent (Bio-Rad, Hercules, CA). For Western blot analysis, equal amounts of protein (20–50 µg) were electrophoresed on 4 –20% gradient pre-cast SDS-PAGE gels (Bio-Rad) and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were probed overnight at 4°C with the specified primary antibodies in 5% BSA in TBST (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20). Primary antibodies were detected using the appropriate anti-mouse, anti-goat or

anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Proteins were detected by chemiluminescence (PerkinElmer) and exposed to X-ray film.

For immunoprecipitations, equal amounts of protein (200–500 µg) were incubated with the required primary antibody and 20 µL of protein A/G bead slurry (GE HealthCare) for 2 h at 4°C. The beads and bound proteins are washed four times with 200 mM NETN containing 0.25 mM NaVO3. Protein complexes were eluted from the beads by boiling in sample buffer, electrophoresed on polyacrylamide gels and transferred onto PVDF membranes as described above. Membranes were probed with the appropriate antibodies as described above to detect proteins.

For SLK *in vitro* kinase assays, the immunoprecipitates were also washed once in kinase buffer (0.25 mM NaVO3, 20 mM Tris–HCl pH 7.4, 1 mM NaF, 10 mM β-glycerophosphate, 1 mM DTT, and 15 mM MgCl2) and autophosphorylation reactions were initiated by the addition of 20 µL kinase buffer containing 1 µL [^{32}P] γ-ATP (5 µCi/µL) to each sample followed by a 30 minute incubation at 30°C. The reactions were terminated and protein complexes eluted from the beads by the addition of a sample buffer and boiling. Proteins were electrophoresed and transferred to PVDF membranes as above. Membranes were exposed to X-ray films and target proteins were visualized by probing the membranes with the appropriate antibodies as described above. Densitometry was performed using the ImageJ software (National Institutes of Health, Bethesda, MD) to quantify images obtained from autoradiography.

2.8 Migration assays

Wound closure assays were performed by seeding MEF3T3 cells in 10% FBS DMEM onto fibronectin-coated (10 µg/mL, Invitrogen) 60 mm 6-well dishes following transfections with the desired siRNAs as described above, and incubating the cells at

37°C and 5% CO_2 for 48 h and/or to confluency. Micropipette tips were then used to create two parallel wounds in the cell monolayer and the growth medium was replaced with fresh 10% FBS DMEM. The wounds were then incubated at 37 °C and 5% CO_2. Phase contrast photomicrographs of predetermined and constant locations along the wound edge were obtained at 0 and 6 h post-wounding using a Nikon Eclipse TE2000-U microscope. Fluorescence images of wounds were obtained as described above. Boyden chamber migration assays were performed as previously described (Quizi, Baron et al. 2012). Scratch wound induced migration analysis was performed as described previously (Wagner, Storbeck et al. 2008). In brief, confluent MEF3T3 cell monolayers were serum-starved overnight in 0.25% FBS DMEM and scratched with a micropipette tip until 50% of the cells were removed from the monolayer. The cells were then incubated and collected at various time points up to 2 h post-wounding for biochemical analysis.

2.9 Immunohistochemistry

Tissues were harvested from euthanized individuals as described above and fixed overnight in 10% formalin, then stored in 70% ethanol. The tissues were paraffin embedded and sectioned at a 4 µm thickness. Sections were deparaffinised, rehydrated and antigen retrieval was performed in 10 mM citrate buffer (pH 6.0), followed by quenching in 3% H_2O_2. Sections were probed with primary antibody followed by incubation with horseradish peroxidase-conjugated secondary antibodies and then, incubated with 3, 3'-diaminobenzidine (DAB) substrate. The slides were then stained with hematoxylin, followed by dehydration with ethanol and clearing in xylene. Finally, the slides were mounted. If the sections were only stained with hematoxilin and eosin, following the rehydration, the slides were stained with hematoxilin, followed by eosin.

The protocol continued with dehydration in ethanol and clearing in xylene prior to mounting the slides.

2.10 Statistical analyses

Representative results from one of three independent experiments are shown for all *in vitro* experiments. Error bars represent the mean +/- the standard error of the mean. P-values to assess differences between groups were determined using two-tailed paired sample Student's *t*-test analysis. Significant results were deemed to be p-values < 0.05.

Chapter 3 – Results:

The role of LMO4 in regulating SLK activity

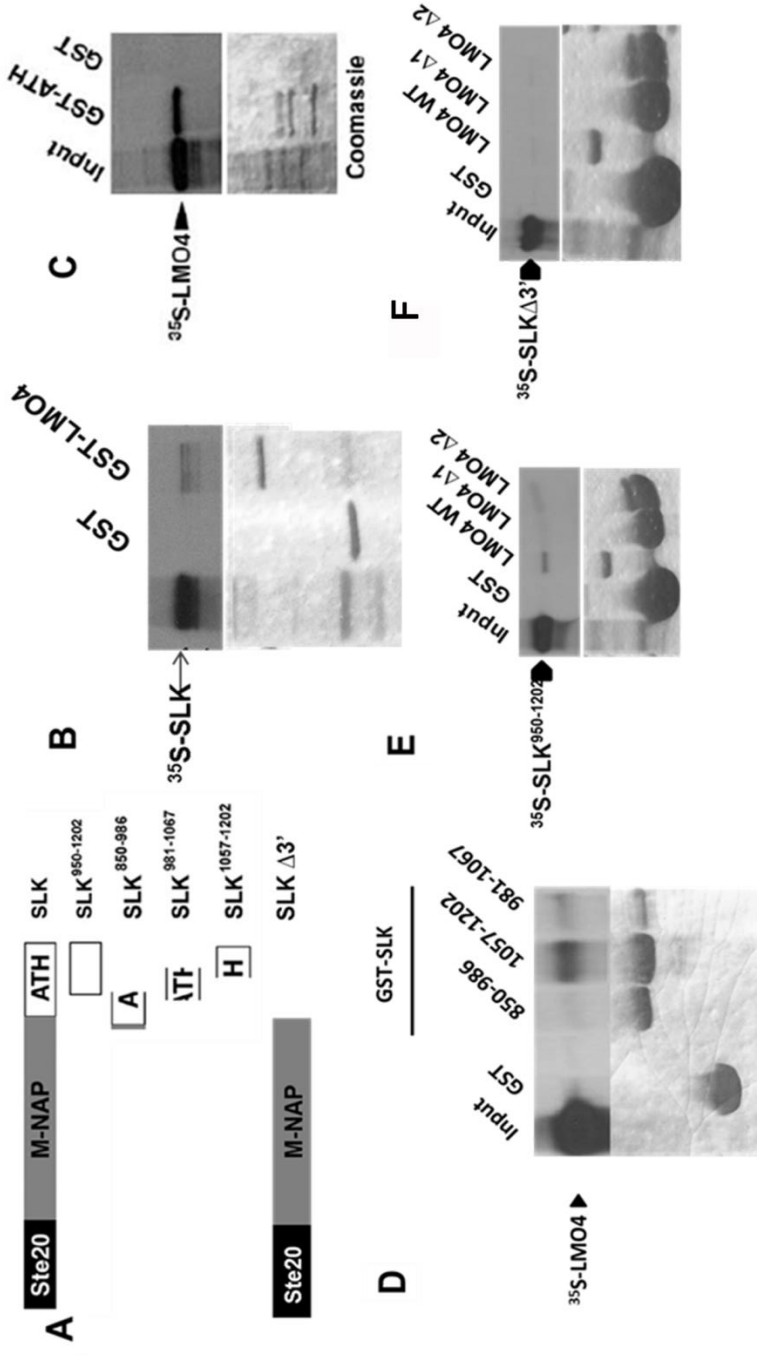
3. Results – The role of LMO4 in regulating SLK activity.

3.1 LMO4 interacts directly with SLK.

We have previously shown that SLK plays a central role in cell migration (Wagner, Flood et al. 2002; Wagner, Storbeck et al. 2008; Wagner and Sabourin 2009; Al-Zahrani, Sekhon et al. 2014) and heregulin-induced motility and invasion (Roovers, Wagner et al. 2009). Interestingly, we have shown that the transcriptional co-activators Ldb1 and Ldb2 (Ldbs) play novel functions in the cytosol by regulating SLK activity and cell migration (Storbeck, Wagner et al. 2009). The Ldbs have been observed to interact with LMO4 (Grutz, Forster et al. 1998; Mizunuma, Miyazawa et al. 2003; Deane, Ryan et al. 2004; Grosveld, Rodriguez et al. 2005). This binding is critical for the organization of transcriptional complexes regulating multiple developmental programs (Mukhopadhyay, Teufel et al. 2003; Meier, Krpic et al. 2006; Wang, Lin et al. 2007; Song, Sun et al. 2009; Narkis, Tzchori et al. 2012; Salmans, Yu et al. 2014). Together, these findings suggest that the LMO4/Ldb complex might regulate numerous cellular processes. As we have shown that SLK activity is regulated by the Ldbs (Storbeck, Wagner et al. 2009), we have tested whether LMO4 is also part of the SLK complex.

To assess whether LMO4 interacts directly with SLK or Ldb1, we performed *in vitro* binding assays using recombinant proteins. Direct GST-pull downs using *in vitro* translated SLK or LMO4 shows that the interaction is mediated by the c-terminal ATH domain of SLK (Figure 4). Specifically, LMO4 preferentially binds to the ATH domain of SLK [amino acids (aa) 950 – 1202] (Figures 4D-G), which is also the site of Ldb1/2 interaction with the kinase (Storbeck, Wagner et al. 2009). Ldb2 preferentially binds to the central portion of the ATH domain (aa 981 – 1067) (Storbeck, Wagner et al. 2009). The interaction between LMO4 and the ATH domain lies within the 1057 – 1202 portion

Figure 4. SLK interacts with LMO4 *in vitro* (Baron, Al-Zahrani et al. 2015). (A) Schematics of full length SLK and the various constructs used in the pull down assays. (B) In vitro translated ^{35}S-labelled SLK was pulled down with a GST-LMO4 fusion construct. (C) In vitro translated ^{35}S-labelled LMO4 was pulled down with a GST-ATH domain construct. (D) In vitro translated ^{35}S-labelled LMO4 interacts preferentially with the C-terminal portion of the ATH region. Normalization to each GST fusion shows a 6- to 8-fold increase in relative binding to GST-SLK 981-1067 when compared to the GST-SLK 850-986 fusion. (E) In vitro translated ^{35}S-labelled ATH domain binds poorly to LMO4 lacking the first or second LIM domain (see Figure 5 for constructs). (F) In vitro translated ^{35}S-labelled SLK lacking the ATH (SLKΔ3') region does not bind to LMO4. Reprinted by permission of Elsevier Ltd.


A
Ste20
M-NAP
ATH
SLK
SLK950-1202
SLK850-986
SLK981-1067
SLK1057-1202
SLK Δ3'
B
GST
GST-LMO4
35S-SLK
C
Input
GST-ATH
GST
35S-LMO4
Coomassie
D
GST-SLK
Input
GST
850-986
1057-1202
981-1067
35S-LMO4
E
35S-SLK950-1202
Input
GST
LMO4 WT
LMO4 Δ1
LMO4 Δ2
F
35S-SLKΔ3'
Input
GST
LMO4 WT
LMO4 Δ1
LMO4 Δ2

of the domain indicating that the site of interaction between the kinase and LMO4 is slightly shifted towards the C-terminal end of the ATH domain (Figure 4E). In addition, our experiments confirmed previous works which determined that LMO4 binds directly to Ldb1 and SLK via its LIM1 domain (Figures 4 & 5) (Grutz, Forster et al. 1998; Mizunuma, Miyazawa et al. 2003; Deane, Ryan et al. 2004; Grosveld, Rodriguez et al. 2005). Deletion constructs eliminating either the LIM1 domain (LMO4 Δ1) or the LIM2 domain (LMO4 Δ2) were constructed to decipher definitively which of the two domains are required for association with Ldb1/2 and SLK (Figures 4F, G & 5C). Deletion of the LIM1 domain of LMO4 abolished the interaction with the ATH domain of SLK; whereas deletion of the LIM2 domain only partially disrupted the interaction (Figure 4F). Previous studies have shown that the LBD domain is required for LMO4 interaction with Ldb1 (Deane, Mackay et al. 2003; Deane, Ryan et al. 2004) and this was confirmed with Ldb2 (Figures 5D & E). Deletion of either LIM1 or LIM2 of LMO4 disrupts the interaction with full length Ldb2 and Ldb2 LBD (Figures 5C, D). Although not tested here, it is therefore likely that in the context of the SLK complex and the Ldbs, this binding could be enhanced by LMO4 (Deane, Mackay et al. 2003; Matthews and Visvader 2003; Deane, Ryan et al. 2004; Matthews, Bhati et al. 2008).

Earlier work in our lab showed that SLK and Ldb1/2 form a complex and co-localize at the membrane ruffle of actively migrating cells (Wagner, Flood et al. 2002; Wagner, Storbeck et al. 2008; Quizi, Baron et al. 2012). As shown in Figure 6A, immunoprecipitation of SLK from exponentially growing MEF3T3 fibroblasts resulted in the co-precipitation of LMO4 suggesting that LMO4 associates with the SLK complex *in vivo*. The co-precipitated LMO4 signal was markedly decreased upon Cre-mediated deletion of LMO4 in conditional fibroblasts, demonstrating the specificity of the anti-LMO4 signal (Figure 6B). Although LMO4 is mostly nuclear, a small fraction was found

Figure 5. LMO4 interacts with both Ldb1 and Ldb2 (Baron, Al-Zahrani et al. 2015). Schematics of in vitro translated ^{35}S-labelled LDB1/2 (A) and GST constructs of LMO4 (B) used in pull down assays. (C) Both LIM domains of LMO4 are required for efficient interaction with Ldb2 in vitro. (D) Deletion of the Ldb2 dimerization domain (DD) markedly reduces its ability to interact with LMO4 (E) Deletion of the Ldb2 LIM binding domain (LBD) abolishes its interaction with LMO4. Identical results were obtained with Ldb1. Reprinted by permission of Elsevier Ltd.

