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14-3-3ζ Regulation of Metastasis Through Mediation of Liprin-α and Liprin-β

Rachel Hynes

A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of

Master of Science

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## ABSTRACT

### 14-3-3 $\zeta$ Regulation of Metastasis Through Mediation of Liprin- $\alpha$ and Liprin- $\beta$

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Master of Science

Cancer is a set of varied and diverse diseases that share common characteristics, such as active proliferation, increased replicative potential, and tissue invasion or metastasis. One protein, 14-3-3 $\zeta$ , is shown to be upregulated in a number of different cancers and also correlates with poor patient prognosis, recurrence, and mortality. This protein comes from a family of adapter proteins known for their scaffolding ability, pro- and anti-oncogenic capabilities, and affinity for phosphorylated substrates. It has been shown previously to participate in cancer progression, subversion of apoptosis, and to increase chemoresistance. Herein we will discuss the ability of 14-3-3 $\zeta$  to promote distant-site metastasis and we propose that it does so through a variety of different mechanisms including the MAPK signaling cascade, HER2/ErbB2 pathway, and by mediation of cell adhesion through regulation of LAR.

Liprin- $\beta$  was identified as a novel 14-3-3 $\zeta$  interactor in a mass spectrometry-based interactomics analysis. 14-3-3 $\zeta$  was found to co-immunoprecipitate with both Liprin- $\alpha$  and Liprin- $\beta$ . We will discuss the identification and mutation of putative 14-3-3 $\zeta$  binding sites on both Liprins, the effect these have on the binding of both Liprins to 14-3-3 $\zeta$  and of Liprin- $\alpha$  to LAR, and the possible downstream consequences of these interactions. The results described herein are inconclusive due in part to our inability to obtain a reliable Liprin- $\beta$  pulldown and in part our inability to identify the 14-3-3 $\zeta$ -binding sites on Liprin- $\alpha$  and Liprin- $\beta$ . The concluding chapter contains a discussion of the possible future directions, including the creation of further Liprin mutants as well as fluorescent imaging of LAR localization and focal adhesion turnover.

Keywords: 14-3-3 $\zeta$ , Liprin, metastasis, breast cancer

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# 1 INTRODUCTION

## 1.1 Background on Cancer and Metastasis

Cancer is a set of diseases caused by mutations in the genetic code that vary by type, location, cause, effect on genetic expression, and drug resistance. Despite these differences, all cancers share six common traits: apoptosis resistance, limitless replicative potential, active proliferation, induced angiogenesis, suppression of growth inhibitors, and tissue invasion and metastasis.<sup>1-2</sup> As with the origin of cancer, the cause of each of these traits varies from one type of cancer to another. In fact, even patients diagnosed with the same type of cancer frequently have tumors that are genetically and phenotypically different. With respect to biomedical research, each trait represents a convoluted puzzle with multiple starting points and many different overlapping and crisscrossing paths, all producing similar outcomes. One of the most intriguing cancer trait puzzles is deciphering how these cells develop the ability to migrate from the site of the original tumor to a distant, noncancerous tissue and give rise to a new tumor.

Metastasis, or the process by which cancerous cells spread from the original tumor to a new site, occurs in nearly all late stage cancers and results in poor prognosis.<sup>1, 3-4</sup> The correlation between metastatic cancer and patient mortality originates from the increased likelihood of cancer recurrence upon formation of secondary tumors. This harmful process of recurrence at a secondary site can occur immediately or even years after removal of the original tumor.

Metastasis plays a leading role in cancer progression, chemoresistance, patient mortality, and has

been reported to be the cause of 90% of solid tumor deaths.<sup>5</sup> Many labs and clinics across the world are researching how metastasis occurs in the various types of cancer. Despite the amount of research taking place, the causes and mechanisms have not yet been completely elucidated and metastatic tumors still develop.

No single cause of metastasis exists, but it occurs when a tumor cell retracts the focal adhesions (FAs) that anchor it to the extracellular matrix (ECM) and adherent junctions (AJs) that link to the surrounding cells and then proceeds to migrate through the bloodstream or lymphatic vessels until it rests at a site distant from the primary tumor.<sup>6</sup> Once the cell settles, it can remain dormant or immediately begin proliferating to form another tumor. This type of invasive cancer is hard to detect and difficult to eradicate because physicians cannot always predict the location of secondary tumor growth and the dormant tumors often remain too small to be identified until after removal of the original tumor. The ability of these cells to evade detection and the detrimental effects of systemic cancer treatment on the patient make evident the necessity of further research to better understand the mechanisms through which tumor cells undergo metastatic transition.

There are many different signaling pathways that lead to metastasis. Nearly every type of cancer employs a distinct pathway to initiate tumor cell metastasis, making the task of finding the cause a daunting undertaking, especially considering that no single cause exists. However, despite their differences many types of cancer share characteristics such as proteins expressed or genes silenced. Similarities such as these aid in the study of tumor cell metastasis because the study of one type of cancer can correlate to other types. One such family of proteins that is differentially expressed in multiple types of cancers is the 14-3-3 family. Recently, their relation to cancer has been increasingly studied. They are known to interact with a myriad of proteins

involved in a variety of different cellular functions, many of which pertain to cancer progression and growth and others to cancer evasion. In this review, we focus on elucidating the role of one member of the family, 14-3-3 $\zeta$ , and how its overexpression leads to cancer metastasis.

## 1.2 Defining 14-3-3 Proteins

Though originally isolated from brain tissue in 1967,<sup>7</sup> the 14-3-3 proteins can be found in a number of organs and tissues throughout all eukaryotic organisms.<sup>8</sup> The name of these proteins comes from the fraction containing the proteins collected after filtration by DEAE column chromatography.<sup>9</sup> The 14-3-3 proteins are a conserved family of acidic proteins made up of seven different isoforms ( $\beta$ ,  $\gamma$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ,  $\sigma$ , and  $\tau$ ) encoded by different genes which can be found in all eukaryotic organisms.<sup>8</sup> Through interactions with a variety of different proteins, 14-3-3 participates in a number of cellular functions, including cell cycle progression, cell survival,<sup>10-11</sup> metabolism,<sup>12</sup> protein trafficking,<sup>13</sup> and signal transduction.<sup>10, 13-22</sup>

14-3-3 family proteins participate in hundreds of different pathways and interactions, despite having no inherent enzymatic activity. Instead they function by first dimerizing then interacting with and modulating the activity of other proteins in a phosphorylation-dependent manner.<sup>23</sup> The phosphorylated interacting partners contain conserved binding motifs, the most common of which is RXXpS/TXP, where pS/T represents the phosphoserine/threonine to which 14-3-3 proteins directly bind.<sup>24</sup> Regulation on a given protein by 14-3-3 can cause scaffold-like activity, sequestration, activation or inhibition, conformational change, or complex formation.

### 1.3 14-3-3 Proteins and Cancer

In general, members of the 14-3-3 family are somewhat polarized when it comes to their role in cancer: for example, the sigma isoform generally acts in a tumor suppressor role while many of the other isoforms have pro-oncogenic effects. Some studies show, however, that this division is not as black and white as it first appears and that certain isoforms can play both pro- and anti-oncogenic roles depending on the expression level and profile.<sup>25-27</sup> The 14-3-3 family of proteins has been shown to play a role in almost every type of cancer (reviewed in reference 23). Each 14-3-3 dimer pair (including homo- and heterodimerizations) has a unique set of binding partners with few overlapping interactors, which results in each set having a distinct impact on cancer.<sup>28-30</sup> Many of these interacting partners participate either directly or indirectly in cancer progression. For example, interaction with BAD, Cdc25, and  $\beta$ -integrins leads to their altered localization; binding to Raf-1, CHK1, WEE1, and serotonin-N-acetyl transferase alters their enzymatic function; finally, binding to proteins such as RAF-BCR and RAF-A20 causes complex formation.<sup>10, 31-38</sup> 14-3-3 proteins also interact with the type 1 insulin-like growth factor receptor (IGFIR), a protein that regulates growth and development upon binding of either IGF-I or IGF-II in both normal and cancerous cells.<sup>39</sup> Binding of 14-3-3 to IGFIR induces cancer cell transformation, whereas blocking 14-3-3 binding to IGFIR by mutation causes the cells to lose the ability to form soft agar colonies.<sup>40</sup>

The zeta isoform of the 14-3-3 family has been shown to correlate with multiple types of cancer, including colon,<sup>41</sup> lung,<sup>42-43</sup> stomach,<sup>44</sup> and breast cancer.<sup>45-47</sup> In breast cancer specifically, the overexpression of 14-3-3 $\zeta$  has been linked to poor patient prognosis, patient mortality, increased recurrence, and greater chemoresistance.<sup>48-51</sup> Many studies suggest that 14-3-3 $\zeta$  plays a role in transformation, cancer progression, tumor cell survival, invasion, and has

been well characterized as an inhibitor of apoptosis. It is known to aid in cell survival by interacting with a network of proteins involved in apoptosis, including BAD, Bax, caspase-2, and Raf kinases.<sup>52</sup> Subversion of apoptosis allows cancerous cells to grow under conditions that would normally cause cell death, such as limited nutrient and oxygen supply or high levels of genetic mutations.

In addition to subversion of apoptosis, 14-3-3 $\zeta$  also participates in various stages of tumor growth. This can be seen by observing the effects of 14-3-3 $\zeta$  in different cancers and how they related to the hallmarks of cancer: replicative potential, apoptosis resistance, proliferation, angiogenesis, suppressed growth inhibitors, and invasiveness. In human esophageal cancer, 14-3-3 $\zeta$  expression is associated with tumorigenesis.<sup>53</sup> In breast cancer, 14-3-3 $\zeta$  overexpression inhibited apoptosis.<sup>54</sup> In prostate cancer it activates proliferation through association with the androgen receptor in the nucleus.<sup>55</sup> In multiple myeloma, siRNA knockdown of 14-3-3 $\zeta$  caused a downregulation of various functions related to angiogenesis.<sup>56</sup> In transformed oncogenic mouse mammary epithelial cells, 14-3-3 $\zeta$  was shown to have an opposing role to 14-3-3 $\sigma$  and stimulated growth by negatively modulating TGF- $\beta$ 1 growth inhibition.<sup>57</sup> In intrahepatic cholangiocarcinoma (ICC) it enhanced the tumor cell invasiveness as well as increasing their proliferative capacity.<sup>58</sup> Considering these data, 14-3-3 $\zeta$  likely acts as a coordinator for the various stages and pathways involved in cancer development and progression.

#### **1.4 14-3-3 $\zeta$ and Metastasis**

The ability of 14-3-3 $\zeta$  to induce apoptosis has been researched extensively, while its role in cell adhesion and motility is not as well understood. 14-3-3 $\zeta$ , as with all the 14-3-3 family proteins, is generally known as a scaffolding protein, though it can also act to sequester,

activate/inactivate, or induce conformational changes as previously mentioned. Many of the signaling pathways that 14-3-3 $\zeta$  participates in and proteins it has been shown to interact with take part in cancer, specifically in cancer metastasis. It has been shown to increase distant site metastasis, cancer recurrence, and increased invasion in multiple types of cancer, including breast, lung, esophageal, ICC, and colon. Here we will discuss a few of the different metastasis-promoting pathways and proteins that 14-3-3 $\zeta$  interacts with, including MAP kinase cascade signaling, HER2/ErbB2 pathway, and LAR-mediated cell adhesion.