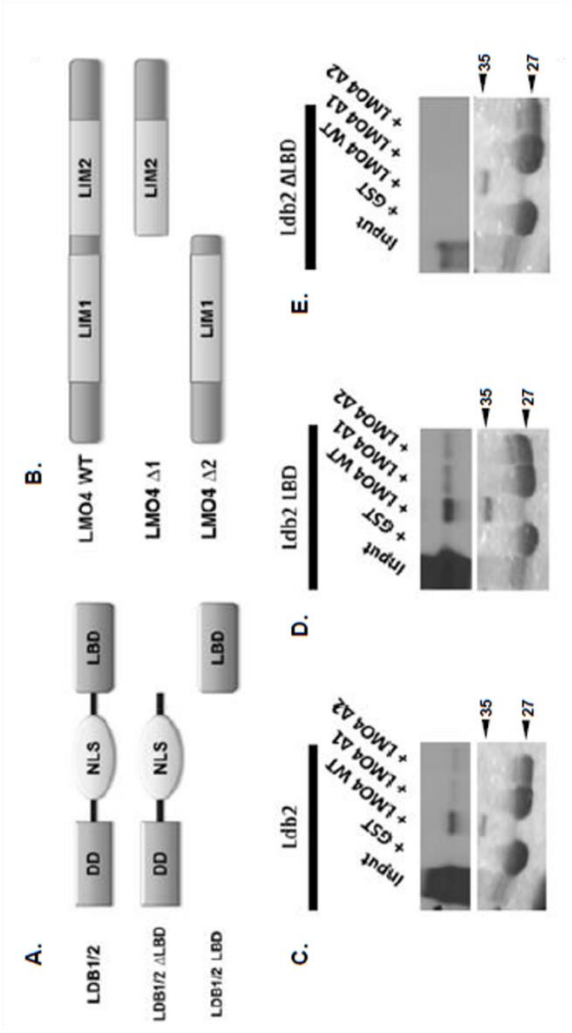
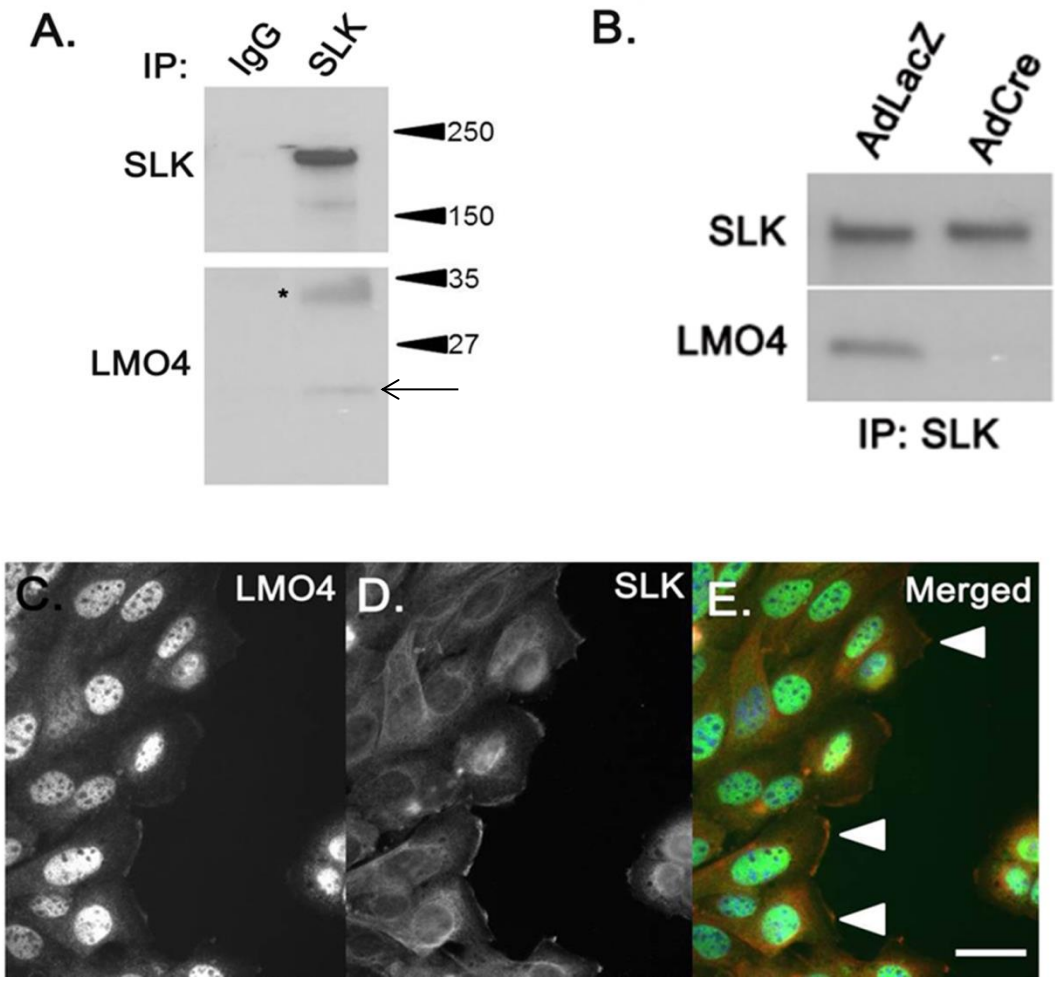

A.
LDB1/2
DD
NLS
LBD
LDB1/2 ΔLBD
DD
NLS
LDB1/2 LBD
LBD
B.
LMO4 WT
LIM1
LIM2
LMO4 Δ1
LIM2
LMO4 Δ2
LIM1
C.
Ldb2
Input
+ GST
+ LMO4 WT
+ LMO4 Δ1
+ LMO4 Δ2
35
27
D.
Ldb2 LBD
Input
+ GST
+ LMO4 WT
+ LMO4 Δ1
+ LMO4 Δ2
35
27
E.
Ldb2 ΔLBD
Input
+ GST
+ LMO4 WT
+ LMO4 Δ1
+ LMO4 Δ2
35
27

Figure 6. LMO4 associates with SLK in vivo and colocalizes with the kinase at the leading edge of migrating cells (Baron, Al-Zahrani et al. 2015). (A) Endogenous SLK was immunoprecipitated (IP) from sub-confluent MEF3T3 cells and blotted back for LMO4 (polyclonal antibody; pAb). LMO4 (arrow) was found to co-precipitate with SLK. A non-specific signal (*) is also observed. The co-precipitated LMO4 signal was abolished following deletion of LMO4 (B). MEF3T3 cells were seeded on fibronectin-coated coverslips, grown to confluency and scratch wounded. Scratched cells were fixed with 4% PFA 60 minutes post-wounding and immunostained. Individual coverslips were stained for LMO4 (monoclonal antibody) (C) and SLK (D). Cells showed co-localization of SLK and LMO4 at the leading edge (arrowheads) (E). Scale bar 10µm. Reprinted by permission of Elsevier Ltd.


A.
IP:
IgG
SLK
SLK
250
150
LMO4
35
27
B.
AdLacZ
AdCre
SLK
LMO4
IP: SLK
C.
LMO4
D.
SLK
E.
Merged

to co-localize with SLK at the leading edge of migrating cells following scratch wounding (Figures 6C - E). As for SLK, LMO4 also co-localized with paxillin in membrane ruffles but not in focal adhesion complexes (Wagner, Storbeck et al. 2008) as well as with Ldb1 at the leading edge of migrating cells (Wagner, Storbeck et al. 2008; Storbeck, Wagner et al. 2009) (Figure 7). The SLK and LMO4 patterns were lost upon SLK knockdown or LMO4 deletion (Figure 8). Together these data suggest that LMO4 interacts with the SLK/Ldb complex *in vivo*.

3.2 LMO4 is required for SLK activity and activation in migrating cells.

Following scratch wounding, SLK is activated and localizes to the leading edge of migrating cells within 60 min (Wagner, Storbeck et al. 2008; Storbeck, Wagner et al. 2009; Quizi, Baron et al. 2012). As LMO4 can directly associate with SLK and localize to the leading edge of cells, we investigated whether this interaction is affected by scratch wounding. Confluent fibroblast monolayers were wounded (Etienne-Manneville and Hall 2001) and SLK immunoprecipitates were surveyed for LMO4 association. Scratch wounding resulted in a 2-fold up-regulation in SLK activity (Figures 9A & B). This was also accompanied with an increase in co-precipitated LMO4. The migration-dependent SLK activation and complex formation were also confirmed in the murine mammary carcinoma cell line 4T1 (Figure 9C). Complex formation peaked at 60 min when kinase activity was maximal. These results suggest that LMO4 is recruited to SLK during cell migration. Although total SLK levels are similar in fibroblasts (not shown), the peak SLK kinase activity is higher in 4T1 cells, suggesting potential differences in cancer cells.

As LMO4 deletion results in SLK down-regulation in exponentially growing cells (Figure 9D), we tested whether the loss of LMO4 would also affect SLK activation following scratch wounding. Although kinase activity was not affected in confluent cells

Figure 7. **LMO4 co-localizes with Ldb1 and Paxillin in membrane ruffles (Baron, Al-Zahrani et al. 2015).** (A-I) MEF3T3 cells were seeded on fibronectin-coated coverslips, fixed with 4% PFA when sub-confluent and immunostained. Individual coverslips were stained for SLK (A) and LMO4 (D and G). Cells showed co-localization of SLK and LMO4 with paxillin (Pxn; C and F) at the leading edge. LMO4-stained cells were also co-stained with Ldb1 (H). In addition to nuclear staining, both SLK-binding proteins could be localized at the leading edge of migrating cells (I). Scale bar 10μm. Reprinted by permission of Elsevier Ltd.

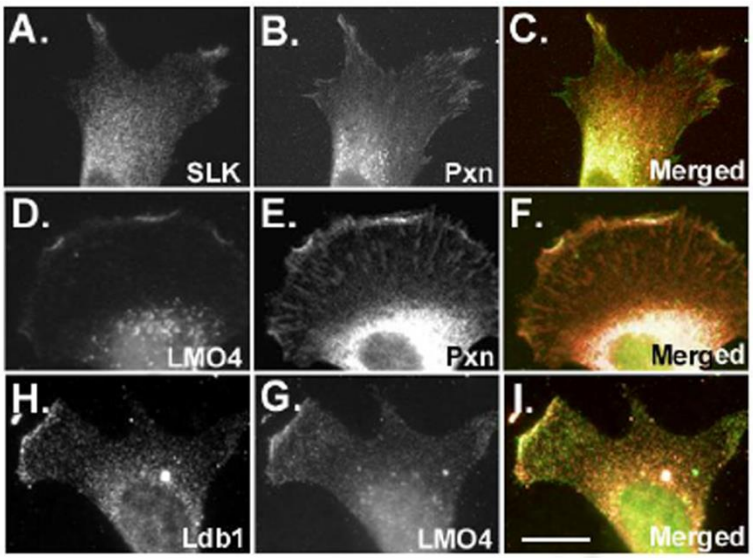

A.
SLK
B.
Pxn
C.
Merged
D.
LMO4
E.
Pxn
F.
Merged
H.
Ldb1
G.
LMO4
J.
Merged

Figure 8. **LMO4 and SLK staining is lost upon knock down or gene deletion (Baron, Al-Zahrani et al. 2015).** MEF3T3 cells were stained for SLK following infection with control adenovirus (A) or an shSLK virus (B). Loss of staining was observed upon expression of AdshSLK. LMO4-floxed fibroblasts were immunostained for LMO4 following infection with a control virus (C) or AdCre (D). Staining was lost upon LMO4 deletion. Reprinted with permission from Elsevier Ltd.

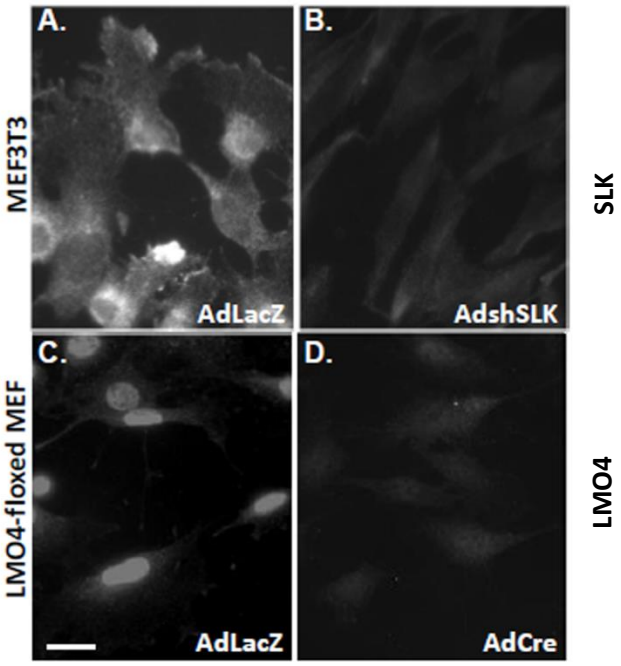
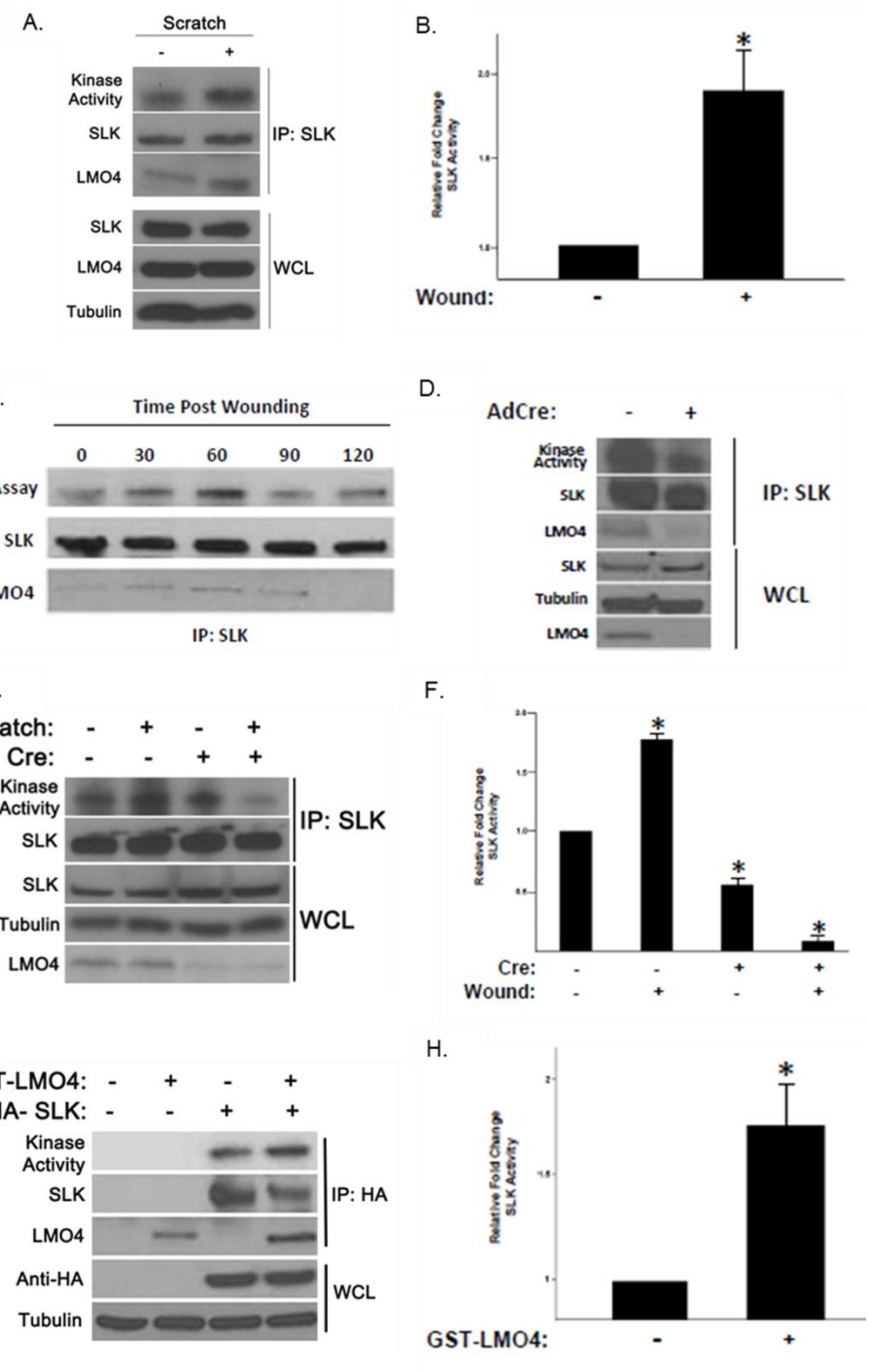