#### **1.4.1 MAP Kinase Signaling Cascade**

In order for a tumor cell to initiate metastasis it must undergo a series of events leading to its retraction from the surrounding tissue and survival in the vasculature until it reaches its target tissue. This involves the integration of many different signaling pathways, one of which is the mitogen-activated protein kinase (MAPK) cascade. MAPK signaling is a complex network of interconnected pathways that can lead to initiation of many of the steps leading to metastasis. The following paragraphs will discuss the role of MAPK in the epithelial mesenchymal transition (EMT), apoptosis evasion, survival in the vasculature, and initiation of the dormancy stage.

First, the cells must undergo EMT in order to allow them to migrate and invade other tissues.<sup>59</sup> EMT can be induced through various methods, many of which pertain to MAPK and its associated proteins. One such method involves a coordinated effort of transcription factors such as Twist1, Snail, and Slug, that repress e-cadherins, allowing the cell to release its FAs and AJs in order to move away from the surrounding tissue.<sup>59</sup> EMT has been shown to be initiated by direct regulation of these transcription factors or indirectly through reactive oxygen species

(ROS). MAPK-associated proteins have been shown to respond to ROS accumulation in the context of tumor invasiveness and correlate with the expression of both Twist1 and Snail.<sup>60-62</sup> A second method of coordinating the transcription factors is through TGF- $\beta$ 4 which has been shown to be induced by MAPK signaling and to lead to EMT.<sup>63</sup> Lastly, EMT can be induced by hypoxia, a condition common to tumor cells that grow to a size exceeding the limit of nutrient and oxygen delivery. Furthermore, one of the key transcription factors induced by hypoxia, HIF-1 $\alpha$ , is stabilized by the p38 $\alpha$  MAPK, which is sufficient to directly activate Snail and Twist.<sup>64</sup>

Second, the cells that release from their surrounding tissue must circumvent apoptosis while they undergo circulation until they reach the target tissue. In order to prevent the spread of tumors, cells have developed a mechanism to induce apoptosis in cells that release their FAs. This process of anoikis, or anchorage-dependent apoptosis, must be overcome by tumors in order for them to survive once they release from the primary tumor. Interestingly, the suppression of components of the MAPK cascade has been linked to tumor suppression by inducing anoikis and inhibition of these MAPK proteins induces anoikis resistance.<sup>65-67</sup> This seemingly opposing role of MAPK signaling in cancer progression illustrates the need for tight regulation of the MAPK cascade, possibly through a scaffolding protein such as 14-3-3 $\zeta$ . 14-3-3 could potentially cause a tumor cell to release from the primary tumor by activating the MAPK signaling pathway, but then through sequential inactivation, prevent the pathway from inducing apoptosis.

Third, the cancer cells that successfully release from the tumor and survive the transition to mesenchymal cells must enter the blood stream or lymphatic system by degrading the extracellular matrix (ECM). MAPK signaling cascade phosphorylates heat-shock protein 27 to activate its anti-apoptotic and pro-metastatic role in cancer.<sup>68</sup> In order to become invasive cells must become motile, a process that involves the coordination of FA removal and placement as

well as actin polymerization and depolymerization. MAP kinase activity inactivates the actin depolymerizing factor cofilin by phosphorylation, allowing the actin filaments to polymerize and carry out cell motility.<sup>68</sup> Knockdown of MAPK proteins by dominant-negative mutants and siRNA inhibition caused a decrease in cell invasion.<sup>69-73</sup> MAPK modulates the activity of urokinase-type plasminogen activator, uPA, which plays a critical role in the invasion, intravasation, and metastasis.<sup>74-75</sup>

Lastly, once the cells reach the new tissue and before they begin infiltrating and forming a new tumor, the cells generally enter a dormancy stage called metastatic latency.<sup>76</sup> This stage of latency occurs after a cell reaches the target organ and before it receives the proper signals to begin rapid proliferation. Generally, cells accomplish this by arresting the cell cycle at the G0/G1 stage or by equalizing the rate of proliferation and death.<sup>77</sup> Extracellular signals cause the inactivation of ERK1/2 and activation of p38MAPKs resulting in dormancy stage, while reversal of these two opposing states initiates rapid tumor growth of a distant, metastatic tumor.<sup>76</sup>

As shown above, the MAPK cascade participates in many of the steps necessary for a cell to become metastatic. Some of its key regulators, such as the proteins Ras, Raf and mitogen-activated protein kinase kinase (MEK), belong to the family of 14-3-3 $\zeta$ -interacting proteins.<sup>78-80</sup> The presence of 14-3-3 $\zeta$  has been shown to be sufficient and necessary for activation of the MAPK/ERK (extracellular signal-regulated kinase) pathway and overexpression of a dominant/negative 14-3-3 $\zeta$  mutant resulted in p38/MAPK activation and apoptosis induction.<sup>60, 79, 81</sup> These results indicate that 14-3-3 $\zeta$  acts as a regulator between the MAPK-associated p38 and ERK pathways in order to promote cell survival and metastasis. A second method of 14-3-3 $\zeta$  activation of the MAPK/ERK cascade is by binding and activating c-Raf.<sup>82</sup> Activation of this part of the MAPK signal cascade by 14-3-3 $\zeta$  could lead to MAPK-induced metastasis.<sup>83</sup>



Thymosin  $\beta$ 4, another known regulator of the MAPK cascade, initiates its phosphorylation-dependent activation.<sup>84</sup> While no direct correlation between 14-3-3 $\zeta$  and thymosin  $\beta$ 4 has been experimentally proven, both proteins have been shown to correlate with the metastatic potential of tumor cells via MAPK signalling.<sup>85</sup> These two proteins, Thymosin  $\beta$ 4 and 14-3-3 $\zeta$ , likely work in conjunction to regulate this pathway

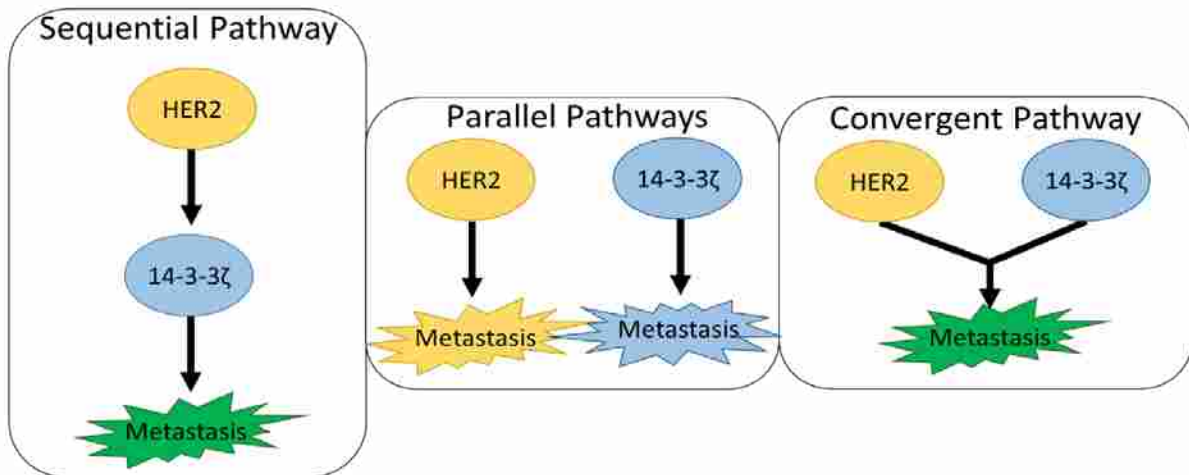
#### **1.4.2 HER2 Pathway**

Human epidermal growth factor receptor 2 (HER2), also known as receptor tyrosine-protein kinase ErbB2, belongs to the human epidermal growth factor receptor tyrosine kinase (RTK) family along with EGFR, ErbB3, and ErbB4. The overexpression of these proteins has been shown to play a role in malignant cancer progression and each are targets for many anti-cancer therapies.<sup>86-87</sup> Interestingly, HER2 has no known ligand but is still crucial to downstream RTK signaling by forming heterodimers with its sister proteins.<sup>88</sup> Each of these receptors can be activated by ligand-binding or in a ligand-independent manner in response to phosphorylation induced by ionizing radiation (IR).<sup>89-92</sup> Oxidative stressors, such as IR exposure, lead to tumorigenicity and increased invasion of tumor cells into the surrounding tissue.<sup>93-95</sup> The HER2 protein is overexpressed in 20% of breast cancers and, like 14-3-3 $\zeta$ , correlates with poor patient prognosis, and increased levels of metastatic cancer.<sup>96-99</sup> Similarly to HER2, 14-3-3 $\zeta$  has been found to be expressed in early stage tumors, to correlate to poor patient prognosis, and to have IR-mediated overexpression. One study showed that both HER2 and 14-3-3 $\zeta$  signaling increase upon IR activation to promote breast cancer metastasis.<sup>100</sup>

As with most cancers, breast cancer progresses through various stages, including invasive breast cancer (IBC) and its noninvasive precursor ductal carcinomas in situ (DCIS). While highly

expressed in DCIS cells, the protein HER2 is found in only approximately 25% of IBC cases.<sup>87, 101-103</sup> These statistics indicate that HER2 expression alone is insufficient to promote the transition from DCIS to the invasive, more lethal form of breast cancer. The question remains as to what cofactor acts in conjunction with HER2 to initiate the transition to metastatic cancer. Though many different candidate proteins or other metastasis-promoting factors could aid in the transition from DCIS to IBC, we propose that this is accomplished through coordinated signaling with 14-3-3 $\zeta$ .

The protein 14-3-3 $\zeta$ , as previously discussed, binds to many different proteins that influence the various hallmarks of cancer.<sup>1, 10, 16</sup> Furthermore, 14-3-3 $\zeta$  was found to be overexpressed in many early stage breast cancer tumors, including DCIS.<sup>104</sup> A recent study looked at 14-3-3 $\zeta$  overexpression in conjunction with HER2 in order to determine whether they work in a cooperative fashion. The data showed that the co-overexpression of 14-3-3 $\zeta$  and HER2 in *in vitro* breast cancer systems led to increased progression to IBC and higher levels of distant site metastasis in mouse models.<sup>50</sup> Though the exact mechanism of HER2-induced metastasis in cancer cells remains unclear, it has repeatedly been shown to correlate with increased metastasis and poor patient prognosis. While breast cancer patients testing positive for either HER2 or 14-3-3 $\zeta$  showed increased likelihood of metastasis, those overexpressing both proteins have a statistically higher likelihood of developing metastatic recurrence. The close correlation between these two proteins and metastasis suggests that the HER2 and 14-3-3 $\zeta$  pathways do not work in parallel, but work in a convergent manner to promote metastasis (Figure 1).



**Figure 1** HER2 and 14-3-3 $\zeta$  work together to promote tumor cell metastasis. Previously it has been proposed that HER2 and 14-3-3 $\zeta$  work in either a sequential or parallel pathway. We propose that they work together in separate but convergent pathways to promote metastasis.

### 1.4.3 LAR-Mediated Adhesion

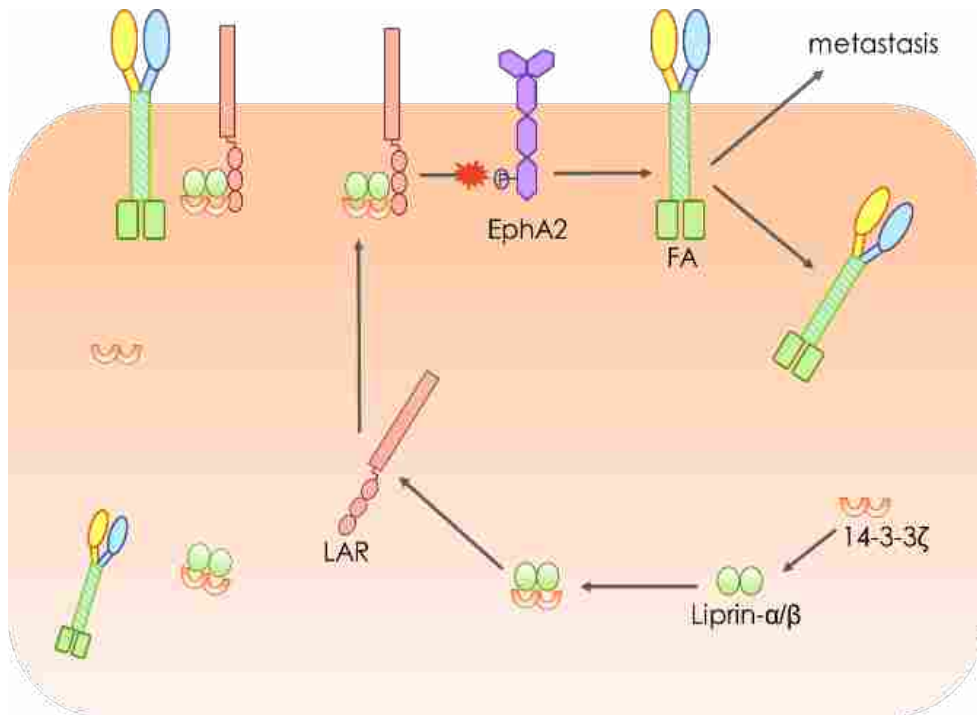
The protein-tyrosine phosphatase family is divided into two groups, or subfamilies, consisting of receptor-like (RPTP) and nonreceptor PTPases, which function by dephosphorylating tyrosyl-phosphorylated proteins.<sup>105-106</sup> Like other RPTPs, leukocyte common antigen related receptor (LAR) possesses a single transmembrane domain and two intracellular phosphatase domains named D1 and D2.<sup>106-107</sup> Unlike most receptors, ligand binding to RPTPs typically causes their inactivation by inducing dimerization which allosterically inhibits the active D1 domain through occlusions by the D1 domain of the opposing subunit.<sup>108-112</sup> The D1 domain is believed to carry out the PTPase activity while the D2 domain typically remains inactive, but plays a key regulatory role in determining substrate specificity.<sup>110, 113-116</sup> Regulation of the D2 domain occurs through the binding of various proteins, such as the scaffolding protein called Liprin- $\alpha$  which is believed to influence LAR's substrate specificity.<sup>117</sup>

A recent 14-3-3 $\zeta$  interactomics study revealed several novel 14-3-3 $\zeta$  binding partners including a metastasis-regulating protein called Liprin- $\beta$ .<sup>118-119</sup> Liprins comprise a gene family that contains six Liprin- $\alpha$  and two Liprin- $\beta$  proteins:  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3,  $\alpha$ 4,  $\beta$ 1, and  $\beta$ 2. This highly conserved family of scaffold proteins was originally identified as interacting partners of LAR in 1998.<sup>117</sup> Since that time, many articles have been published about the binding partners and roles of Liprins in cell migration and adhesion, synapse activity and development, and lymphatic vessel development.<sup>120-129</sup> Though little is known about the regulation of Liprins, their general structure has been well characterized. Each Liprin protein contains a C-terminal liprin homology (LH) domain comprised of three sterile alpha motif (SAM) domains and an N-terminal coiled-coil region.<sup>130</sup> Liprin- $\alpha$  proteins heterodimerize and interact with a variety of different synaptic proteins (CAST, GIT1, RIM, and KIF1A) via interaction at the SAM domain.<sup>129, 131-133</sup> Though the mechanism is still not well understood, Liprin- $\beta$  and Liprin- $\alpha$  heterodimerize by binding at the coiled-coil N-termini,<sup>117</sup> an interaction that could lead to the Liprin-dependent activation of LAR by 14-3-3 $\zeta$ , causing a downstream effect of loss of cell adhesion through indirect interaction with integrins.

Receptor tyrosine kinases (RTKs), the counterpart to RPTPs, act in a manner distinct to that of RPTPs and are activated through ligand-induced dimerization, whereupon each subunit participates in a trans-phosphorylation of tyrosine residues on the opposing receptor.<sup>134</sup> RTKs require a triple phosphorylation in order to remain fully active and often are inactivated through dephosphorylation by either a nonreceptor PTPase or an RPTP. One such RTK substrate of the LAR PTPase is Ephrin type-A receptor 2 (EphA2),<sup>135</sup> also known as epithelial-cell kinase (Eck), which is a member of the largest family of RTKs.<sup>136</sup> Like the other 13 members of the Ephrin family, EphA2 is membrane-bound and activated by ligands called ephrins, specifically ephrin-

A. It is believed that the Eph-ephrin interaction and signaling cascade participate in cell motility by limiting migration through inhibition of integrin-mediated adhesion and spreading, thus obstructing migration.<sup>137-140</sup>

We propose that 14-3-3 $\zeta$  overexpression initiates metastasis by interacting with a Liprin- $\alpha$ /Liprin- $\beta$  heterodimer, which in turn binds to LAR. This interaction recruits LAR to the cell periphery where it can dephosphorylate EphA2, thus inactivating its ability to stabilize integrins and FAs at the cell surface. In turn, this triggers the uptake of FAs into the cell causing a loss of adhesion with the surrounding tissue and ECM, whereupon the cell is free to mobilize and metastasize to a secondary site within the organism (Figure 2).



**Figure 2 Proposed mechanism of 14-3-3 $\zeta$ /LAR-induced cell motility.** Overexpressed 14-3-3 $\zeta$  interacts with Liprins, causing them to interact with LAR and localize to the cell periphery. Once at the cell edge, LAR dephosphorylates and inactivates EphA2, a RTK known to stabilize integrins and inhibit cell motility. Upon EphA2 inactivation, the FAs are internalized, leaving the cell free to migrate or metastasize.

## 2 LIPRINS

### 2.1 Why Liprins

Liprins belong to a family of proteins known as the LAR protein tyrosine phosphatase (PTP)-interacting protein family. This family consists of six proteins split into two distinct classes,  $\alpha$  and  $\beta$ . Four Liprin- $\alpha$  and two Liprin- $\beta$  proteins have been identified in mammals:  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\beta 1$ , and  $\beta 2$ . Each protein within the family is composed of an N-terminal coiled-coil and a C-terminal liprin homology (LH) domain containing three sterile alpha motif (SAM) regions (Figure 3). The Liprin proteins have been shown to homodimerize with proteins of the same class (i.e.  $\alpha$  to  $\alpha$  or  $\beta$  to  $\beta$ ) at their N-terminal coiled coil region and heterodimerize with Liprins of the opposite class ( $\alpha$  to  $\beta$ ) or interact with other proteins at their C-terminal LH domain.<sup>117, 124</sup> Although substantial progress has been made toward understanding the mechanism of Liprin- $\alpha$  signaling and its participation in cell adhesion, elucidation of the role of Liprin- $\beta$  and understanding how it participates in Liprin- $\alpha$ -mediated mechanisms remains an active, though challenging, area of investigation.



**Figure 3 Diagram of Liprin structure.** C-terminal coiled-coil domain where Liprins form homodimers. N-terminal Liprin homology (LH) domain of three sterile alpha motifs where Liprins form heterodimers or bind to other proteins.

### 2.1.1 Liprin- $\alpha$

Liprin- $\alpha$ , also known as LAR interacting protein alpha (PTPRF interacting protein alpha [PPFIA]), plays an integral role in synaptic vesicle trafficking and synapse development and maintenance.<sup>123, 141</sup> Liprin- $\alpha$  proteins bind a number of neuronal proteins, such as GRIP and GIT1, through which they are suspected to coordinate trafficking of proteins like the AMPA receptor in the active zone of synapses.<sup>127, 131</sup> More recently the Liprin- $\alpha$ -GIT1 interaction has been implicated as a positive regulator of motility by preventing the negative regulation of GIT1 on paxillin, a crucial component of FAs.<sup>142</sup>

A second regulatory role of Liprin- $\alpha$  on cell motility involves its effect on the permanence of integrins at the cell surface. Integrins stabilize the actively proliferating actin lamellipodia of migrating cells by anchoring the cytoskeleton to the extracellular matrix.<sup>143</sup> A 2010 study identified Liprin- $\alpha$  as a factor that aids in the stabilization of  $\beta$ 1 integrins by preventing their internalization.<sup>119</sup> Additionally, Liprin- $\alpha$ 1 knockdown was shown to suppress cell spreading and migration, whereas its overexpression promoted cell motility.<sup>126</sup> It was also shown to affect integrin distribution, alter cell morphology, enhance lamellipodia formation, and increase spreading.<sup>118</sup>

Cell motility plays a key role in metastasis, and Liprin- $\alpha$  proteins have also been shown to participate in the motility of cancer cells. The Liprin- $\alpha$  gene was shown to be amplified in about 20% of breast cancers and correlating high protein expression was observed.<sup>144</sup> The researchers further identified Liprin- $\alpha$  as a novel regulator of tumor cell invasiveness and metastasis in breast cancer cells.<sup>144</sup> One way through which Liprin- $\alpha$  proteins affect motility is by interacting with the distal phosphatase domain of LAR and increasing its trafficking to the plasma membrane. Both Liprin- $\alpha$  and LAR have been found to localize to FAs at the cell



periphery and initiate their disassembly and internalization, providing a means through which cells can metastasize.<sup>126</sup>

### 2.1.2 Liprin- $\beta$

Liprin- $\beta$ , also known as LAR interacting protein binding protein  $\beta$  (PTPRF interacting protein binding protein [PPFIBP]), does not directly interact with LAR, but associates with it through interaction with Liprin- $\alpha$ . Other than Liprin- $\alpha$  only one protein interactor has been identified: a metastasis regulating protein named S100A4.<sup>145</sup> As with  $\alpha$ -Liprins, the  $\beta$  subfamily sequence identity is highly conserved across species, suggesting a conserved evolutionary role for both of these proteins. For the most part, the physiological role that Liprin- $\beta$  proteins play in cells has not been well studied,<sup>117, 145</sup> though they were identified as a potential mediator of lymphatic vessel integrity, specifically in intestinal lymphatic endothelial cells.<sup>146</sup>

Of the little that is known about Liprin- $\beta$ , the majority of it shows a strong correlation between this protein and cell motility. It is known to associate directly with Liprin- $\alpha$  and indirectly with LAR, both of which regulate FA and/or AJ stability. Additionally, the study that identified Liprin- $\beta$  as a S100A4 interactor carried out immunofluorescent staining that revealed enhanced Liprin- $\beta$ 1 staining at the cell periphery, predominantly at lamellipodia-like areas of the membrane.<sup>145</sup> Conversely, Liprin- $\beta$ 2 has been identified as a suppressor of motility. Knockdown of ERK2 in invasive breast cancer cells resulted in the indirect upregulation of Liprin- $\beta$ 2 which lead to the inhibition of motility.<sup>147</sup> Together these data suggest that Liprin- $\beta$  is highly dynamic and careful regulation of the different isomers is required for regulation of metastasis. Such intense regulation could be carried out via a scaffolding protein such as 14-3-3 $\zeta$ .