A.
B.
C.
D.
MEF3T3
LMO4-floxed MEF
AdLacZ
AdshSLK
AdLacZ
AdCre
SLK
LMO4

Figure 9. LMO4 is required for SLK activation following scratch wounding (Baron, Al-Zahrani et al. 2015). (A) Confluent monolayers of MEF 3T3 cells were scratch wounded and collected at 0 (-) and 60 (+) minutes post-wounding. Endogenous SLK protein was IP and subjected to an *in vitro* kinase assay. Scratch wounding resulted in an increase in kinase activity as well as higher amounts of co-precipitated LMO4. Blot back for SLK showed equivalent amounts of immunoprecipitated kinase. Whole cell lysates (WCL) immunoblotted for SLK and LMO4 showed no change in expression following wounding. (B) Independent kinase assays (n=3) were subjected to densitometry and the kinase activity signal was normalized to the total SLK in the immunoprecipitations. The averages are shown with standard deviations. SLK activity increased 2-fold following scratch wounding. (C) The murine mammary carcinoma cell line 4T1 grown to confluency was scratch wounded. Samples were obtained at 0, 30, 60, 90 and 120 minutes post-wounding. Endogenous SLK was IP and the *in vitro* kinase assay showed kinase peaking at 60 minutes when LMO4 association is maximal. (D) Subconfluent LMO4-floxed MEFs were infected with AdCre and SLK was IP'd and subjected to an *in vitro* kinase assay. LMO4 deletion resulted in a marked decrease in SLK activity. Co-precipitated LMO4 is also reduced in AdCre-infected samples. Whole cell lysates (WCL) were analyzed by Western blot for the loss of LMO4 protein expression. (E) LMO4-floxed MEF cells were infected with AdCre and allowed to grow to confluency at 37ºC and 5% CO2. Cells were scratch wounded and endogenous SLK was IP and subjected to an *in vitro* kinase assay. LMO4 deletion resulted in a failure to activate SLK following scratch wounding (F), indicating that LMO4 expression is required for SLK activation in migrating cells. Knockdown of LMO4 expression was validated by Western blot analysis of the cell lysates (WCL). (G) HA-SLK was IP from transfected HEK293 cells (12CA5) and subjected to an *in vitro* kinase assay in the presence or absence of recombinant GST-LMO4. WCL were analyzed by Western blot for the presence of transfected HA-tagged protein or tubulin as a loading control. (H) Densitometry showed a 2-fold increase in SLK activity in the presence of GST-LMO4. Kinase assays were performed in triplicates and one representative is shown. Statistical significance was calculated using the Student's T-test (* $p < 0.05$). Modified and reproduced by permission of Elsevier Ltd.


A.
Scratch
-
+
Kinase Activity
SLK
LMO4
IP: SLK
SLK
LMO4
Tubulin
WCL
B.
Relative Fold Change SLK Activity
2.0
1.5
1.0
*
Wound:
-
+
C.
Time Post Wounding
0
30
60
90
120
Kinase Assay
Blot: SLK
Blot: LMO4
IP: SLK
D.
AdCre:
-
+
Kinase Activity
SLK
LMO4
IP: SLK
SLK
Tubulin
LMO4
WCL
E.
Scratch:
-
+
-
+
Cre:
-
-
+
+
Kinase Activity
SLK
IP: SLK
SLK
Tubulin
LMO4
WCL
F.
Relative Fold Change SLK Activity
2.0
1.5
1.0
0.5
*
*
*
Cre:
-
-
+
+
Wound:
-
+
-
+
G.
GST-LMO4:
-
+
-
+
HA- SLK:
-
-
+
+
Kinase Activity
SLK
LMO4
IP: HA
Anti-HA
Tubulin
WCL
H.
Relative Fold Change SLK Activity
2
1.5
1
*
GST-LMO4:
-
+

prior to wounding, Cre-mediated deletion of LMO4 resulted in a failure to activate SLK upon scratch wounding (Figures 9E & F). More importantly, deletion of LMO4 led to a further decrease in SLK activity after wounding, suggesting that LMO4 is required to activate and maintain SLK activity during cell migration. Supporting a role for LMO4 in SLK activation, addition of exogenous GST–LMO4 to SLK immunoprecipitates resulted in a ~2-fold increase in SLK activity when normalized to total SLK levels (Figure 9G & H).

Chapter 4 – Results:

The regulation of SLK-mediated cell migration by LMO4 & Src-family kinases

4. Results – The regulation of SLK-mediated cell migration by LMO4 and Src-family kinases

4.1 LMO4 is required to recruit the Ldb1-SLK complex to the leading edge to facilitate cell migration.

LMO4 has been shown to interact directly with Ldb1 (Matthews and Visvader 2003). In addition, we have shown that the Ldb1/2 co-factors play a role in cell migration and the regulation of SLK kinase activity (Storbeck, Wagner et al. 2009). Therefore, we investigated the effect of LMO4 deletion on cell motility. To investigate the role of LMO4 on cell migration, both siRNA (Figures 10A & B) and Cre-mediated LMO4 deletion (Figures 10C & D) were used. As shown in Figure 10, both Cre recombinase treatment of conditional LMO4 fibroblasts or siRNA knockdown leads to a marked decrease in LMO4 expression combined with a significant delay in wound closure (50–60% reduction) and transwell migration (not shown). Overall, these results suggest that LMO4 is required for SLK activity and efficient cell migration.

The failure to activate SLK in LMO4-deficient cells suggests that its recruitment to the complex is required to induce kinase activity. Alternatively, LMO4 is critical for the localization of SLK at the leading edge, where it can be activated. To investigate this, we performed immunofluorescence for SLK on wounded monolayers 60 min following Cre-mediated deletion of LMO4. As we have previously shown (Wagner, Storbeck et al. 2008; Quizi, Baron et al. 2012), SLK was found to be recruited to the leading edge of migrating cells 60 min following monolayer wounding (Figures 11A & G). Surprisingly, deletion of LMO4 resulted in a marked reduction (~6-fold) in the proportion of cells that displayed SLK staining to the leading edge following scratch wounding (Figures 11D & G), suggesting that LMO4 is necessary for the recruitment of SLK to the front of

Figure 10. LMO4 is required for cell migration (Baron, Al-Zahrani et al. 2015). (A) LMO4 was knocked down using siRNA and confluent monolayers were scratched wounded. The extent of wound closure was photographed 6h post-wounding. Measurements showed a 50% decrease in wound closure upon LMO4 knock down. (B) Western blot showing the expression of LMO4 following a 48h knock down. (C) LMO4-floxed MEF cells were infected with AdCre and allowed to grow to confluency at 37ºC and 5% CO2. Phase contrast photographs of 5 predetermined and constant locations along the wound edge were obtained at 0 and 6 hours post-wounding. Percent closure was calculated from wound width measurements at both time points. Statistical significance was calculated using the Student's T-test (* $p < 0.05$). (D) Knock out of LMO4 expression was validated by Western blot analysis. Reprinted by permission of Elsevier Ltd.

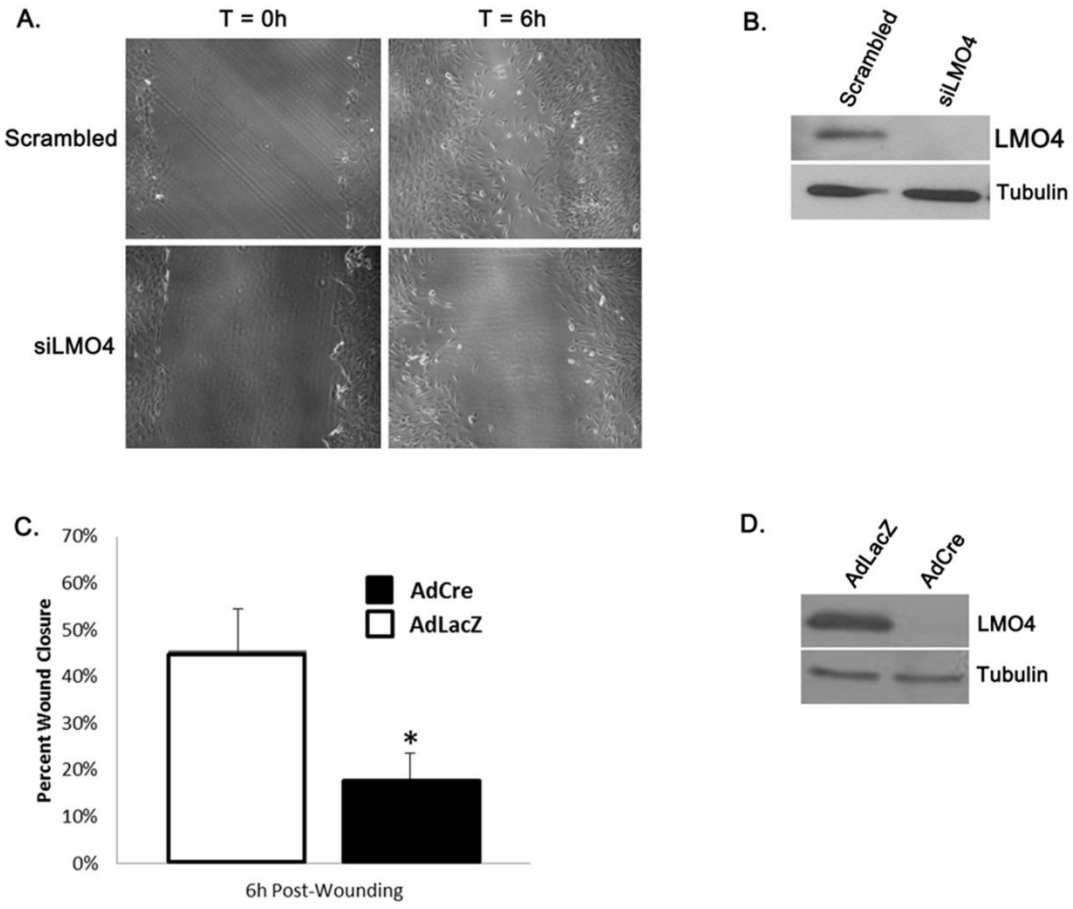
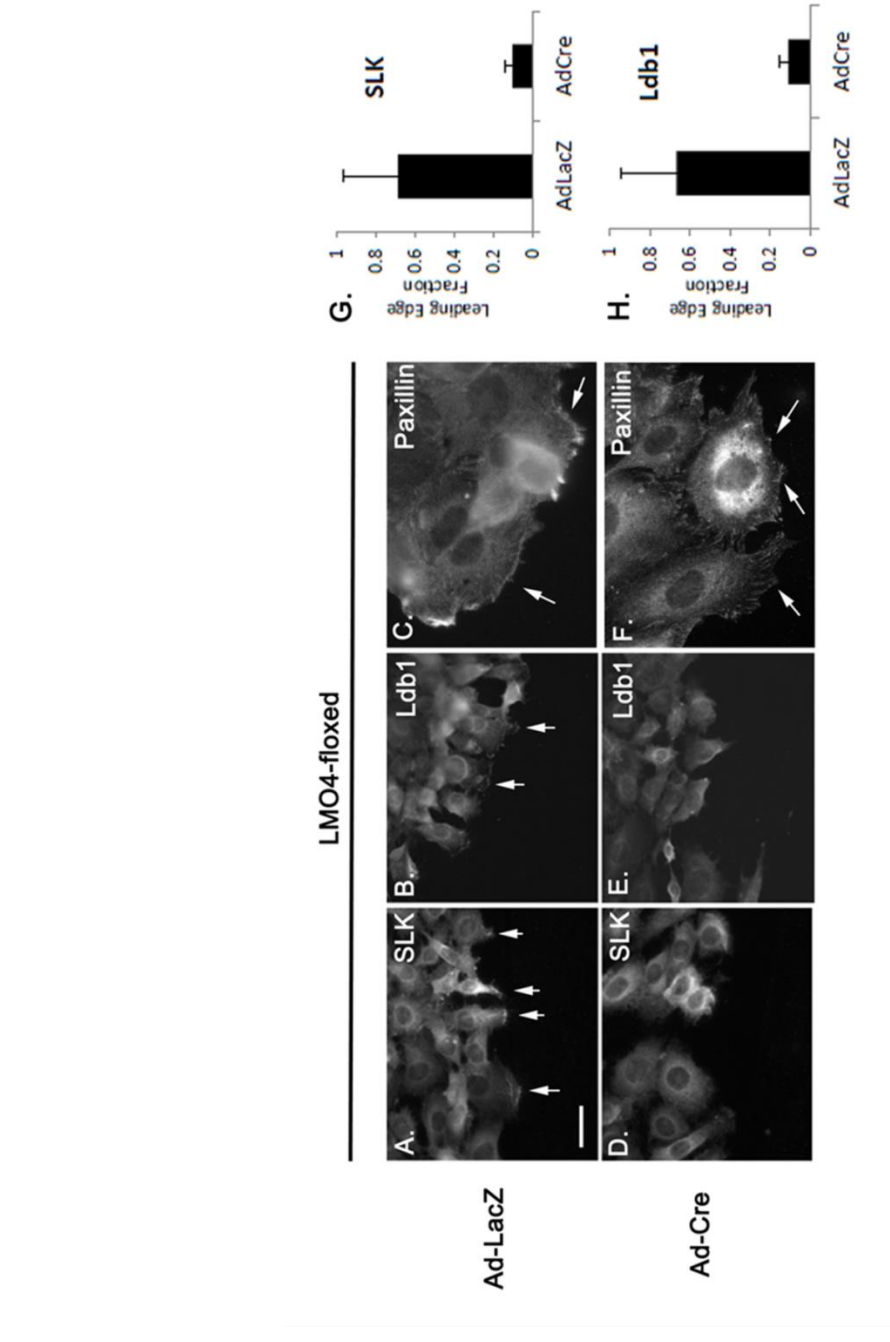

A.
T = 0h
T = 6h
Scrambled
siLMO4
B.
Scrambled
siLMO4
LMO4
Tubulin
C.
70%
60%
50%
40%
30%
20%
10%
0%
Percent Wound Closure
AdCre
AdLacZ
*
6h Post-Wounding
D.
AdLacZ
AdCre
LMO4
Tubulin

Figure 11. LMO4 is required for SLK and Ldb1 localization at the leading edge of migrating cells (Baron, Al-Zahrani et al. 2015). LMO4-floxed MEF cells were seeded on fibronectin-coated coverslips, infected with AdCre or control (AdLacZ; -Cre), and allowed to grow to confluency. Cell monolayers were scratched and incubated for 60 minutes prior to fixing using 4% PFA. Uninfected (wildtype) cells showed SLK (A), Ldb1 (B) and paxillin (C) staining at the leading edge of migrating cells (arrows) whereas positive staining was markedly reduced in cells lacking LMO4 (D and E). Paxillin was found predominantly in focal adhesions (arrowheads) in LMO4 deleted cells (F). Scale bar is at 10µm for panels A-E and 20µm for panel C and F. (G and H). The proportion of cells with positive staining for either SLK (G) or Ldb1 (H) at the leading edge was quantified. The percentage of positive cells was calculated by counting the number of cells at the wound face with positive staining in 6 individual fields of view and statistical significance was found in both cases using the Student's T-test (* $p < 0.05$). Reprinted by permission of Elsevier Ltd.


LMO4-floxed
Ad-LacZ
Ad-Cre
A.
SLK
B.
Ldb1
C.
Paxillin
D.
SLK
E.
Ldb1
F.
Paxillin
G.
SLK
leading Edge fraction
1
0.8
0.6
0.4
0.2
0
AdLacZ
AdCre
H.
Ldb1
leading Edge fraction
1
0.8
0.6
0.4
0.2
0
AdLacZ
AdCre

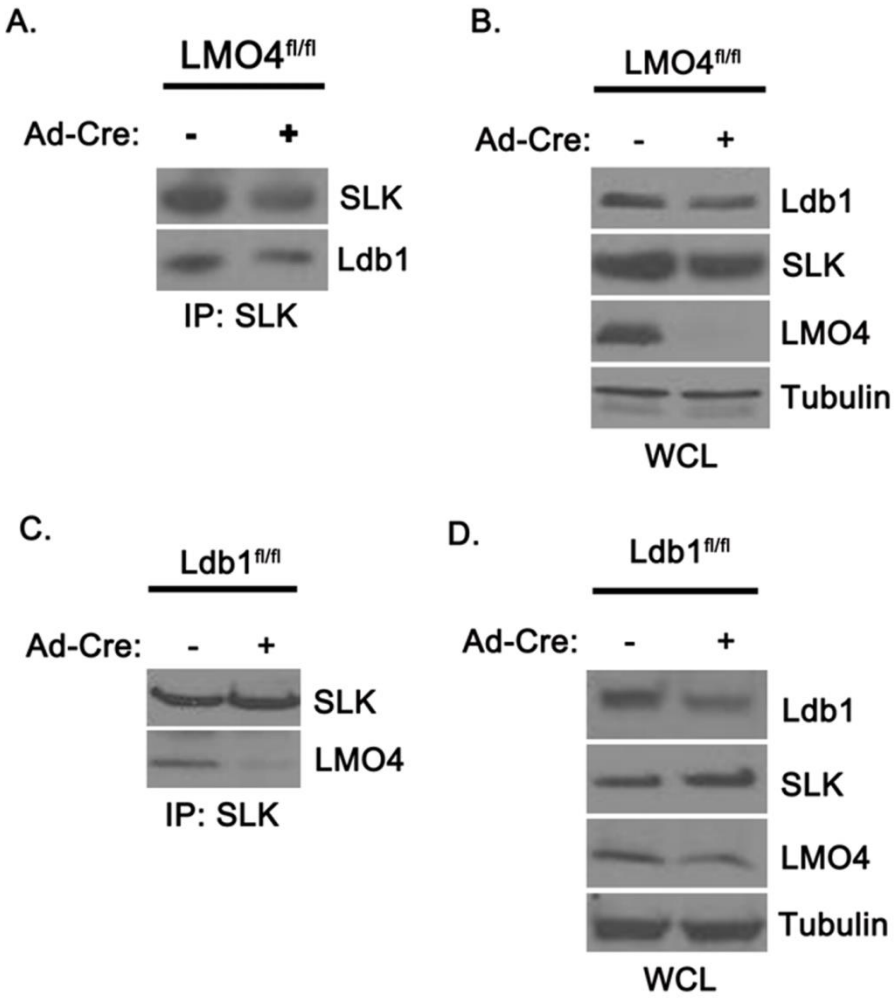
migrating cells. Our previous findings showed that the Ldb1/2 co-activators directly interact with SLK to regulate its kinase activity and cell migration (Storbeck, Wagner et al. 2009). Interestingly, staining for Ldb1 also revealed a 5-fold decrease in the proportion of cells recruiting it to the leading edge (Figures 11E & H), suggesting that LMO4 deletion also alters the recruitment of SLK-associated proteins. Although wild-type cells showed focal adhesion and lamellipodial staining of paxillin, Cre-mediated deletion of LMO4 resulted in enlarged focal adhesions with an absence of paxillin-positive lamellipodiae, suggesting that focal adhesion turnover is impaired (Figures 11C & F). This has also been observed in SLK-deficient cells (Wagner, Storbeck et al. 2008; Wagner and Sabourin 2009; Quizi, Baron et al. 2012). Together, these data suggest that LMO4 is required to recruit the SLK complex to the leading edge of migrating cells. Furthermore, the LMO4-mediated localization of SLK at the leading edge appears to be critical for its activation during cell migration.

As LMO4 deletion also impairs the recruitment of Ldb1 to the leading edge, we tested whether the SLK–Ldb1 association was affected by the loss of LMO4. LMO4-floxed fibroblasts growing exponentially were infected with AdCre and subjected to IP-westerns. Deletion of LMO4 did not affect the ability of Ldb1 to associate with the SLK complex *in vivo* (Figures 12A & B). However, deletion of Ldb1 in conditional fibroblasts abrogates the ability of LMO4 to interact with SLK *in vivo* (Figures 12C & D), suggesting that Ldb1 is critical for the LMO4–SLK interaction.

4.2 LMO4 expression and SLK recruitment to the leading edge require c-Src activity.

We have previously observed a defect in SLK leading edge recruitment in cells lacking the Src-family kinases c-Src, Yes and Fyn [SYF;

Figure 12. Ldb1 deletion impairs SLK-LMO4 association (Baron, Al-Zahrani et al. 2015). (A) LMO4 was deleted from LMO4-floxed fibroblasts by the addition of Ad-Cre and SLK immunoprecipitates were probed for Ldb1. The knock out of LMO4 had no effect on the interaction between SLK and Ldb1. (B) Whole cell lysates (WCL) were probed for proteins in the SLK complex. LMO4 expression was undetectable. (C) Ldb1 was deleted from Ldb1 conditional fibroblasts by the addition of Ad-Cre and SLK immunoprecipitates were assessed for LMO4. Knock out of Ldb1 ablated the association between SLK and LMO4. (D) Probing of whole cell lysate (WCL) showed marked Ldb1 knock down but no change in SLK or LMO4. Reprinted by permission of Elsevier Ltd.


A.
LMO4fl/fl
Ad-Cre: - +
SLK
Ldb1
IP: SLK
B.
LMO4fl/fl
Ad-Cre: - +
Ldb1
SLK
LMO4
Tubulin
WCL
C.
Ldb1fl/fl
Ad-Cre: - +
SLK
LMO4
IP: SLK
D.
Ldb1fl/fl
Ad-Cre: - +
Ldb1
SLK
LMO4
Tubulin
WCL

(Klinghoffer, Sachsenmaier et al. 1999; Wagner, Storbeck et al. 2008)]. In SYF cells, re-expression of c-Src rescued SLK localization upon wounding (Wagner, Storbeck et al. 2008). Therefore, we investigated whether SYF cells were also deficient in LMO4 redistribution to the leading edge of migrating cells. Surprisingly, Western blot analysis revealed that SYF cells expressed markedly reduced levels of LMO4 (Figure 13A). Re-expression of c-Src restored LMO4 expression back to levels comparable to those detected in MEF3T3 fibroblasts (Figure 13B), suggesting that c-Src activity is required to maintain LMO4 expression. Although the LMO4 exposure times are 10 times longer than SLK, neither protein could not be detected at the leading edge of migrating SYF cells (Figure 13C & F) or MEF3T3 treated with the Src inhibitor PP2 (Figures 14A & B). These data suggest that, in SYF cells or cells lacking active c-Src, the low levels of LMO4 are not sufficient to drive SLK recruitment to the leading edge. To test this, epitope-tagged Myc–LMO4 was expressed in SYF cells and the SLK and LMO4 distributions were assessed by immunofluorescence. Staining with LMO4 or anti-Myc antibodies (Figure 13I & J) revealed that Myc–LMO4 was expressed in SYF cells and localized to the leading edge in addition to the nucleus (Figure 13D). Similarly, c-Src re-expression restored LMO4 levels and leading edge staining (Figure 13B & E). This suggests that LMO4 expression requires Src-family kinases but that its recruitment to the leading edge is Src-independent.

Supporting a role for LMO4 in the localization of the SLK complex at the leading edge, expression of Myc–LMO4 in SYF cells restored SLK staining at the front of migrating cells (Figure 13G), similar to the pattern seen in cells re-expressing c-Src (Figure 13E & H). These data strongly suggest that Src-family kinases are required for LMO4 expression which in turn recruits the SLK complex to the leading edge of migrating cells. To determine whether Src family kinase activity is required for SLK

Figure 13. c-Src regulates LMO4 protein levels, SLK subcellular localization in migrating cells and the LMO4-SLK interaction (Baron, Al-Zahrani et al. 2015). (A) Src/Yes/Fyn-deficient (SYF) cells and SYF+c-Src populations were immunoblotted to evaluate LMO4 protein levels. The SYF cell line was found to express very low levels of LMO4 protein that were restored upon c-Src or Myc-LMO4 re-expression (B). (C, D, F and G) SYF cells were seeded on fibronectin coated coverslips, transfected with Myc-LMO4 (D, G) and allowed to grow to confluency. The monolayers were scratched and incubated for 60 minutes prior to staining for LMO4 (C, D) or SLK (F, G). No SLK or LMO4 staining could be detected at the leading edge of untransfected SYF cells (C, F). Capture time for LMO4 was 10 times longer in untransfected SYF cells than that of SLK or Myc-LMO4 (600 ms vs 60 ms). SLK leading edge staining was restored upon expression of exogenous LMO4 (arrowheads in D and G). (E and H) SYF+c-Src cells on fibronectin-coated coverslips show SLK and LMO4 staining in membrane ruffles and leading edge. Scale bar, 20µm. (I and J) SYF cells transfected with Myc-LMO4 were immunostained with an anti-Myc (9E10) to assess LMO4 localization. Modified and reprinted by permission of Elsevier Ltd.

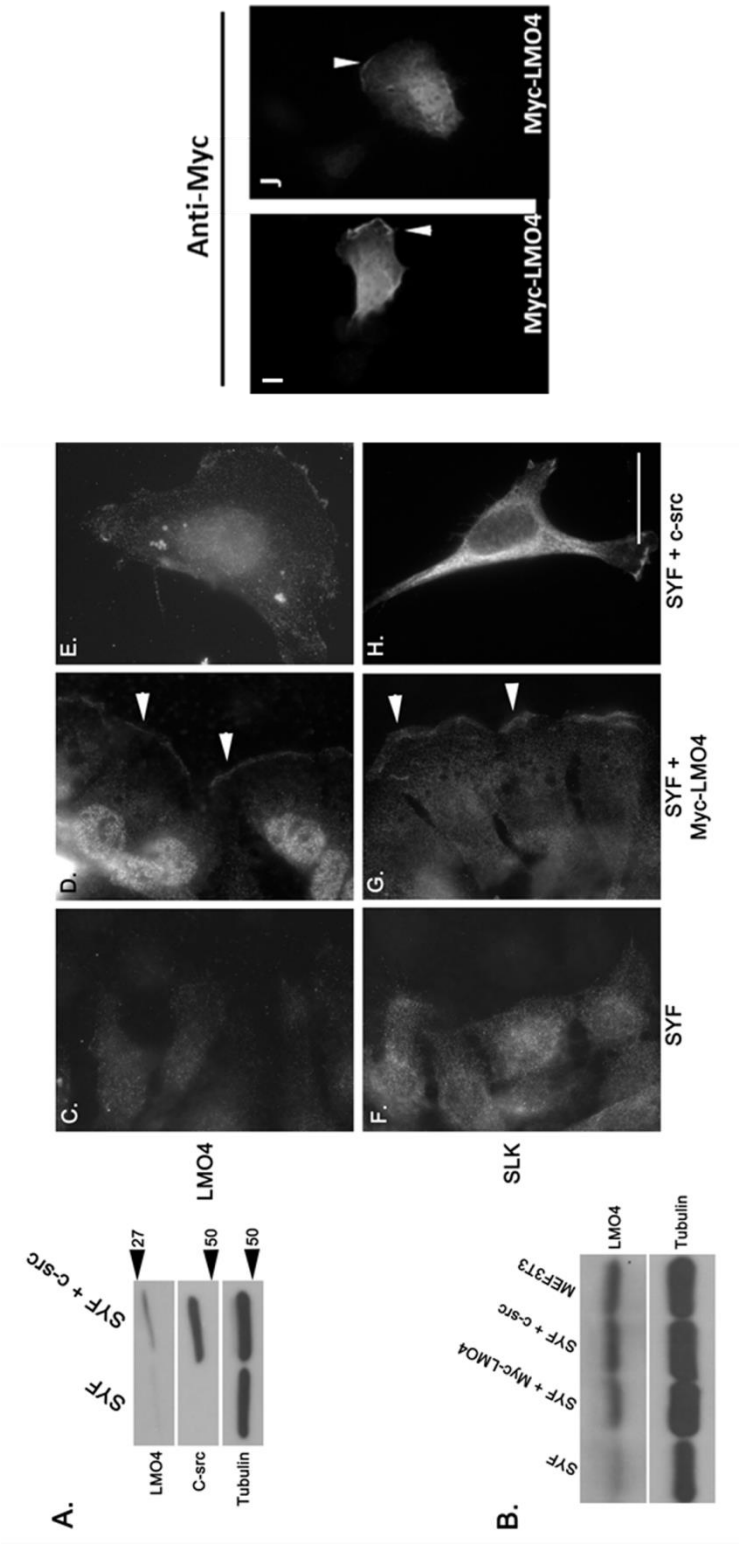
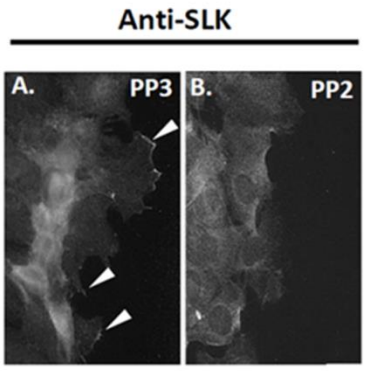
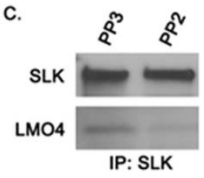
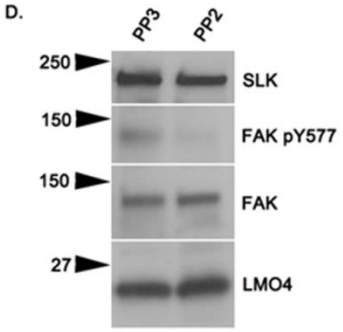
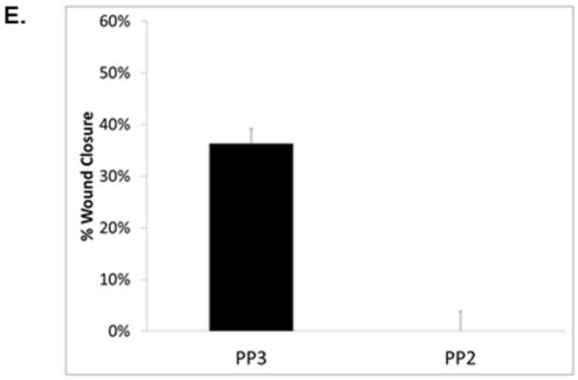