### 2.1.3 14-3-3 $\zeta$ Interactomics

Overexpression of the protein 14-3-3 $\zeta$  in breast cancer tumors has been identified as a marker for poor patient prognosis and increased chemoresistance.<sup>23, 43, 51, 148</sup> One method through which 14-3-3 $\zeta$  promotes cancer cell survival and chemoresistance comes from the ability of this protein to aid in the subversion of apoptosis through the regulation and expression of proteins such as Bcl-xL, Mcl-1, and Caveolin-1, and 14-3-3 $\zeta$ .<sup>149</sup> Other known binding partners of 14-3-3 $\zeta$  include Atg9,<sup>150</sup> the Bcl2 family protein Bad, and Raf kinases. Given the chemoresistance-promoting nature of 14-3-3 $\zeta$  and its phosphorylation-dependency, an interactomics study can be used as a tool to identify novel phosphorylation-mediated interactions that regulate cancer cell movement and survival.

Considering the cancer-promoting role of 14-3-3 $\zeta$ , we can utilize a mass spectrometry-based analysis of the protein-protein interactions to identify possible mechanisms of 14-3-3 $\zeta$ -induced metastasis and chemoresistance. Often 14-3-3 $\zeta$  is considered a scaffolding protein because it has no inherent activity. Instead it relies on its interactions with phosphorylated serine/threonine motifs on its binding partners in order to have an effect within the cell. Using this interactomics tool, we can identify novel binding partners in order to determine the possible effects of 14-3-3 $\zeta$  on the cells.

Our recent mass spectrometry (MS)-based 14-3-3 $\zeta$  interactomics work revealed a number of new interacting partners including the metastasis-regulating protein called Liprin- $\beta$ .<sup>118-119</sup> The ability of 14-3-3 $\zeta$  to bind to Liprin- $\beta$  is particularly interesting because very little is known about the Liprin family of proteins, in particular Liprin- $\beta$ . One particularly concerning effect of 14-3-3 $\zeta$  on cancerous cells is its ability to aid tumors in developing invasive cells,<sup>50</sup> which increases the risk of cancer recurrence and chemoresistance. While 14-3-3 $\zeta$  is known to increase the

occurrence of metastasis, the exact mechanism by which it does so remains unknown. One possible method of promoting cell motility is through its interaction with Liprins.

#### **2.1.4 Workflow**

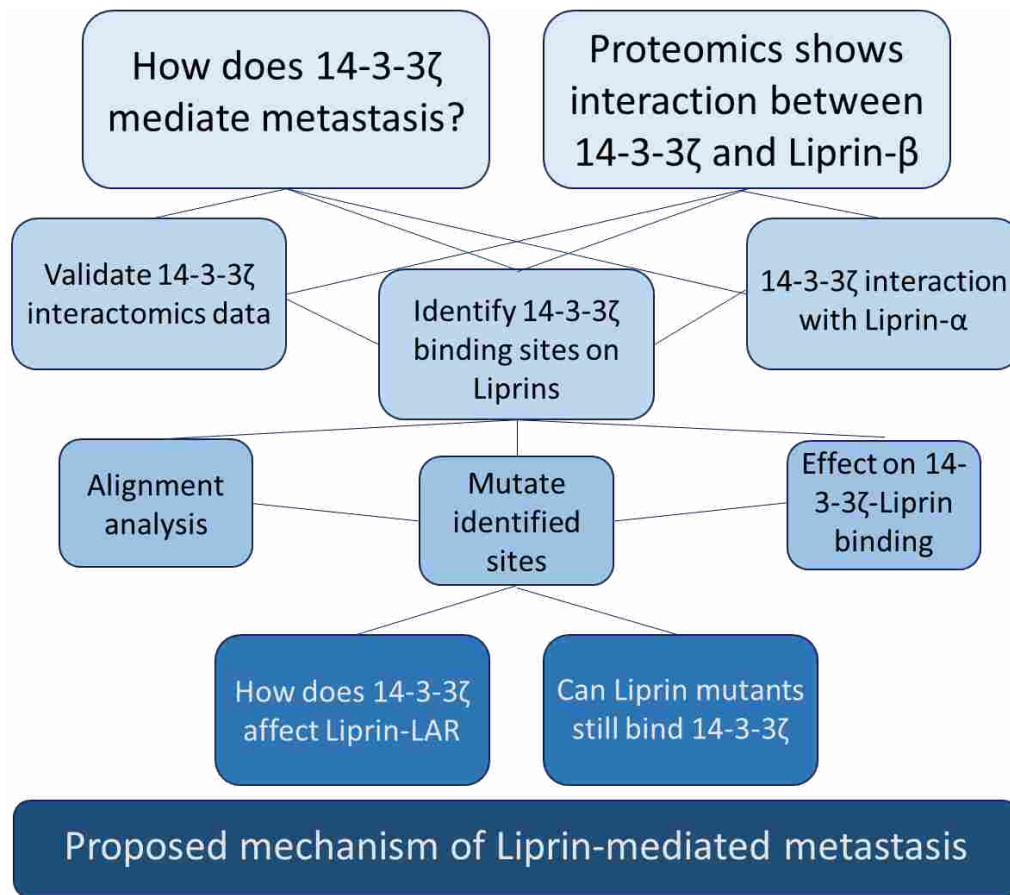
Many questions arise from the aforementioned information about Liprins and 14-3-3 proteins. Foremost among these questions is how does 14-3-3 $\zeta$  induce tumor cell metastasis? To better understand this, we must discover how 14-3-3 $\zeta$  interacts with Liprins and the mechanism by which this interaction induces motility and metastasis. The data discussed in this chapter will be examined according to the following workflow (Figure 4).

## **2.2 Validation of the Interactomics Data**

14-3-3 $\zeta$  interacts with many different proteins, as discussed previously, and can have a variety of functions, the primary one being scaffolding. Considering that 14-3-3 $\zeta$  binds with such specificity, the overexpression of 14-3-3 $\zeta$  has been linked to poor patient prognosis, many different proteins in the cell, a conclusive co-immunoprecipitation is difficult to obtain. In order to validate the interactomics data, we overexpressed tagged Liprin constructs in HEK 293 cells and ran an immunoprecipitation/Western Blot analysis to determine whether they pull down 14-3-3 $\zeta$ .

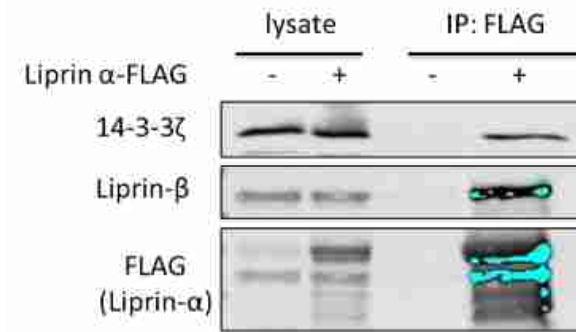
### **2.2.1 Liprin- $\alpha$ Co-Immunoprecipitation**

As described above, the interactomics study revealed that 14-3-3 $\zeta$  binds to Liprin- $\beta$ , which is known to interact with Liprin- $\alpha$ . In order to determine whether Liprin- $\alpha$  also interacts



**Figure 4 Workflow of the experimentation and results discussed in this chapter.** Together with a proteomic analysis showing an interaction between 14-3-3 $\zeta$  and Liprin- $\beta$ , the question “How does 14-3-3 $\zeta$  mediate metastasis?” leads to further research into the exact method of protein binding and the mechanism through which it promotes metastasis.

with 14-3-3 $\zeta$ , we immunoprecipitated overexpressed Liprin- $\alpha$ -FLAG from transfected HEK 293 cell extracts and ran a Western blot analysis and blotted for 14-3-3 $\zeta$  as well as LAR and Liprin- $\beta$ . The results show that 14-3-3 $\zeta$  and endogenous Liprin- $\beta$  will co-immunoprecipitate (co-IP) with overexpressed Liprin- $\alpha$  (Figure 5). This does not, however, indicate a direct interaction between 14-3-3 $\zeta$  and Liprin- $\alpha$ , the two could be linked by complex formation with Liprin- $\beta$ .



**Figure 5 14-3-3 $\zeta$  co-immunoprecipitates with Liprin- $\alpha$ .** HEK 293 cells expressing Liprin- $\alpha$ -FLAG were immunoprecipitated with anti-FLAG agarose beads and the bound proteins visualized through Western blot analysis. Cells treated with Liprin- $\alpha$ -FLAG (lane 4) showed an increase in 14-3-3 $\zeta$  pulldown when compared to the untreated control (lane 3). As has been shown previously, Liprin- $\beta$  also immunoprecipitates with tagged Liprin- $\alpha$ .

### 2.2.2 Liprin- $\beta$ Plasmid Construction

The untagged Liprin- $\beta$  construct was originally in a vector that only expresses in low levels in both prokaryotic and eukaryotic cells. Bacterial transformations and HEK 293 co-transfections with this plasmid showed no expression of Liprin- $\beta$ . In order to use the Liprin- $\beta$  construct we needed to add a tag for immunoprecipitation and clone it into a eukaryotic expression vector. We did this in two steps: first we added an N-terminal Myc tag using the “Myc add” forward and reverse primers (Table 1).