A.
LMO4
C-src
Tubulin
SYF
SYF + c-src
27
50
50
LMO4
SLK
B.
LMO4
Tubulin
SYF
SYF + Myc-LMO4
SYF + c-src
MEF3T3
C.
D.
E.
F.
G.
H.
SYF
SYF + Myc-LMO4
SYF + c-src
Anti-Myc
I
J
Myc-LMO4
Myc-LMO4

Figure 14. SLK recruitment and interaction with LMO4 is Src-dependent (Baron, Al-Zahrani et al. 2015). Confluent MEF3T3 monolayers were pre-treated with the Src inhibitor PP2 (A) or control PP3 (B) (1h, 10μM) and scratch wounded and immunostained for SLK. PP2-treated cells did not show positive SLK staining at the leading edge of cells (B) in contrast to control cultures (A) (arrowheads). (C) Scratch wounded MEF3T3 monolayers pre-treated with the src inhibitor PP2 or control PP3 (1h, 10 μM) were allowed to recover for 60 min in the presence of inhibitors. Endogenous SLK was immunoprecipitated (SLK IP) and blotted back for LMO4. (D) Whole cell lysate (WCL) was probed with anti-FAK pY577 to confirm Src inhibition in the PP2-treated cells. No change in LMO4 expression was observed. (E) Quantitation of wound closure (n=3) in control (PP3) or PP2-treated MEF3T3 cells were obtained at 0 and 6 hours post-wounding. Percent closure was calculated from wound width measurements at predetermined locations and statistical significance was calculated using the Student's T-test (* $p < 0.005$). Modified and reprinted by permission of Elsevier Ltd.


Anti-SLK
A.
PP3
B.
PP2


C.
PP3
PP2
SLK
LMO4
IP: SLK

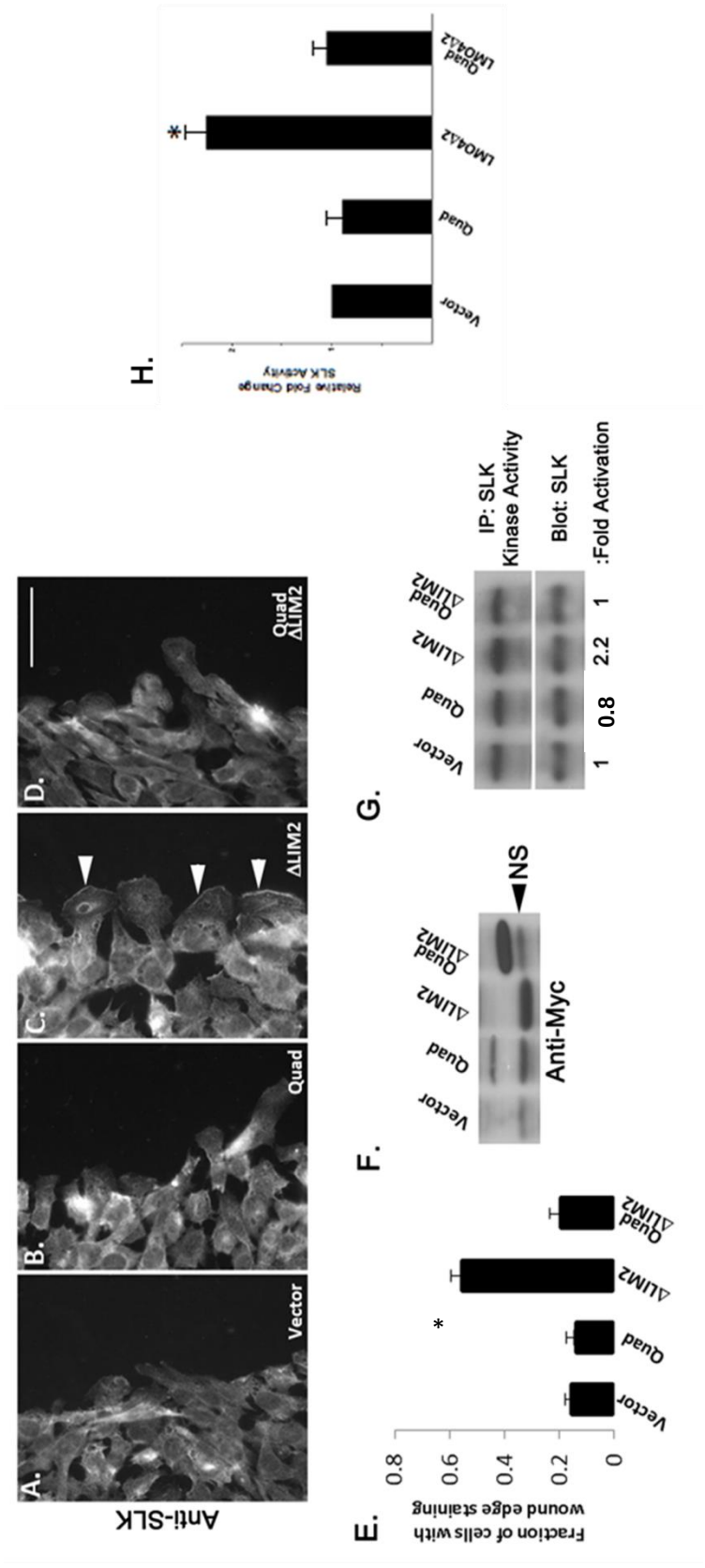

D.
PP3
PP2
250
SLK
150
FAK pY577
150
FAK
27
LMO4


E.
60%
50%
40%
30%
20%
10%
0%
% Wound Closure
PP3
PP2

localization, confluent MEF3T3 cells were treated with the Src inhibitor PP2 or its control PP3, scratched wounded and allowed to migrate for 60 min in the presence of the inhibitor or control. Immunofluorescence showed that SLK was detected at the leading edge of cells treated with PP3 (Figure 14A) but not in cells treated with the Src inhibitor PP2 (Figure 14B). Immunoprecipitation of SLK in PP2 treated cells showed a marked reduction in SLK-associated LMO4 (Figure 14C). Whole cell lysate analysis showed that short-term inhibition of c-Src activity was not sufficient to decrease LMO4 protein expression but resulted in a block of FAK tyrosine phosphorylation at the c-Src target site (Y577), indicating that c-Src activity was inhibited (Figure 14D). In addition, PP2 treatment also inhibited MEF3T3 cell migration (Figure 14E). These results indicate that SLK recruitment at the leading edge and cell migration are dependent upon LMO4 association with SLK, which is regulated by c-Src activity.

To better define the domains that mediate the recruitment of the SLK complex, LMO4 mutants and truncations were expressed in SYF cells and the proportion of cells recruiting SLK to the leading edge was quantified from random fields along the wound edge. Critical LMO4 residues involved in the Ldb1–LMO4 interface have been identified (Deane, Ryan et al. 2004). Expression of a quadruple LMO4 point mutant (Quad) that lacks critical LIM1 domain residues for interaction with the Ldb1–LDB domain (Deane, Ryan et al. 2004) could not rescue SLK recruitment (Figures 15B & E). However, expression of a mutant that lacked the second LIM domain (LMO4Δ2) showing interaction with both SLK and Ldb1/2 [Figures 4 & 5 and (Deane, Ryan et al. 2004)] resulted in SLK re-localization to the leading edge (Figures 15C & E). Expression of a Quad-LMO4Δ2 mutant binding to SLK but not to Ldb1 could not rescue SLK relocalization. These results suggest that SLK recruitment is dependent on its direct interaction with LMO4 but also requires LMO4 binding to Ldb1/2. Supporting the

Figure 15. SLK recruitment and activation requires the LMO4 LIM1 domain (Baron, Al-Zahrani et al. 2015). (A – D) SYF cells were seeded on fibronectin-coated coverslips and following overnight incubation, were transfected with empty vector or LMO4 constructs (Quad, LMO4Δ2 or QuadLMO4Δ2). Confluent coverslips were scratch wounded and incubated for 60 minutes at 37ºC prior to fixing and staining for SLK. SLK protein was found to be absent from the leading edge in the vector- (A), Quad- (B), and QuadLMO4Δ2- (D) expressing SYF cells. Positive leading edge staining was observed in LMO4Δ2-expressing cells (C). (E) Quantification of positively stained cells was obtained by counting the number of SLK-positive leading edge cells at the wound face in 10 fields of view and statistical significance was calculated using the Student's t-test (* $p < 0.01$). (F) Western blot analysis showing expression of the LMO4 constructs in transfected SYF cells. A non-specific (NS) anti-myc reactive band was consistently observed in all samples. (G) SLK kinase assays showing the relative fold-increase in kinase activity upon expression of the various LMO4 constructs and scratch wounding. Kinase assays were performed in triplicates and one representative is shown. Transfection of the LMO4Δ2 constructs increased SLK kinase activity 2.2-fold. (H) Quantitation of endogenous SLK activity following expression of various LMO4 deletion constructs. Statistical significance was calculated using the Student's T-test (* $p < 0.05$). Modified and reprinted by permission of Elsevier Ltd.

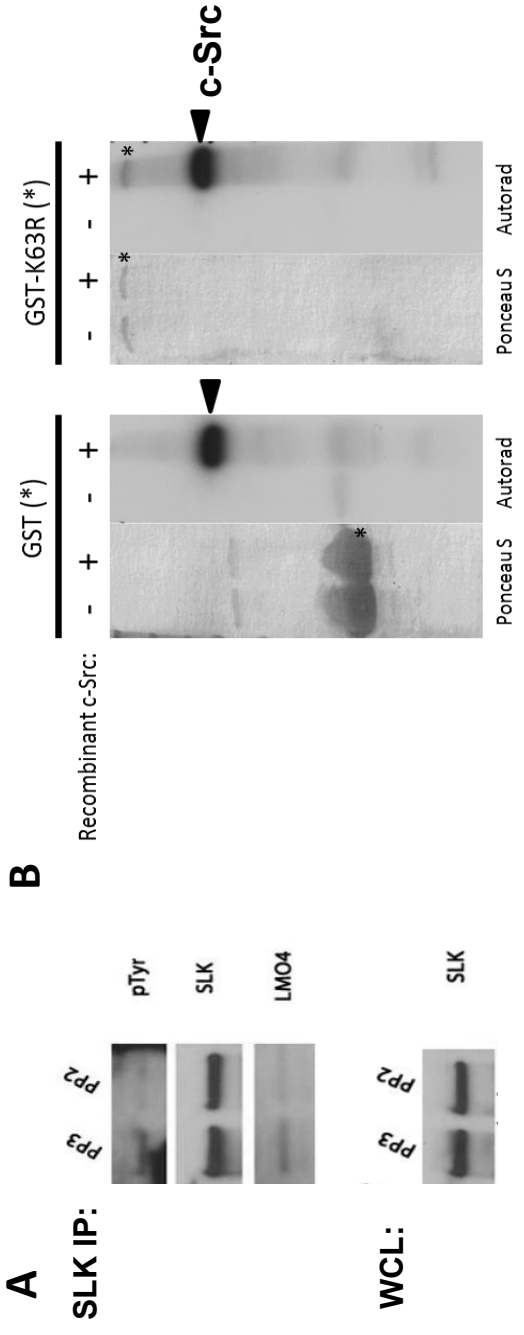

A.
Vector
B.
Quad
C.
ΔLIM2
D.
Quad ΔLIM2
Anti-SLK
E.
Fraction of cells with wound edge staining
0
0.2
0.4
0.6
0.8
Vector
Quad
ΔLIM2
Quad ΔLIM2
*
F.
Anti-Myc
NS
Vector
Quad
ΔLIM2
Quad ΔLIM2
G.
IP: SLK Kinase Activity
Blot: SLK
:Fold Activation
1
0.8
2.2
1
Vector
Quad
ΔLIM2
Quad ΔLIM2
H.
Relative Fold Change SLK Activity
Vector
Quad
LMO4Δ2
Quad LMO4Δ2
*

observation that the LMO4Δ2 mutant can rescue SLK recruitment, SLK activation following wounding was also restored in SYF cells expressing this mutant (Figures 15G & H). These results are also in agreement with the observation that deletion of Ldb1 results in a loss of interaction between SLK and LMO4 (Figure 12C).

4.3 Phosphorylation of SLK by c-Src may modulate the SLK-LMO4 interaction.

Src-dependent activation and localization of the kinase may be a result of indirect signaling or direct phosphorylation of the kinase. The target sequence motif for c-Src has been documented as Tyr-X-X-Ile/Pro (Songyang, Shoelson et al. 1993). A c-Src target site on SLK has not previously been confirmed in our lab. A mass spectrometry screen using the N-terminal region of the kinase (1-373) did not turn up any possible target sites in this region (data not shown). It is possible that c-Src could be phosphorylating SLK on a tyrosine residue located at the C-terminal end of the ATH domain (Y1182) which follows the c-Src consensus motif as it lies within a Tyr-Pro-Ile-Pro sequence. As this is within the site of LMO4-SLK interaction (aa 1057 – 1202; Figure 4), perhaps this phosphorylation triggers LMO4 binding to the kinase in order for SLK to localize to the membrane ruffle and activate in response to cell migration cues. To address this hypothesis, treatment with the c-Src inhibitor PP2 not only prevented co-precipitation of LMO4 with the kinase (Figure 14D & 16A), a pan-phospho-tyrosine antibody showed a decrease in signal from SLK in the PP2-treated sample as compared with the sample treated with the small molecule control PP3 (Figure 16A). In addition, an *in vitro* experiment was designed where the glutathione (GST)-tagged kinase-dead SLK mutant (K63R; it is unable to auto-phosphorylate (Sabourin and Rudnicki 1999)) was purified and incubated with recombinant c-Src and radiolabelled γP-ATP. The addition of c-Src caused an increase in the level of phosphorylated K63R (Figure 16B). These results

Figure 16. c-Src phosphorylates SLK *in vitro*, regulating the association between LMO4 and the kinase. (A) Sub-confluent MEF3T3 cells were treated with the small molecule c-Src inhibitor PP2 or its control PP3 (both at 10 µM) for two hours at 37ºC prior to harvesting. Endogenous SLK was immunoprecipitated and blotted back for LMO4 as well as with a pan-phospho-tyrosine monoclonal antibody. Inhibition of c-Src resulted in a reduction in both kinase-associated LMO4, as well as phospho-tyrosine signal (pTyr), suggesting that active c-Src is required for SLK-LMO4 complex formation through possible phosphorylation of the kinase. (B) Recombinant c-Src (arrowheads) was incubated for 30 minutes at 30ºC with GST alone (*) and GST-tagged SLK kinase-dead mutant (K63R) that is unable to autophosphorylate (*). c-Src showed the ability to phosphorylate the K63R mutant while it did not phosphorylate GST alone, indicating that c-Src may target SLK for phosphorylation.

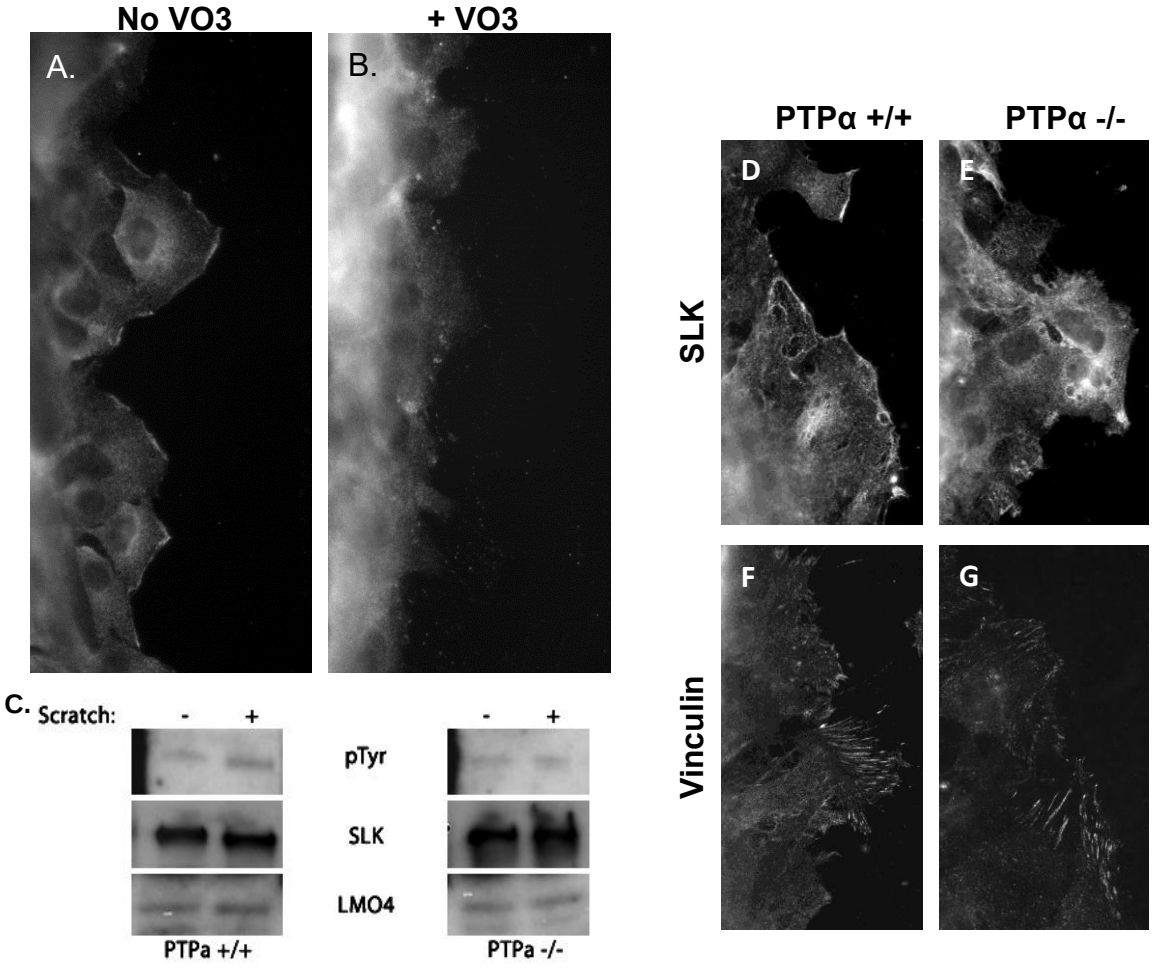

A
SLK IP:
pTyr
SLK
LMO4
PP3
PP2
WCL:
SLK
PP3
PP2
B
Recombinant c-Src:
GST (*)
- + - +
Ponceau S
Autorad
GST-K63R (*)
- + - +
Ponceau S
Autorad
c-Src

suggest that c-Src may phosphorylate SLK *in vivo* and *in vitro*. In order to determine whether Y1182 is a true c-Src target site, this tyrosine residue was mutated to an unphosphorylatable phenylalanine (F) residue. The GST-tagged ATH Y1182F mutant was subjected to the *in vitro* kinase assay with recombinant c-Src and radiolabeled γP^{32}-ATP. It would be expected that c-Src would be unable to phosphorylate the ATH fragment if Y1182 was the target site, however this experiment showed that c-Src phosphorylated the mutant ATH fragment, indicating that Y1182 is not phosphorylated by c-Src (data not shown). As this site is the only putative c-Src consensus site in the SLK amino acid sequence, this would suggest that c-Src phosphorylates the kinase on a non-consensus site or that the phosphorylation is indirect, through another signaling kinase.

4.4 PTPα regulates the c-Src-dependent SLK-LMO4 association and complex localization in migrating cells.

Further investigation into the mechanism of c-Src regulation of the LMO4-SLK complex in response to migratory cues revealed an upstream enzyme which results in impaired cell migration, actin fiber dissolution, and impaired focal adhesion turnover when deleted (Su, Muranjan et al. 1999; Zeng, Si et al. 2003; Cheng, Sun et al. 2014), similar to the deletion of SLK or the lack of SLK activity (Wagner, Flood et al. 2002; Wagner, Storbeck et al. 2008; Quizi, Baron et al. 2012). Protein-tyrosine phosphatase-α (PTPα) is activated during cell migration through integrin-induced phosphorylation in response to migratory signals (Chen, Chen et al. 2006). Recently, PTPα has been shown to mediate the activation of c-Src, through the dephosphorylation of its inhibitory phosphate on the Y527 residue. The dephosphorylation of Y527 is required for the downstream phosphorylation of FAK on Y397, leading to the formation of the Src-FAK complex and full activation of the Src-FAK kinase complex to orchestrate cytoskeletal

Figure 17. Protein tyrosine phosphatase α acts upstream of c-Src to regulate the SLK-LMO4 complex formation, SLK phosphorylation and intracellular localization. (A and B) Confluent MEF3T3 monolayers were treated for two hours with 10μM of the pan-tyrosine phosphatase inhibitor sodium orthovanadate (VO3) prior to scratch wounding and incubated for 60 minutes at 37ºC in the presence of VO3 post-wounding. Cells were fixed with 4% PFA and stained with the anti-SLK polyclonal antibody. Treatment with the tyrosine phosphatase inhibitor prevented the localization of SLK at the leading edge of migrating cells (B) in contrast to untreated cells (A). (C) Confluent PTPα +/+ and PTPα -/- monolayers were scratch wounded and incubated for 60 minutes at 37ºC. Cells were harvested and endogenous SLK was immunoprecipitated. Wild-type cells showed an increase in phospho-tyrosine (pTyr) signal from SLK and increased amounts of associated LMO4 in the scratched sample, whereas PTPα -/- cells showed neither in its scratched sample. (D – G) Confluent PTPα +/+ and PTPα -/- monolayers were scratch wounded on coverslips and incubated at 37ºC for 60 minutes, prior to fixing with 4% PFA. Cells were stained with either anti-SLK (D, E) or anti-vinculin (F, G). SLK was present at the leading edge of migrating cells in the PTPα +/+ cells (D) but not in the PTPα -/- cells (E). Vinculin staining showed no change between either cells (F, G). Magnification 400x.


No VO3
+ VO3
A.
B.
C.
Scratch:
-
+
pTyr
SLK
LMO4
PTPa +/+
PTPa -/-
PTPα +/+
PTPα -/-
D
E
F
G
SLK
Vinculin

reorganization, FA turnover, and ultimately, cell migration (Cheng, Sun et al. 2014). To determine whether a phosphatase is involved in the Src signaling mechanism seen here, MEF3T3 cell monolayers were treated with the nonspecific tyrosine phosphatase inhibitor sodium orthovanadate ($NaVO_3$) prior to scratch wounding and staining for SLK localization. Immunofluorescence showed that treatment with $NaVO_3$ prevented the redistribution of SLK to the leading edge of migrating cells (Figure 17B). In order to specifically target the role of PTPα, we obtained PTPα +/+ and -/- fibroblasts from Dr. C. Pallen (Children & Family Research Institute, University of British Columbia). Scratch wounding of cell monolayers showed that LMO4 could not associate with SLK in the PTPα -/- cells, nor was the phospho-tyrosine signal on SLK increased following scratch activation (Figure 17C). Furthermore, immunofluorescence showed that SLK does not localize to the leading edge of the PTPα -/- cells when stimulated to migrate (Figure 17E). Vinculin staining showed no change in these cells (Figures 17F and G). These data support the hypothesis that the LMO4 association with SLK, activation of SLK, and recruitment of the SLK-LMO4 complex to the leading edge of migrating cells is dependent upon activated c-Src, downstream of PTPα and perhaps, a combination of signaling molecules.

Chapter 5 – Results:

The effect of LMO4 deletion on murine mammary tumorigenesis

5. Results – The effect of LMO4 deletion on murine mammary tumorigenesis

5.1 LMO4 expression in the developing mammary gland and ErbB2-positive tumors

LMO4 is widely expressed within murine tissues, mostly in cells of epithelial lineage and especially in proliferating epithelial cells (Sum, O'Reilly et al. 2005). As such, overexpression of the protein in transgenic mouse models results in mammary epithelial hyperplasia and intraepithelial neoplasias (Sum, Segara et al. 2005). LMO4 (-/-) mice are viable and pregnancy-associated phenotypes have been reported within the mammary gland epithelium (Visvader, Venter et al. 2001; Wang, Kudryavtseva et al. 2004; Sum, Shackleton et al. 2005). Because LMO4 overexpression in the mammary epithelium results in a hyperplastic phenotype, we investigated the effect of LMO4 deletion on mammary tumor initiation and progression. LMO4 flox/flox ($LMO4^{fl/fl}$) animals were crossed with mice overexpressing a mouse mammary tumor virus (MMTV)-driven Cre recombinase (MMTV-Cre). Prior to our tumorigenesis study, we performed an analysis of mammary development in the absence of LMO4 expression. Sample size was three virgin mice each per genotype (ie. homozygous $LMO4^{+/+}$, heterozygous $LMO4^{fl/+}$ [not shown], and homozygous $LMO4^{fl/fl}$) per cross with MMTV-Cre (Figure 18A). Virgin $LMO4^{fl/fl}$ MMTV-Cre females displayed normal mammary tree and normal arborisation (Figure 18B), confirming previous studies (Sum, Shackleton et al. 2005). Analysis of ErbB2+ mammary tumors showed abundant expression of LMO4 in the tumor cells at end point (Figure 19A), as previously shown (Montanez-Wiscovich, Seachrist et al. 2009). Loss of LMO4 protein expression was readily observed by Western blot analysis in $LMO4^{fl/fl}$ MMTV-Cre mammary gland tissue (Figure 19B). LMO4 protein expression was analysed via IHC and as expected, LMO4 protein was present in

Figure 18. LMO4(fl/fl) mammary glands show no overt defects. (A) PCR genotyping of LMO4-floxed females. (B) Whole mount and paraffin sections H&E of mammary gland from the various LMO4 genotypes. No overt phenotypes were detected.