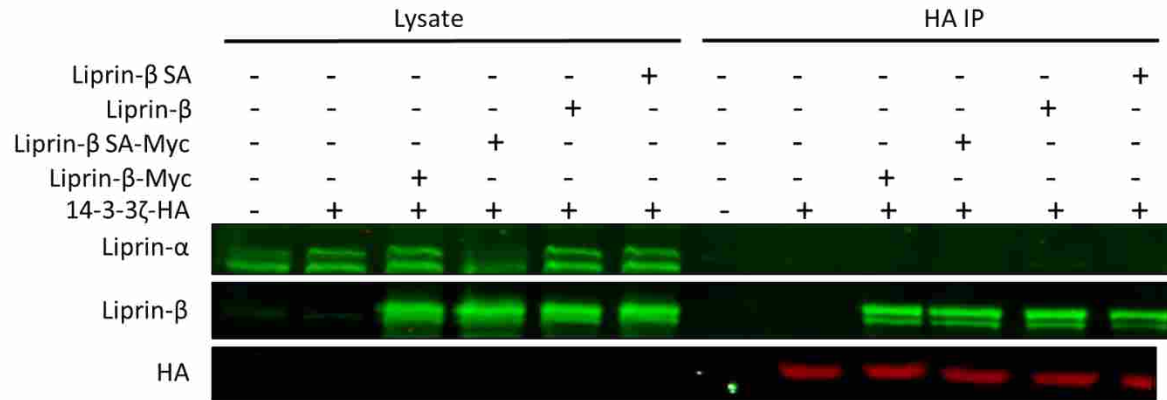
**Table 1: Liprin- $\beta$  Myc-Tag and Restriction Site Addition Primers**

<b>Primer</b>	<b>Sequence</b>
Myc add_F	ttctgaagaagattgATGAGTGATGCAAGTGAC
Myc add_R	atcaatttctgttccatTTTATTATTTGTCAGGGGC
LipB_RE BamHI_F	attataggatccATGGAACAGAAATTGATTTCTGAAG
LipB_RE EcoRV_R	tacaatgatcTCAAACGTTTGAGTCTTCATCTG
Myc del_F	ATGAGTGATGCAAGTGACATG
Myc del_R	CATGGACCGAGCTCGTAC
Myc C-term add_F	tgaagaagattgtgaGATATCCAGCACAGTGGC
Myc C-term add_R	gaaatcaatttctgttcAACGTTTGAGTCTTCATCTG

### 2.2.3 Liprin- $\beta$ Co-Immunoprecipitation

Once the tag was added, we amplified the Myc-tagged Liprin- $\beta$  fragment using primers containing BamHI and EcoRV restriction digestion sites in order to facilitate cloning into the pcDNA3.1 vector. After cloning into the expression vector, we also made clones in which we removed the N-terminal Myc tag and another in which added the tag to the C-terminal. This was done to ensure that any inhibition of binding to Liprin- $\alpha$  or 14-3-3 $\zeta$  was not an artefact of blockage by the N-terminal Myc-tag. The cloning results were validated using DNA sequencing analysis. A co-transfection with 14-3-3 $\zeta$ -HA and Liprin- $\beta$ -Myc or untagged Liprin- $\beta$  demonstrated an interaction between both the tagged and untagged Liprin- $\beta$  constructs and 14-3-3 $\zeta$ , but no interaction with Liprin- $\alpha$  (Figure 6).

Knowing that 14-3-3 $\zeta$  will precipitate with Liprin- $\alpha$ , the lack of interaction demonstrated in this blot could be an artefact of the endogenous signal being covered up by the strong fluorescence from the overexpressed Liprin- $\beta$  proteins. We know from the proteomic analysis that endogenous Liprin- $\beta$  interacts with 14-3-3 $\zeta$ , however we do not see that interaction here (Lane 8). Lanes 1 and 2 show that endogenous Liprin- $\beta$  is present in the cell lysate, so the lack of interaction seen in Lane 8 is likely caused because 14-3-3 $\zeta$  interacts with so many other proteins



**Figure 6 14-3-3ζ and Liprin-β co-transfection shows interaction.** Lysate and HA tag immunoprecipitate from cells co-transfected with 14-3-3ζ-HA and Liprin-β -Myc or 14-3-3ζ-HA and untagged Liprin-β show interaction between Liprin-β and 14-3-3ζ. Immunoprecipitate with the 14-3-3ζ -HA/Liprin-β construct did not co-precipitate Liprin-α. Lanes 1 and 2 show the presence of endogenous Liprin-β but Lane 8 shows that there is not enough present to give a strong signal when co-precipitated with 14-3-3ζ-HA.

that the amount of endogenous Liprin-β pulled down is too little to be observed on a Western blot analysis

### 2.3 Identification of 14-3-3ζ Binding Motifs

The 14-3-3 family of protein is known to bind other proteins at specific motifs conserved across species. Analyses carried out using synthetic phosphopeptides revealed that 14-3-3 proteins bind in one of two ways: to canonical motifs such as RSXpSXP and RX(F/Y)XpSXP or to a phosphorylated residue that is the penultimate amino acid of the C-terminus (<http://scansite.mit.edu>).<sup>151-152</sup> The pS represents the phosphoserine to which the 14-3-3s bind and X represents any amino acid<sup>24</sup>. Though other binding motifs exist, such as a substitution of phosphothreonine for the serine or even non-motif binding, RXXpSXP is the most common.<sup>24</sup> 14-3-3 proteins have been shown to bind to two separate proteins, such as in the plant plasma membrane proton pump where it binds two phosphorylated tails of adjacent subunits.<sup>153</sup>

However, 14-3-3s more commonly bind two phosphorylated sites within the same protein in a cooperative fashion.<sup>152, 154</sup> We searched for these motifs within the Liprin- $\alpha$  and - $\beta$  primary protein structure to identify the putative 14-3-3 $\zeta$  binding sites.

### 2.3.1 Liprin Sequence Alignment

In order to locate the binding sites for 14-3-3 $\zeta$  on each of the Liprins we carried out an alignment of amino acid sequences from various eukaryotic species. We identified the conserved serines and threonines from each alignment and analyzed them for their likelihood of binding to 14-3-3 $\zeta$ . The possible serine and threonine binding sites were analyzed based on the criteria found in Table 2.

**Table 2: Criteria for 14-3-3 $\zeta$  Binding Motif Identification**

<b>Criterion</b>	<b>Explanation</b>
Conserved	It must be conserved across most, if not all, species
RXXpS	It is preceded by an arginine or other positively charged amino acid at least three but no more than five residues upstream
pSXP	It is followed by a proline two residues downstream
Location	It cannot be located within the coiled coil region or any of the SAM domains

On Liprin- $\beta$  we identified one site at S540 that follows the exact RXXpSXP pattern and met all of the criteria (Figure 7A). None of the serines nor the threonines on Liprin- $\alpha$  followed the pattern as closely as did Liprin- $\beta$ , nevertheless four other possible binding sites were identified that are similar, though not identical, to the 14-3-3 $\zeta$  binding motif and met the criteria (Figure 7B and C). In one case, at S239, the serine was followed by a +2 glycine instead of a proline. We accepted this as a possible motif because both of these hydrophobic residues allow for kinks in the secondary and tertiary protein structure. The proline has been shown to twist the peptide out





**Figure 7 Locating the putative 14-3-3 $\zeta$  binding motifs on Liprins.** **A** Alignment of a possible binding site on Liprin- $\beta$  at S540 and **B** on Liprin- $\alpha$  at T387. The red box denotes the conserved motif containing a pS or pT where 14-3-3 $\zeta$  could bind. All serines and threonines highlighted blue. **C** Table listing all the possible 14-3-3 $\zeta$  binding sites identified on both Liprins. Column one indicates the Liprin family member, column two lists the amino acid residue according to the sequence found on uniprot.org, column three shows the whole motif surrounding the phosphorylation site.

of the 14-3-3 docking site, a bond rotation that glycine is also capable of supporting due to its small size.<sup>26</sup> The necessity of this twist away from the 14-3-3 dimer remains unknown, but the substitution of a bulky or charged residue for the proline could disrupt the binding ability.<sup>24, 36</sup>

### 2.3.2 Mutation of the Binding Sites

The phosphorylation of 14-3-3 $\zeta$  motifs is required for binding of 14-3-3 $\zeta$  to target proteins. By generating alanine mutations at the serine or threonine, as identified and described in Figure 7, we created Liprin mutants that cannot be phosphorylated at those residues. The inability of the mutants to be phosphorylated will allow us to identify the exact phosphoserine or threonine to which 14-3-3 $\zeta$  binds on one or both of the Liprins. The sequence of the final Liprin- $\alpha$  mutant contained two serines, one with an arginine at the -3 position and the other with a proline at +2. As either serine could be the target of phosphorylation and 14-3-3 $\zeta$  binding, both residues were mutated to alanine.

The Liprin- $\beta$  and Liprin- $\alpha$  S530A mutants (denoted with \*) were prepared using the Agilent QuikChange Site-Directed Mutagenesis Kit and primers designed with the QuikChange® Primer Design Program (Agilent Technologies, California). The remaining three mutant Liprin- $\alpha$  constructs (denoted with †) were prepared via site-directed mutagenesis using the Q5® Site-Directed Mutagenesis Kit and primers designed using the NEBaseChanger™ (New England Biolabs, Massachusetts). The mutant Liprin- $\beta$  S540A construct is herein referred to only as Liprin- $\beta$  SA, as there was only a single mutant construct designed. The four Liprin- $\alpha$  SA mutants will be identified by the base number of the mutation (i.e. Liprin- $\alpha$  S239A). See Table 3 for a list of the primers used in site-directed mutagenesis, lower case letters indicate the bases altered to obtain the alanine mutant, results were validated with sequencing.

**Table 3: Primers for Site-Directed Mutagenesis**

<b>Mutation</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
*Liprin- $\beta$ SA	AACAAGAGAACAGCAgcTGC ACCAAACCTTAGC	GCTAAGTTTGGTGCAgcTG CTGTTCTCTTGTT
†Liprin- $\alpha$ S239A	AAAGAGATCTgCTGATGGTT CTTTAAG	CCACTCGTGCTTGGTGTA
†Liprin- $\alpha$ T387A	GAAGGCAGAGgCGCTCCCGG	CTCAGTGTCTGTTGCAGCT TTTGC
*Liprin- $\alpha$ S530A	CCCCACTTGGGCgcTGTCCCA GATTT	AAATCTGGGACAgcGCCCA AGTGGGG
†Liprin- $\alpha$ SS679- 680AA	TAGATCTATGgccgCCATTCC CCCC	AAACGACCAAGATTGTCTAG

\* Agilent QuikChange Site-Directed Mutagenesis Kit

† Q5® Site-Directed Mutagenesis Kit

## 2.4 Proposed Effects of the Mutant Liprin Constructs

FLAG-tagged Liprin- $\alpha$  (WT, SA, and TA) constructs and Myc-tagged Liprin- $\beta$  (WT and SA) constructs were transfected into HEK 293 cells. After a 48-hour incubation period the cells

were lysed using a 1% Triton-X100 lysis buffer and the proteins co-immunoprecipitated from the extract. The relative quantity of 14-3-3 $\zeta$  that co-precipitated with the tagged constructs was measured by separating the proteins on a Western blot and immunoblotting the precipitate with anti-14-3-3 $\zeta$  antibody. Equal loading was determined by immunoblot using an anti-FLAG (Liprin- $\alpha$ ) or anti-Myc (Liprin- $\beta$ ) antibody. The 14-3-3 $\zeta$  bands from the lanes treated with the mutant constructs were compared to the WT constructs to determine whether the alanine substitution disrupted the binding of 14-3-3 $\zeta$  to the Liprins.