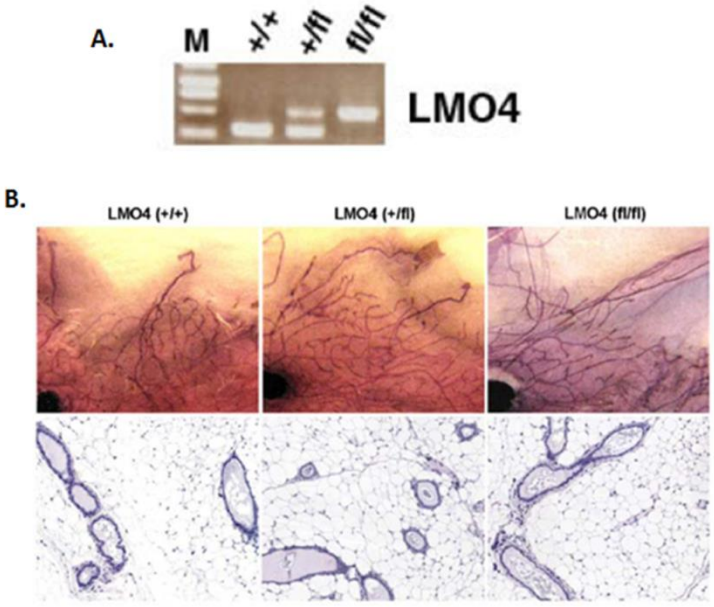
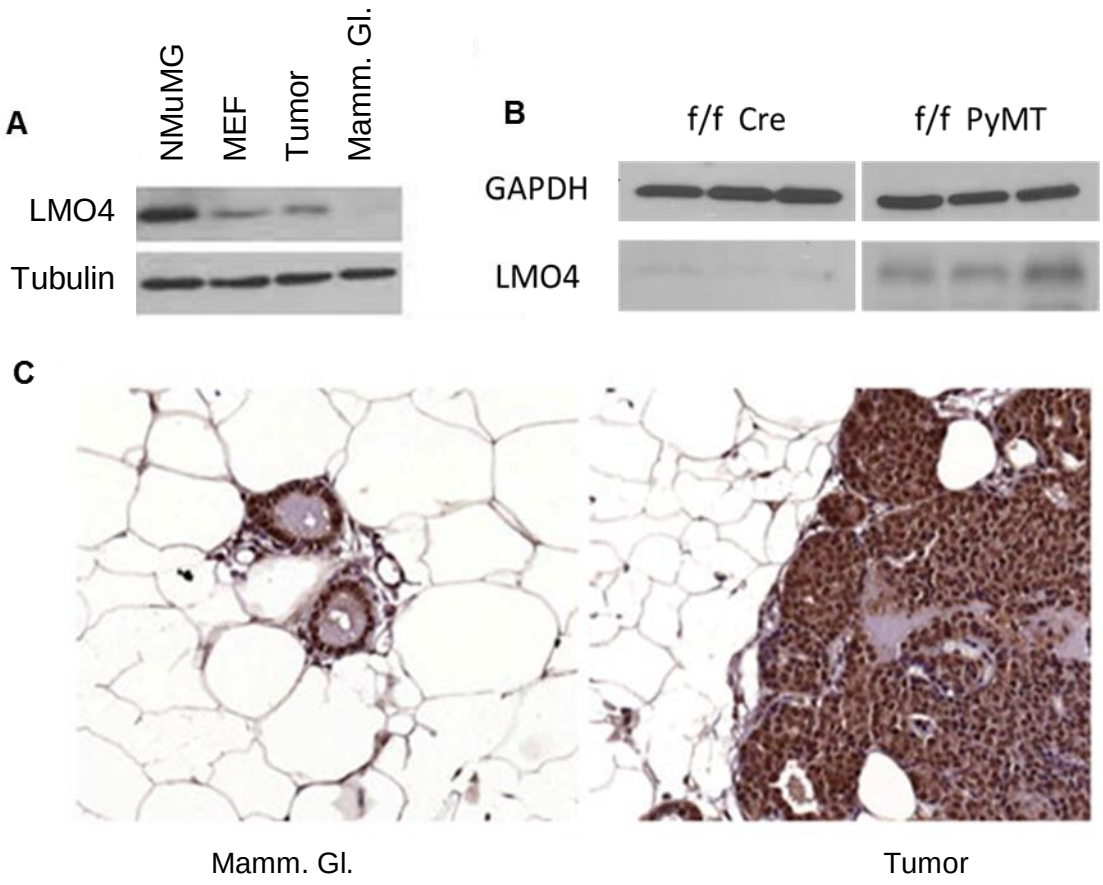

A.
M
+/+
+/fl
fl/fl
LMO4
B.
LMO4 (+/+)
LMO4 (+/fl)
LMO4 (fl/fl)

Figure 19. LMO4 expression in ErbB2+ tumors. (A) Western blot showing expression of LMO4 in non-transformed MEF, NMuMG breast epithelium, normal murine mammary gland (Mamm. Gl.), and murine mammary tumor tissue (tumor). Higher levels are found in NMuMG epithelial cell line as compared to the MEF fibroblast cell line and in ErbB2-induced murine mammary tumor tissue as compared to normal murine mammary gland tissue. (B) Western blot showing loss of LMO4 protein expression in normal LMO4 f/f Cre+ mammary tissue and the presence of LMO4 protein in Cre-negative LMO4 f/f PyMT tumor tissue. The blots show the successful excision of the *lmo4* gene in the mammary glands of Cre+ animals. (C) Immunohistochemistry showing LMO4 expression in mammary epithelium and ErbB2-induced tumors.

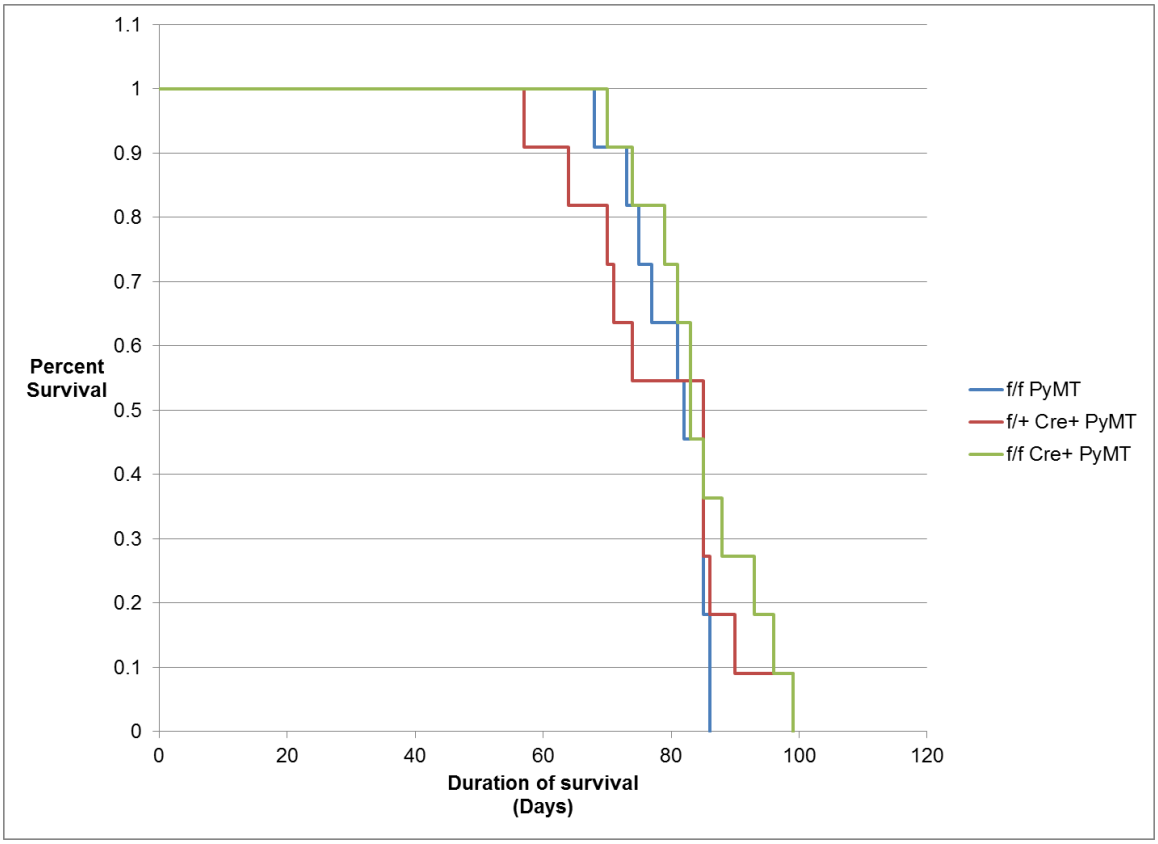

A
NMuMG
MEF
Tumor
Mamm. Gl.
LMO4
Tubulin
B
f/f Cre
f/f PyMT
GAPDH
LMO4
C
Mamm. Gl.
Tumor

the luminal epithelium of the normal mammary gland and in ErbB2-induced tumor tissue (Figure 19C).

5.2 LMO4 protein expression is required for murine mammary tumorigenesis.

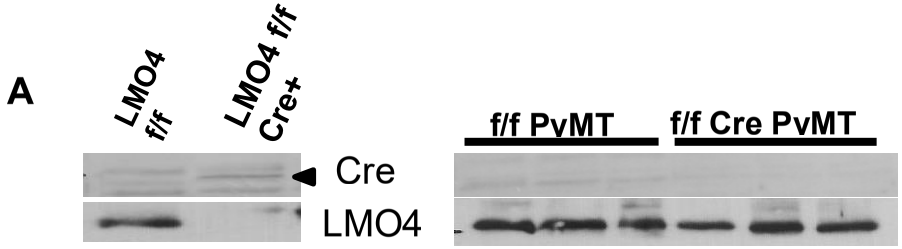
The transgenic mouse model expressing the polyoma virus middle T oncoprotein (PyMT) does so under the mouse mammary tumor virus LTR such that the expression of the oncoprotein is restricted to the mammary epithelium (Guy, Cardiff et al. 1992). The PyMT model is considered a useful and accurate model of the biology of human breast carcinoma progression from premalignant to metastatic disease and relevant to our study, PyMT females develop mammary tumours via up-regulation of the HER2/Neu/ErbB2-pathway signaling by approximately 10 weeks of age (Ichaso and Dilworth 2001; Maglione, Moghanaki et al. 2001; Lin, Jones et al. 2003). Thus, tumor formation was monitored from weaning onward in the LMO4$^{fl/fl}$ or LMO4$^{fl/fl}$ MMTV-Cre cohorts. In previous works, the repression of LMO4 protein expression results in reduced migration and invasion, and induced apoptosis in breast carcinoma cells (Sum, Segara et al. 2005; Tian, Wang et al. 2010). To assess the effect of LMO4 ablation on mammary tumorigenesis and progression *in vivo*, cohorts of PyMT/MMTV-Cre x LMO4$^{fl/fl}$ virgin females were subjected to Kaplan-Meier analysis for Overall Survival (OS). The average OS for the LMO4$^{fl/fl}$ PyMT cohort was calculated to be 80.3 days. The average OS for the LMO4$^{fl/+}$ MMTV-Cre PyMT and LMO4$^{fl/fl}$ MMTV-Cre PyMT cohorts were 78.7 and 84.3 days, respectively (Figure 20). Tissue samples from the LMO4$^{fl/fl}$ cohort (Cre-negative) were obtained after euthanasia at approximately one year of age and no tumors were present in any of the mice in this cohort, as was expected (data not shown). Tumor tissue samples were lysed and analyzed for LMO4 protein expression to confirm LMO4 excision. Surprisingly, despite genotyping results (data not shown), LMO4 protein levels in the LMO4$^{fl/fl}$ MMTV- Cre PyMT cohort were unchanged

Figure 20. Kaplan-Meier analysis shows no difference in the Overall Survival of the three major tumor cohorts. Kaplan-Meier estimates were calculated for a total of 11 mice per tumor cohort ($LMO4^{fl/fl}$ PyMT, $LMO4^{fl/+}$ Cre PyMT, and $LMO4^{fl/fl}$ Cre PyMT) and plotted (y axis) versus duration of survival to humane endpoint in days (x axis). No statistical difference was found using the Log-Rank Test between the control cohort ($LMO4^{fl/fl}$ PyMT) and either of the sample cohorts $LMO4^{fl/+}$ Cre PyMT (p value = 0.349 > 0.05) and $LMO4^{fl/fl}$ Cre PyMT (p value = 0.774 > 0.05).


1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Percent Survival
0
20
40
60
80
100
120
Duration of survival (Days)
f/f PyMT
f/+ Cre+ PyMT
f/f Cre+ PyMT

compared to the Cre-negative LMO4$^{fl/fl}$ PyMT control cohort (Figure 21A). IHC on tumor tissue sections confirmed the expression of LMO4 protein in tumors from mice genotyped as Cre positive (Figure 21B - D). The pattern of LMO4 expression in the tumors as seen by IHC (Figure 21B – D), confirms that seen by previous groups which indicates a mixture of primarily nuclear or cytosolic LMO4 (Visvader, Venter et al. 2001). The tumors shown in panels B and D show primarily nuclear LMO4 expression, whereas the tumor shown in C has primarily cytosolic LMO4 expression. The expression of LMO4 in animals genotyped Cre-positive explains the similarities in OS between the cohorts of mice seen in Figure 20. As no tumors arose consisting of cells lacking LMO4, our result could propose that expression of LMO4 may be required for tumor initiation in the MMTV-PyMT model.

Figure 21. Cre-positive tumors express LMO4 protein. (A) Normal mammary tissue from Cre-negative and Cre-positive LMO4$^{fl/fl}$ mice were were lysed and assayed for Cre protein expression and LMO4 protein expression. The Cre-positive sample (LMO4$^{fl/fl}$ Cre+) showed Cre protein expression (arrowhead) and lack of LMO4 protein and as expected, the Cre-negative sample did not express Cre protein and did express LMO4. Next, PyMT-positive mammary tumors from LMO4$^{fl/fl}$ Cre-positive and Cre-negative animals were lysed and assayed for LMO4 protein expression. Unexpectedly, LMO4$^{fl/fl}$ Cre+ PyMT mammary tumor tissue showed the presence of LMO4 protein. (B – D) Immunohistochemistry on LMO4$^{fl/fl}$ Cre PyMT mammary tumor tissue samples further demonstrated the incomplete excision of the *lmo4* gene by the presence of LMO4 protein in the tumor (arrowheads).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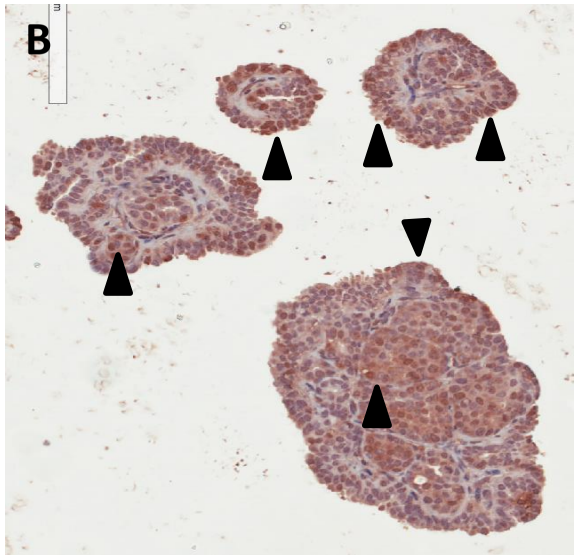
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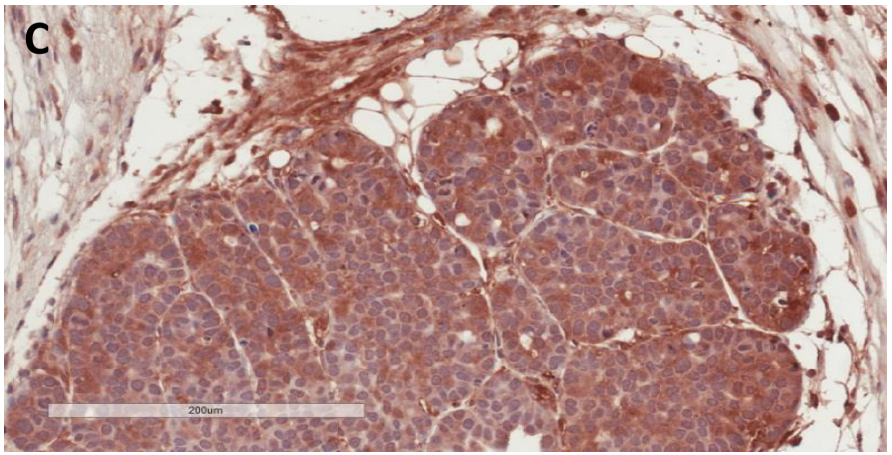
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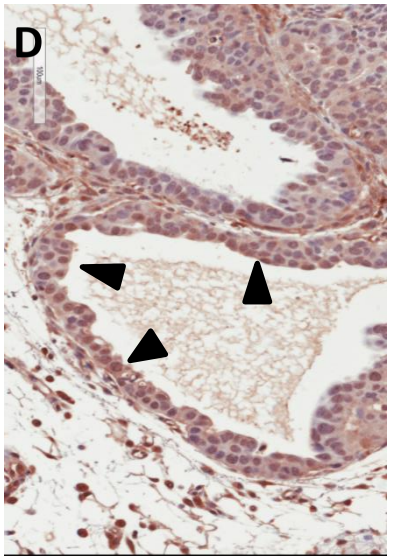
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Chapter 6 –

General Discussion

6. General Discussion

6.1 Recruitment and activation of SLK at the leading edge of migrating cells requires Src family kinase activity and the LIM-only protein 4.