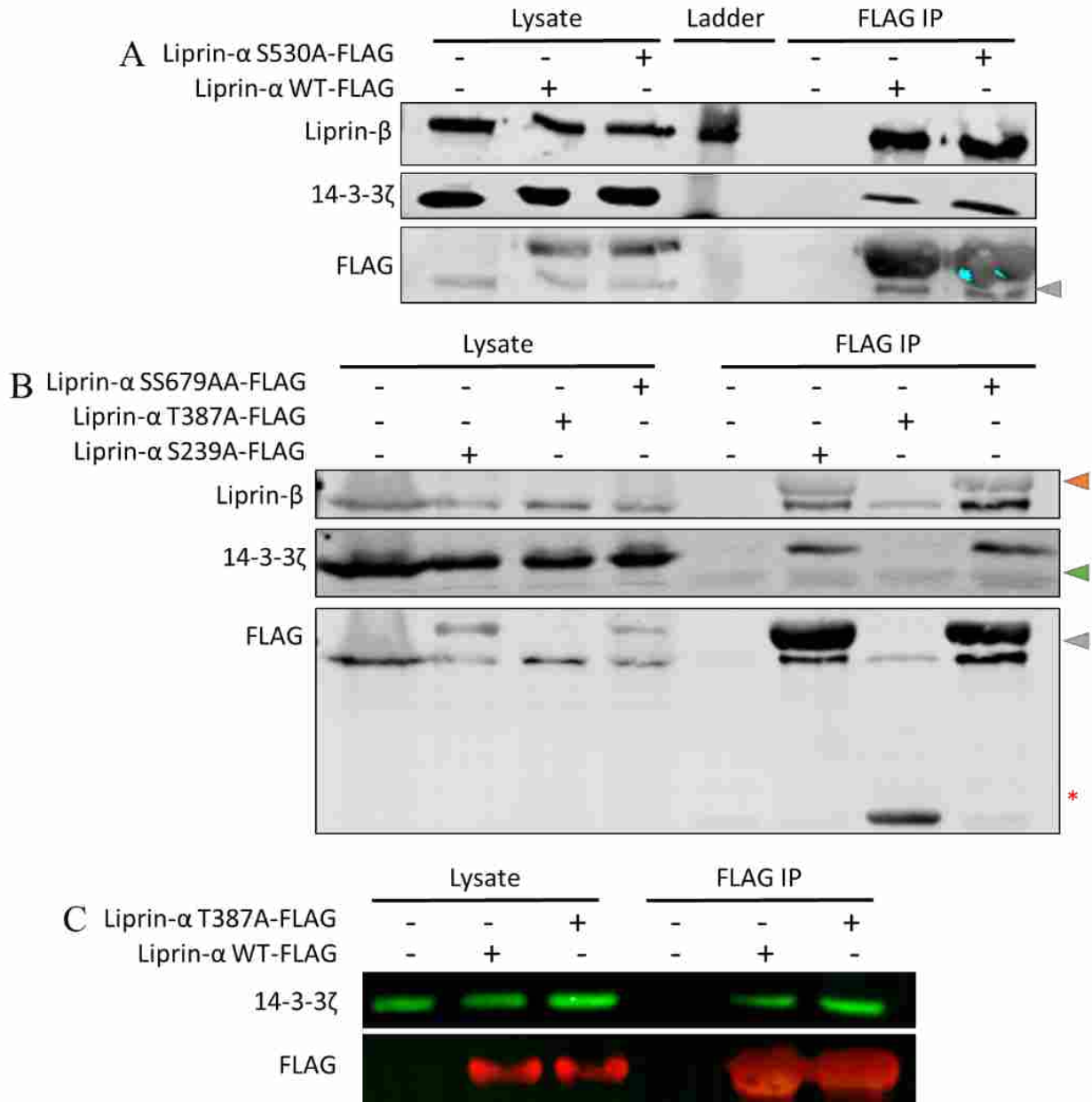
There were two possible outcomes for the binding of 14-3-3 $\zeta$  to the Liprins mutants: 1) 14-3-3 $\zeta$  would not IP as strongly with the Liprin S/TA mutant, or 2) minimal to no change would be seen in the binding of the Liprin mutant when compared to the WT. Results showing the first outcome would indicate that the mutated residue plays an important role in the binding of 14-3-3 $\zeta$  to Liprin. The second outcome would indicate that either 14-3-3 $\zeta$  does not interact with the mutated residue or that the binding between 14-3-3 $\zeta$  and the mutated serine or threonine is a weak interaction and not necessary to maintain the Liprin-14-3-3 $\zeta$  interaction.

The outcome would depend on the direct or indirect interaction between 14-3-3 $\zeta$  and the Liprin subunits. It could bind to two residues within one of the Liprins or one residue on each of the Liprins. The 14-3-3 $\zeta$  interactomics data supports the first hypothesis in that only Liprin- $\beta$  was identified as a binding partner. Liprin- $\alpha$  could still be part of the complex but have a direct interaction with Liprin- $\beta$  and an indirect interaction with 14-3-3 $\zeta$ . The second hypothesis is supported by the data in Figure 5 that illustrates how 14-3-3 $\zeta$  co-immunoprecipitates from cells treated with WT Liprin- $\alpha$ -FLAG. Additionally, the alignment data from Liprin- $\alpha$  indicate the possibility that Liprin- $\alpha$  binds to 14-3-3 $\zeta$  at one of the amino acid residues listed in Figure 7C. Though not an exact match to the most common RXXpS/TXP motif, the putative binding sites

on Liprin- $\alpha$  differ by only a few amino acids. For example, the T387A mutant differs only in the first amino acid, K, which is the second most common amino acid found at the -4 position. Furthermore, in the bovine Liprin- $\alpha$  protein, the lysine is an arginine, making it an exact match to the common 14-3-3 $\zeta$  binding motif.

## 2.5 Liprin- $\alpha$ Mutants

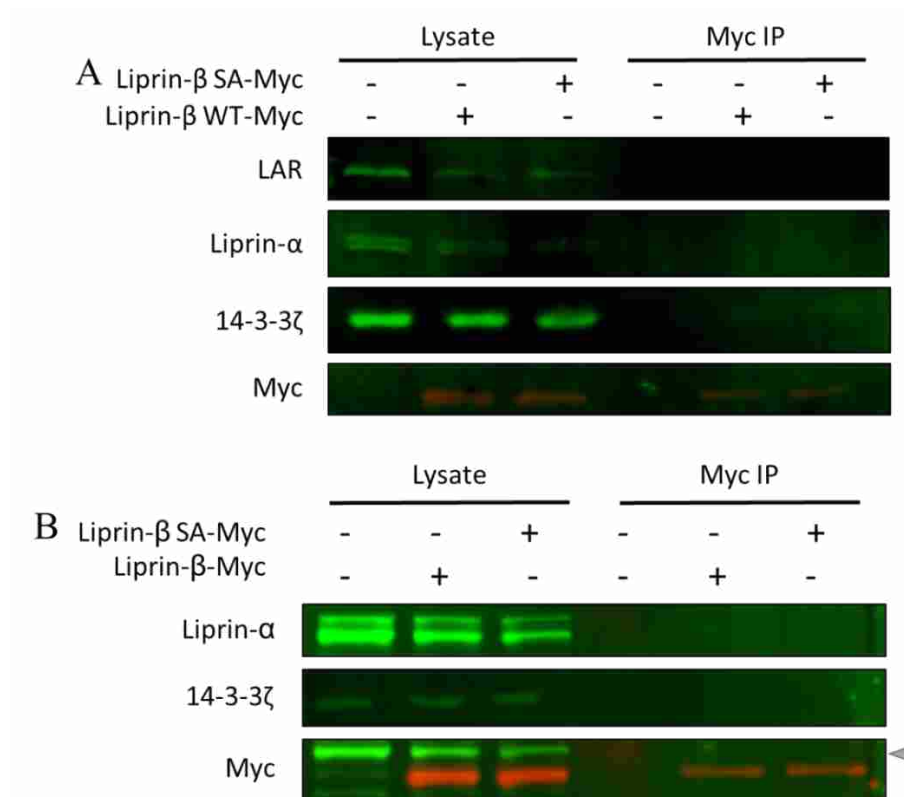
We first tested the Liprin- $\alpha$  mutants because the Liprin- $\alpha$  construct contained a FLAG tag to aid in immunoprecipitation. Initially only the S530A mutant was tested, but it showed no decrease in the amount of 14-3-3 $\zeta$  binding, the amount of 14-3-3 $\zeta$  pulled down by the Liprin- $\alpha$  mutant compared in quantity to that of the wild type (WT) (Figure 8A). Following the S530A mutation, we mutated the WT Liprin- $\alpha$  construct with three separate sets of primers to obtain the S239A, T387A, and SS679-680AA mutants. Initially the T387A construct obtained was truncated (Figure 8B), which did not pull down 14-3-3 $\zeta$  and only minimally interacted with Liprin- $\beta$  so the WT Liprin- $\alpha$  was mutated once again with the Q5® Site-Directed Mutagenesis kit to obtain the complete, mutated Liprin- $\alpha$ . As seen in Figure 8C and D, none of these constructs were able to inhibit the co-immunoprecipitation of 14-3-3 $\zeta$  with Liprin- $\alpha$ . These results indicate that the interaction between 14-3-3 $\zeta$  and the Liprin complex occurs either completely or primarily on Liprin- $\beta$ .



**Figure 8 Liprin- $\alpha$  SA mutants do not inhibit 14-3-3 $\zeta$  co-immunoprecipitation.** A S530A mutant is able to co-IP Liprin- $\beta$  and 14-3-3 $\zeta$  in comparative quantities, gray arrow indicates Liprin- $\beta$ . B SS679-680AA and S239A mutants also pull down 14-3-3 $\zeta$  and Liprin- $\beta$ . Orange arrow indicates Liprin- $\alpha$ , green arrow indicates IgG, and gray arrow indicates Liprin- $\beta$ . Red \* denotes truncated T387A mutant. C T387A mutant is also able to pull down 14-3-3 $\zeta$  in quantities comparative to the WT.

## 2.6 Liprin- $\beta$ Mutants

Initially, the putative 14-3-3 $\zeta$  interaction with Liprin- $\beta$  was the more promising of the two Liprins for two reasons: first, the beta isoform was the only Liprin originally identified in the interactomics study and second, it contains a conserved 14-3-3 phospho-binding motif at serine 540. To determine whether the Liprin- $\beta$  SA mutant construct is able to interact with 14-3-3 $\zeta$  in vivo, we overexpressed and immunoprecipitated N-terminal Myc-tagged Liprin- $\beta$  (referred to herein as Liprin- $\beta$ -Myc) from HEK 293 cells. The resulting Western blot showed little binding of 14-3-3 $\zeta$  and no difference between the mutated and WT forms (Figure 9A).



**Figure 9** 14-3-3 $\zeta$ , Liprin- $\alpha$ , and LAR do not co-immunoprecipitate with Liprin- $\beta$  WT or SA mutants. **A** Liprin- $\beta$  containing a Myc tag at the N-terminal transfected into HEK 293 cells only co-precipitates 14-3-3 $\zeta$  to a limited degree. **B** Validation of A indicates that Liprin- $\beta$  pulldown does not co-precipitate 14-3-3 $\zeta$ . Grey arrows indicate Liprin- $\alpha$  bands.