Previous work in our lab has demonstrated that SLK is required for focal adhesion turnover and cell migration (Wagner, Flood et al. 2002; Wagner, Storbeck et al. 2008; Roovers, Wagner et al. 2009; Wagner and Sabourin 2009; Quizi, Baron et al. 2012). We have also shown that the Ldb1/2 transcriptional co-activator can negatively regulate SLK to control cell motility (Storbeck, Wagner et al. 2009). Here we report a novel cytosolic function for the transcriptional co-factor LMO4, known to interact with Ldb1/2. As for Ldb1/2, we show that LMO4 can also interact directly with SLK. Similarly, it can be localized to the leading edge of migrating cells with paxillin, SLK and Ldb1. Deletion of LMO4 in conditional $LMO4^{fl/fl}$ MEFs results in an inhibition of cell migration and a failure to recruit and activate SLK at the leading edge of migrating cells. Surprisingly, deletion of Ldb1 results in a loss of interaction between LMO4 and SLK. Interestingly, SYF cells express very low levels of LMO4 and cannot recruit SLK to the leading edge. This can be rescued by re-expression of wildtype LMO4 or a mutant that binds both SLK and Ldb1 but not to SLK alone. Combined with the observation that PP2 impairs the SLK-LMO4 interaction and SLK recruitment to the leading edge, our data suggest that SLK activation and recruitment to the leading edge of migrating cells is dependent on its interaction with LMO4 in a Src-dependent manner. Furthermore, this binding is required for efficient cell migration.

LIM domain containing proteins represent a large family encompassing cytosolic adapters and transcriptional regulators [extensively reviewed in (Zheng and Zhao 2007)]. The LIM domain is essentially a protein interaction motif and is involved in diverse

biological functions. Surprisingly, a number of LIM proteins have been shown to regulate both cytoskeletal remodeling and transcription. LIM domain proteins such as paxillin, zyxin, PINCH and FHLs have been shown to play a role in focal adhesion turnover and cytosolic signaling (Zheng and Zhao 2007). They have also been shown to shuttle into the nucleus, suggesting a dual role in cytoskeletal remodeling and transcription. Interestingly, several LIM domain containing proteins have been implicated in cancer progression or invasion. Nuclear localization of paxillin is postulated to play a role in prostate cancer through interaction with the androgen receptor (Sen, O'Malley et al. 2010; Sen, De Castro et al. 2012). Similarly, zyxin-related proteins appear to play a role in tumor cell invasion (Grunewald, Willier et al. 2013). Furthermore, the SLK-binding proteins Ldb1/2 have also been shown to play a role in tumorigenesis and to regulate cell migration (Grutz, Forster et al. 1998; Mizunuma, Miyazawa et al. 2003; Storbeck, Wagner et al. 2009; Salmans, Yu et al. 2014). Together, these observations highlight novel and important roles for LIM-domain adapter proteins.

Up to now, LMO4 had been characterized as a neuronal signaling adapter (Schaffar, Taniguchi et al. 2008; Gomez-Smith, Qin et al. 2010; Qin, Zhou et al. 2012) with a role in synaptic regulation and axon guidance. The regulation of cell migration is a novel cytosolic function for the LMO4 protein. Interestingly, a large number of signaling molecules implicated in neuronal activity and guidance also play regulatory roles in cell migration (Luo, Jan et al. 1996; Fournier, Kalb et al. 2000; Ivins, Yurchenco et al. 2000). Mechanistically, the regulation of SLK appears to be quite complex. Its activity has been shown to be dependent on phosphorylation (Delarosa, Guillemette et al. 2011; Luhovy, Jaberi et al. 2012) as well as interaction with adapter proteins (Storbeck, Wagner et al. 2009). Although its activation by migratory signals requires FAK (Wagner, Storbeck et al. 2008; Roovers, Wagner et al. 2009), its recruitment to the leading edge is dependent on

Src-family kinase activity [Figures 13 and 14 and (Wagner, Storbeck et al. 2008)]. Surprisingly, the levels of LMO4 are markedly reduced in SYF cells. However, expression of Myc-LMO4 in SYF cells results in its recruitment the leading edge. These data suggest that its leading edge localization does not require Src activity (see Figure 13 and 14) but rather its expression is Src-dependent.

Inhibition of c-Src by PP2 treatment results in a loss of LMO4-SLK interaction (Figure 14) and an absence of SLK at the leading edge. This suggests that the activity of SLK can be modulated by both the levels of LMO4 and Src-dependent SLK re-localization. Surprisingly, although PP2 inhibits SLK recruitment in cells expressing LMO4, overexpression of LMO4 in Src-deficient cells is sufficient to rescue SLK recruitment (Figure 15). One possibility is that over-expression of LMO4 in the absence of c-Src can induce its binding to Ldb1, resulting in SLK recruitment. Alternatively, the localization of LMO4 at the leading edge is Src-independent but SLK recruitment to LMO4 requires the activation of cell migration. Interestingly, deletion of LMO4 also impairs the recruitment of Ldb1 at the leading edge but not its association with SLK. However, deletion of Ldb1 markedly reduces the association of LMO4 with SLK suggesting that Ldb1 is required for LMO4 binding and recruitment of the SLK complex at the leading edge. Our preliminary data suggest that SLK can be tyrosine phosphorylated by recombinant c-Src *in vitro* (Figure 16), suggesting that c-Src may phosphorylate SLK directly to regulate its subcellular localization. Most likely however, the recruitment of SLK to the leading edge of migrating cells involves intermediate signaling molecules in a Src-dependent manner.

Treatment with the tyrosine phosphatase inhibitor sodium orthovanadate, prevented SLK localization to the leading edge of migrating cells indicating that dephosphorylation may also be activating this pathway (Figures 17A & B). Interestingly,

the tyrosine phosphatase PTPα is responsible for c-Src activation via dephosphorylation in response to migratory cues from integrin-mediated signalling (Chen, Chen et al. 2006; Cheng, Sun et al. 2014). Investigation of this candidate phosphatase showed that in migrating PTPα (-/-) cells, SLK was not present at the leading edge and that the phosphorylated tyrosine residue(s) on the kinase did not increase in response to stimulation, nor did the levels of kinase-associated LMO4 (Figure 17C – E). These results support the vanadate data (Figure 17A & B) implicating one or several phosphatases upstream of the SLK – LMO4 migratory cascade.

There is mounting evidence that LMO4 expression is an important indicator of disease outcome in breast cancer patients (Visvader, Venter et al. 2001; Sum, Segara et al. 2005; Wang, Lin et al. 2007; Montanez-Wiscovich, Shelton et al. 2010). Overexpression of LMO4 in mammary epithelium has been shown to induce mammary hyperplasia and promote cell migration (Sum, Segara et al. 2005). In addition, overexpression of LMO4 has been observed in about 60% of all human breast tumors and in over 60% of HER2-positive tumors. This overexpression has also been correlated with high grade tumors and poor clinical outcome (Visvader, Venter et al. 2001; Sum, Segara et al. 2005). Importantly, increased cytosolic LMO4 staining in metastatic human breast tumor tissue has been presented (Visvader, Venter et al. 2001; Sum, Segara et al. 2005; Montanez-Wiscovich, Shelton et al. 2010). Supporting this, deletion of LMO4 in fibroblasts results in decreased SLK activity and reduced cell migration (Figure 10). The observation that SLK activity is also enhanced by activated HER2/ErbB2 (Roovers, Wagner et al. 2009) raises the possibility that LMO4 over-expression in those tumors might be mediating the increase in SLK activity.

LMO4 has been shown to be required for ErbB2/HER2/Neu-induced cell cycle progression through G2/M by regulating Cyclin D expression, downstream of PI3K

(Montanez-Wiscovich, Seachrist et al. 2009; Montanez-Wiscovich, Shelton et al. 2010). Interestingly, we have also shown that SLK is required for cell cycle progression through G2/M (O'Reilly, Wagner et al. 2005). Expression of dominant negative SLK results in a delay in G2/M progression and a failure to downregulate cyclin A with normal levels of cyclin D. This suggests that SLK and LMO4 might regulate cell cycle progression differently. Alternatively, the transcriptional role of LMO4 is independent of its cytosolic functions that involve SLK regulation. Our data show that SLK also displays a perinuclear pattern of staining. Although SLK-LMO4 co-localization is also observed at the leading edge, the interaction could also take place in the perinuclear space. One possibility is that the perinuclear interaction may be more relevant for G2/M transit and cell cycle progression. Further studies will be required to dissect its specific functions in the different sub-cellular compartments.

6.2 LMO4 protein expression may be required for murine mammary tumorigenesis.

LMO4 was initially described as highly expressed in proliferating uncommitted embryonic cells (Kenny, Jurata et al. 1998), in proliferating adult epithelial cells (Sum, O'Reilly et al. 2005), responsible for clonal expansion of undifferentiated haematopoietic cells (Grutz, Forster et al. 1998), and the proliferation of mammary epithelial lobuloalveolar cells during pregnancy (Visvader, Venter et al. 2001; Sum, O'Reilly et al. 2005). Further evidence showed that these characteristics of LMO4 are mirrored in transformed counterparts of these tissues, with its expression highly upregulated in haematopoietic cancers (Grutz, Forster et al. 1998) and in particular, mammary cancers (Racevskis, Dill et al. 1999; Visvader, Venter et al. 2001; Sum, Segara et al. 2005). Forced overexpression of LMO4 leads to mammary hyperplasia and neoplasia or adenosquamous carcinoma through increased rates of proliferation and cellular

migration and invasion (Sum, Segara et al. 2005). Therefore, we investigated whether LMO4 deletion through MMTV-Cre-driven excision would impair mammary tumorigenesis in a PyMT model of breast cancer. Our model showed no difference in Overall Survival between animals with and without LMO4 expression. Western blot analysis and IHC showed that all tumors that arose expressed LMO4 protein in animals genotyped as Cre-positive (Figure 21). This suggests that cells that had escaped Cre-mediated excision proceeded to form tumors. The failure to detect LMO4-null tumors also supports the notion that LMO4 could be required for initiation and progression. Previously, the expression of a dominant-negative Engrailed-LMO4 fusion protein in breast cancer cells induced apoptosis (Tian, Wang et al. 2010). Therefore, one possible explanation is that the expression of functional LMO4 is absolutely required for tumor initiation and to suppress apoptosis. Incomplete Cre excision has also been seen in PyMT transgenic models using Cre-mediated ablation of β1 integrin, FAK, and c-Src expression, respectively (White, Kurpios et al. 2004; Lahlou, Sanguin-Gendreau et al. 2007; Marcotte, Smith et al. 2012). Closer inspection of these models showed that escape from excision was due to just 50 – 80% of mammary luminal epithelial cells expressing the Cre protein, indicating that tumors had formed from the 20 – 50% of cells which did not express Cre (White, Kurpios et al. 2004; Lahlou, Sanguin-Gendreau et al. 2007; Marcotte, Smith et al. 2012). Newly generated MIC mice, a murine model in which the oncogene and *cre* gene are linked via a bi-cistronic mRNA will ensure that the subsequent mammary epithelial tumors consist only of cells expressing Cre, such as in the NIC (Neu-driven) model. In addition, crossing this model with the GTRosa26 transgenic line (Dr. Soriano, Mount Sinai Hospital, New York) would enable the investigator to track the loss of Cre-expressing cells as tumors progress. These models would be beneficial to confirm LMO4 is absolutely required for tumorigenesis, further supporting its role as a key breast cancer oncogene.

The ultimate goal of elucidating the mechanism of this pathway is to uncover novel therapeutic strategies for human breast carcinomas which may improve upon current approaches. This study has established a role for a recognized mammary oncogene LMO4, in the regulation of SLK, a kinase integral for cell migration and invasion. We have determined that the interaction between LMO4 and SLK is required for activation and localization of the kinase, resulting in efficient cell migration. Therefore, a viable therapeutic strategy could be the disruption of this interaction as this was shown to not only affect localization of the kinase but also, its activation in response to migratory cues. Development of therapeutics targeted to this pathway such as those which interrupt the SLK complex, could supplement or even replace current ErbB2-targeted therapeutics such as Herceptin (Trastuzumab), to reduce the morbidity and mortality associated with breast cancer. Herceptin is effective when used in combination with conventional chemotherapeutics in ErbB2 positive tumors but it is not without severe side effects and the development of resistance (McLean, Komiyama et al. 2004; Lan, Lu et al. 2005). The disruption could be engineered to occur in one of three places to inhibit this pathway: between LMO4 and SLK, SLK and Ldb1 or Ldb1 and LMO4 as we have shown that Ldb1 is required for optimal interaction between LMO4 and the kinase. The binding locations between LMO4 and Ldb1, and Ldb1 and SLK are already known and this study has mapped the interaction between LMO4 and SLK, thus the development of small molecule inhibitors to disrupt any or all of these interactions could theoretically be possible. Such a drug would not only target and prevent the ability of mammary tumors to expand and invade the surrounding tissue, but also induce the apoptosis of tumor cells.

6.3 Conclusion

In summary, we have shown that SLK redistribution and activation during cell migration is dependent upon its interaction with LMO4 and that LMO4 is required for murine mammary tumorigenesis. As an oncogene, LMO4 may therefore, contribute to the invasive potential of breast cancers through the regulation of SLK activity and localization. The association between these two proteins requires c-Src activity and is critical for cell migration. The effect of LMO4 deletion or overexpression of LMO4 on SLK activity specifically in breast cancer *in vivo* remains to be investigated, possibly via murine models mentioned above. In addition, further identification of upstream SLK regulators and additional binding proteins will advance our understanding of its molecular regulation. Nevertheless, the modulation of SLK activity by a confirmed oncogene such as LMO4 makes the kinase an attractive therapeutic target. The overall conclusion of this project as reconciled with previously known SLK functions is as follows: LMO4 is recruited to SLK downstream of of c-Src activation, to bring SLK to the leading edge of cells where the active kinase phosphorylates paxillin, resulting in FA turnover and cell migration. Furthermore, this study establishes a novel cytosolic role for the transcriptional co-activator LMO4.

Appendices

Appendix A:

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