Interestingly, neither Liprin- $\alpha$  nor LAR precipitated with Liprin- $\beta$ . Though we did not anticipate observing an interaction with LAR, as it directly interacts with Liprin- $\alpha$ , we expected that the Liprin- $\alpha$  co-precipitation could act somewhat as a positive control. As was discussed in the previous section, Liprin- $\beta$  is able to co-precipitate with as Liprin- $\alpha$ -FLAG pulldown. Previous reports conclude that these two Liprins interact via their C-terminal LH domain, yet we were unable to observe that interaction in any of our Liprin- $\beta$  co-IP Western blot analyses, as will be shown hereafter.

The data shown in Figure 9A seem to contradict our prediction and preliminary data that indicate a tight interaction between 14-3-3 $\zeta$  and Liprin- $\beta$ , though we did observe limited binding between both the WT and mutant Liprin- $\beta$  SA constructs. Thinking that perhaps there was an error in how the co-IP was performed, we repeated the experiment, taking care to follow the protocol exactly and observed no co-precipitation of 14-3-3 $\zeta$  with a Liprin- $\beta$  pulldown (Figure 9B). Additionally, we observed that the Myc IP expressed lower levels than the Myc in the lysate. This is not normally the case with IPs, but could be due to inaccessibility of the Myc tag or poor affinity for the antibody expressed on the beads. Though these data oppose our hypothesis, possible explanations include obstruction of the binding site by the Myc tag, or too-harsh lysis conditions. In order to rule out these as possibilities of interference we carried out further tests and accounted for these variables, as described in the following sections.

### **2.6.1 Liprin- $\beta$ -CMyc**

The lack of binding between Liprin- $\beta$  and Liprin- $\alpha$ /14-3-3 $\zeta$  could be due to steric hindrance or occlusion of the binding site by the Myc tag. This was the most likely factor affecting the binding because, as previously mentioned, the Myc IP was not as strong as the Myc

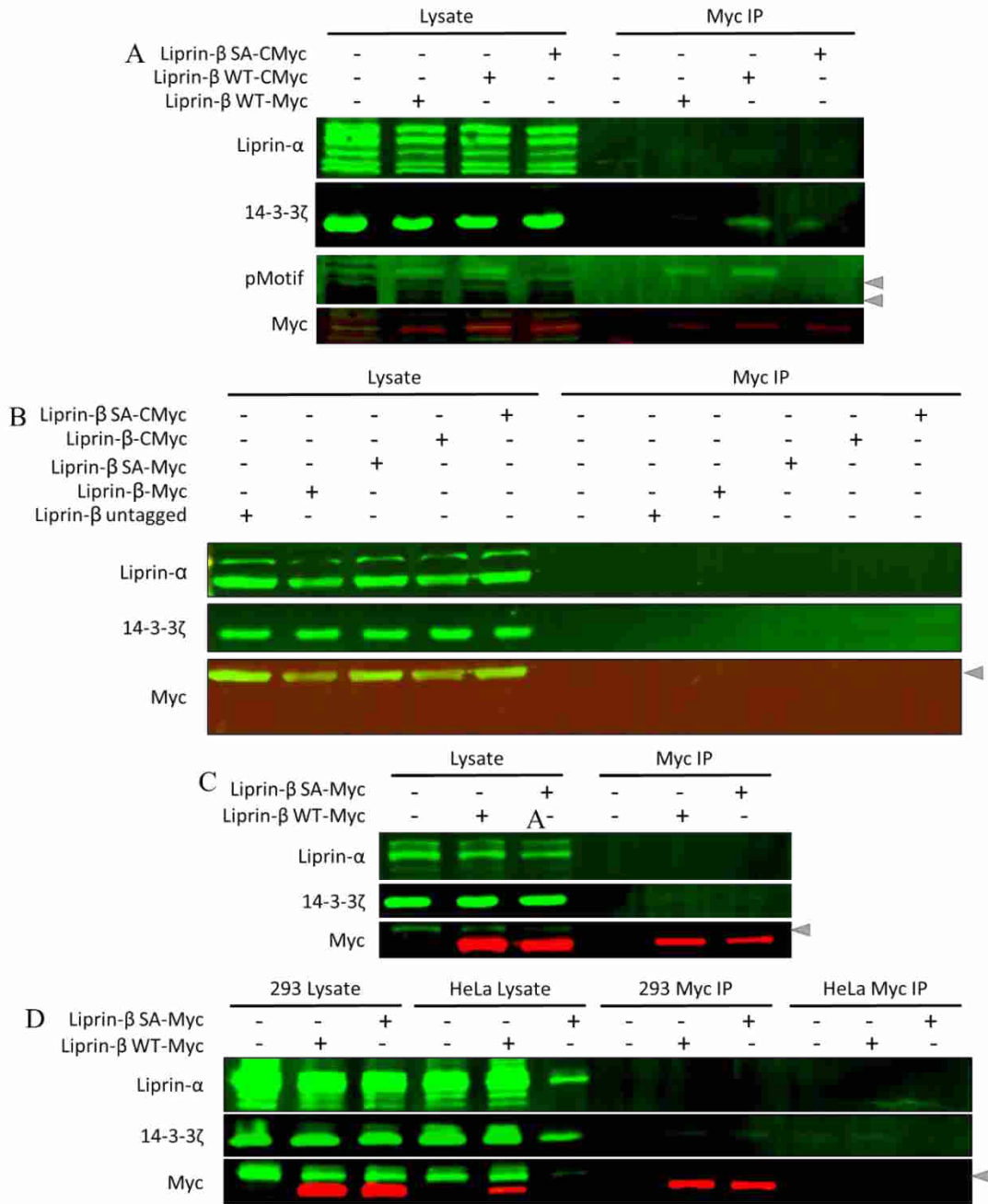
in the total lysate. To ensure that the weak or nonexistent interaction between Liprin- $\beta$  and 14-3-3 $\zeta$  was not due to the placement of the Myc tag, we created a Liprin- $\beta$  construct with a C-terminal Myc tag, hereafter referred to as CMyc. The Liprin- $\beta$ -CMyc constructs (both WT and SA) were transfected into HEK 293 cells, co-immunoprecipitated, and run on a Western blot (Figure 10A). Placement of the Myc tag on the C-terminal did not increase Liprin- $\alpha$  or 14-3-3 $\zeta$  binding. Again we saw less Myc in the IP than we did in the total lysate. We also saw limited pulldown of 14-3-3 $\zeta$ .

This blot was the most promising, as there appeared to be a stronger interactions between Liprin- $\beta$ -CMyc and 14-3-3 $\zeta$  than between Liprin- $\beta$ -Myc and 14-3-3 $\zeta$ . Additionally, the Liprin- $\beta$  SA-CMyc appeared to have a weaker interaction with 14-3-3 $\zeta$  than the WT construct, though we are unable to conclusively determine that the SA mutation diminishes binding with 14-3-3 $\zeta$  because we were unable to validate this in subsequent co-IP/WB analyses (Figure 10B). We blotted using an antibody specific to the phospho 14-3-3 motif (pMotif) and saw that the antibody specifically recognized WT Liprin- $\beta$  but not Liprin- $\alpha$  nor the Liprin- $\beta$  SA mutant. We were unable to conclude that the CMyc tag was more effective than the Myc tag on the N-terminus due to our inability to validate the IP.

## 2.6.2 Liprin- $\beta$ IP with a Gentle Lysis Buffer

Another possible source of disruption on the interaction between these proteins comes from the lysis buffer. The results previously described were obtained using a harsh lysis buffer capable of extracting the membrane-bound LAR from the phospholipid bilayer. In these Liprin- $\beta$  immunoprecipitations we are not looking for interaction with LAR (as it binds directly to Liprin- $\alpha$  but not Liprin- $\beta$ ) so we carried out a lysis using a different, less harsh, lysis buffer (Figure





**Figure 10 Co-IP of Liprin-β not improved by CMyc tag, lysis buffer, or cell type. A** Liprin-β containing a CMyc tag cells only minimally co-precipitate 14-3-3ζ but not Liprin-α. WT Liprin-β is recognized by the pMotif antibody (lanes 6 and 7). **B** A second co-IP and Western blot using Liprin-β-CMyc showed no transfection. **C** Cells transfected with WT and Liprin-β SA were lysed with a gentle lysis buffer, this still does not significantly co-precipitate 14-3-3ζ or Liprin-α with Liprin-β. **D** Liprin-β does not significantly co-precipitate 14-3-3ζ or Liprin-α from either HEK 293 or HeLa cells. Gray arrow indicates Liprin-α immunofluorescence.

10C). Despite using a gentler lysis buffer and procedure, 14-3-3 $\zeta$  only showed minimal co-precipitation (with no difference between WT and SA Liprin- $\beta$  constructs) and Liprin- $\alpha$  still did not precipitate with the Myc-tagged Liprin- $\beta$ . Though minimal amounts of 14-3-3 $\zeta$  co-IP were observed, there was no measurable difference between the WT and SA Liprin- $\beta$  constructs. We concluded that the lysis conditions between the two buffers had little to no effect on the amount of Myc-tagged proteins that precipitated with the myc antibodies conjugated to agarose beads (Figure 10B lanes 5 and 6), nor did it appear to greatly affect the co-precipitation of 14-3-3 $\zeta$ .

### **2.6.3 Liprin- $\beta$ Transfection in HEK 293 and HeLa Cells**

In a final attempt to precipitate Liprin- $\alpha$  and 14-3-3 $\zeta$  with Liprin- $\beta$ , we transfected both HEK 293 and HeLa cells with the WT and mutant Liprin- $\beta$ -Myc constructs and carried out a lysis, immunoprecipitation, and Western blot analysis. As with the previous attempts, neither of the target proteins measurably co-precipitate (Figure 10D). Strangely, we were able to observe a transfection of Liprin- $\beta$ -Myc into the HeLa cells, but it did not appear in the IP. This leads us to believe that the IP of Liprin- $\beta$  with Myc antibodies conjugated to agarose beads is inefficient and needs to be optimized.

### **2.6.4 Liprin- $\beta$ Conclusion**

The results of the interactomics study along with research in primary literature lead us to hypothesize that Liprin- $\beta$  and 14-3-3 $\zeta$  would have a strong interaction observable by co-IP and Western blot analysis. Despite the promising preliminary data we collected using Liprin- $\alpha$ -FLAG precipitations and 14-3-3 $\zeta$ /Liprin- $\beta$  co-transfections (Figure 6) we were unable to obtain a valid, replicable co-IP using Liprin- $\beta$  pulldown using C-terminal and N-terminal Myc tags.

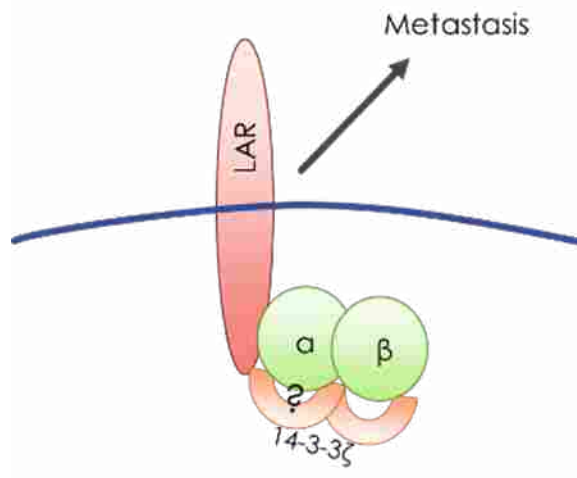
We chose to use Myc tags on the Liprin- $\beta$  constructs because our Liprin- $\alpha$  had a FLAG tag and 14-3-3 $\zeta$  an HA tag. By tagging Liprin- $\beta$  with Myc, we would be able to easily distinguish between all three proteins in the event of a co-transfection. However, the IPs run with Myc-tagged proteins were inefficient when compared to those done with FLAG- and HA-tagged proteins. When adding the Myc tag onto the Liprin- $\beta$  construct, we also made a construct with a FLAG tag, in case the Myc tag addition did not work properly. However, sequencing data validated that both constructs had the proper tag added to the plasmid containing Liprin- $\beta$ . We determined that the best course of action would be to use the Myc-tagged construct, as it would help us distinguish exogenous Liprin- $\beta$  from exogenous Liprin- $\alpha$  or 14-3-3 $\zeta$  when running co-transfections. The Liprin- $\beta$ -FLAG construct was never prepared past addition of the tag, though for future work the FLAG-tagged construct may prove more reliable than the Myc-tagged Liprin- $\beta$ . When considering the data from Figure 6 in addition to that presented in Figures 9 and 10, we can see that the Liprin- $\beta$ -Myc and -CMyc immunoprecipitations are unreliable and the data inconclusive.

## **2.7 Proposed Mechanism of Liprin-Mediated Metastasis**

LAR is a PTPase known to dephosphorylate and interact with proteins that participate in cell adhesion. The PTPase family is divided into two groups, or subfamilies, consisting of receptor-like (RPTP) and nonreceptor PTPases, which function by dephosphorylating tyrosyl-phosphorylated proteins.<sup>105-106</sup> Like other RPTPs, LAR possesses a single transmembrane domain and two intracellular phosphatase domains named D1 and D2.<sup>106-107</sup> Unlike most receptors, ligand binding typically causes inactivation of the RPTPs through dimerization and inhibition of the active D1 domain by occlusion from the D1 domain of the opposing subunit.<sup>108-</sup>  
<sup>112</sup> The D1 domain is believed to carry out the PTPase activity while the D2 domain typically

remains inactive but plays a key regulatory role in determining substrate specificity.<sup>110, 113-116</sup>

Liprin- $\alpha$  binds to the D2 domain of LAR PTPase and is believed to influence which substrates LAR dephosphorylates when bound to Liprins.<sup>117</sup> Published findings and our data indicate that both 14-3-3 $\zeta$  and LAR participate in the activation of cell motility. We posit that 14-3-3 $\zeta$  utilizes LAR-mediated responses in order to cause an increase in the ability of a cell to migrate. We propose that 14-3-3 $\zeta$  plays a scaffolding role with Liprin- $\beta$ , allowing it to bind Liprin- $\alpha$ , thus aiding in the localization to the cell periphery where it recruits LAR (Figure 11).



**Figure 11 Proposed model of 14-3-3 $\zeta$ -induced and Liprin-mediated metastasis.**

The 14-3-3 $\zeta$  homodimer binds to a phosphorylated motif on Liprin- $\beta$  and putatively on Liprin- $\alpha$ . This interaction facilitates the recruitment of LAR to the cell periphery where the complex initiates metastasis.

Receptor tyrosine kinases (RTKs), the counterpart to RPTPs, act in a manner distinct to that of RPTPs and are activated through ligand-induced dimerization whereupon each subunit participates in a trans-phosphorylation of tyrosine residues on the opposing receptor.<sup>134</sup> RTKs

require a triple phosphorylation in order to remain fully active and often are inactivated through dephosphorylation by either a nonreceptor PTPase or an RPTP. One such RTK substrate of the LAR PTPase is Ephrin type-A receptor 2 (EphA2),<sup>135</sup> also known as epithelial-cell kinase (Eck), which is a member of the largest family of RTKs.<sup>136</sup> Like the other 13 members of the Ephrin family, EphA2 is membrane-bound and activated by ligands called ephrins, specifically ephrin-A. It is believed that the Eph-ephrin interaction and signaling cascade participate in cell motility by limiting migration through inhibition of integrin-mediated adhesion and spreading, thus obstructing migration.<sup>137-140</sup>

We propose that 14-3-3 $\zeta$  overexpression initiates metastasis by recruiting LAR to the cell periphery where it can dephosphorylate EphA2, thus inactivating its ability to stabilize integrins and FAs at the cell surface. This, in turn, triggers the uptake of FAs into the cell causing a loss of adhesion with the surrounding tissue and ECM, whereupon the cell is free to mobilize and metastasize to a secondary site within the organism as depicted in Figure 2.

### **2.7.1 Effect of 14-3-3 $\zeta$ on Liprin- $\alpha$ -LAR Binding**

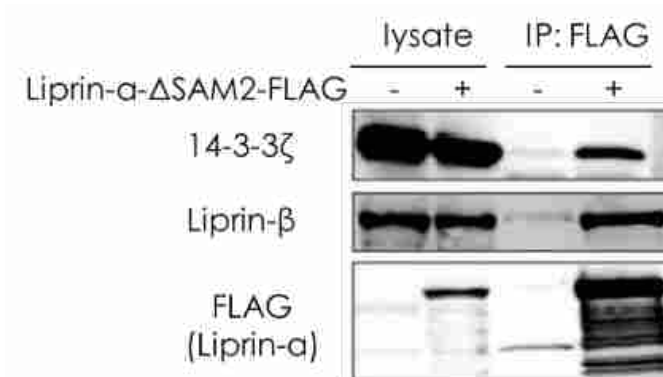
Considering the role of Liprins in cell motility and given that 14-3-3 $\zeta$  has been shown to initiate cell movement and play a part in invasion, the 14-3-3 $\zeta$ -binding deficient mutants should disrupt the ability of the cells to metastasize.<sup>23, 50-51, 155</sup> However, in order to ensure that the effect of 14-3-3 $\zeta$ -Liprin binding plays a role in metastasis, we must measure the ability of these mutant proteins to interact with LAR. 14-3-3 $\zeta$  has been shown to interact with Liprin- $\beta$ , which is known to bind Liprin- $\alpha$ . Liprin- $\alpha$ , in turn binds LAR and the two have been shown to work cooperatively to induce cell motility.<sup>118-119, 144</sup> It has been shown that the overexpression of full length Liprin- $\alpha$  is sufficient to drive motility and cause a change in the distribution of FAs at the

cell surface, however, truncated versions of the same protein were unable to do so.<sup>119</sup>

Furthermore, Liprin- $\alpha$ - $\Delta$ SAM2 mutants, which lack the domain of the LH region where LAR interaction occurs, were unable to increase cell spreading of COS-7 cells.<sup>118</sup> Considered together, all of this data leads to the conclusion that Liprin- $\alpha$  plays a role in cell motility but alone is insufficient to induce it.

As noted above, Liprin- $\alpha$ - $\Delta$ SAM2 mutants are unable to bind LAR and many of the Liprin-mediated effects on cell motility are undetected in cells transfected with  $\Delta$ SAM2 mutants. Liprin- $\alpha$  and Liprin- $\beta$  have been shown to form heterodimers through their C-terminal LH domains though the exact location of binding remains unknown.<sup>117</sup>

In accordance with this data and as seen in Figure 12, Liprin- $\alpha$ - $\Delta$ SAM2 can still co-immunoprecipitate Liprin- $\beta$ , indicating that it does not bind to the SAM2 domain. This does not



**Figure 12 Liprin- $\alpha$ - $\Delta$ SAM2 can bind Liprin- $\beta$ .** Cells transfected with a mutant form of Liprin- $\alpha$  lacking the second SAM domain for the LH region are able to co-IP both Liprin- $\beta$  and 14-3-3 $\zeta$  (lane 4) despite its inability to interact with LAR.

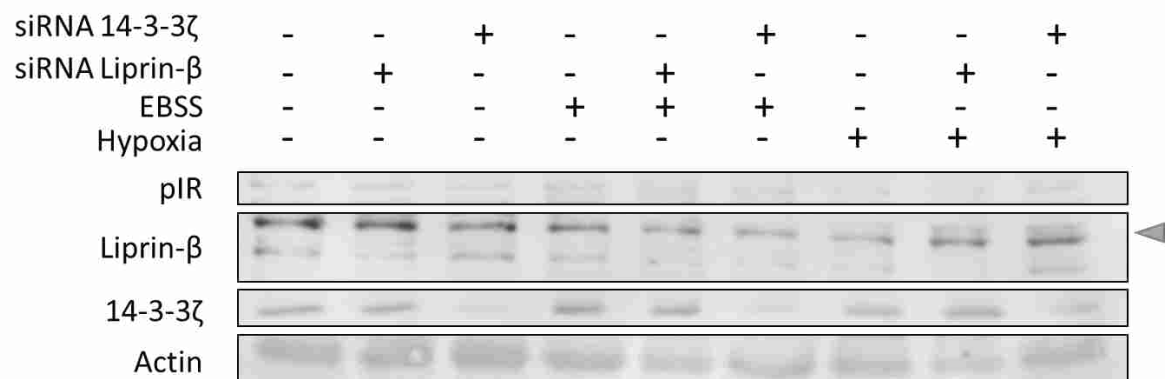
directly indicate that Liprin- $\alpha$  can bind both LAR and Liprin- $\beta$  but rather it shows that they do not bind to the same SAM domain within Liprin- $\alpha$ , which suggests that Liprin- $\alpha$  could possibly bind to both proteins. Additionally, the results show that Liprin- $\alpha$  binds both Liprin- $\beta$  and 14-3-3 $\zeta$  simultaneously. Previous research insinuates that the formation of Liprin- $\alpha/\beta$  heterodimers at the C-terminal LH domain precludes them from binding to other proteins.<sup>117</sup> Our results, however, show that this is not the case, as both 14-3-3 $\zeta$  and Liprin- $\beta$  co-immunoprecipitate with Liprin- $\alpha$ .

### **2.7.2 Effect of 14-3-3 $\zeta$ on EphA2 and IR $\beta$ Phosphorylation**

LAR is a membrane-bound protein tyrosine phosphatase (PTPase) known to dephosphorylate Insulin Receptor  $\beta$  (IR $\beta$ ) and EphA2.<sup>135, 156</sup> As a phosphatase we can visualize LAR activity by measuring the amount of phosphorylation on its substrates. Through interaction with LAR, we believe that Liprins and 14-3-3 $\zeta$  cause an increase in the dephosphorylation activity of LAR, though this theory remains to be tested. Additionally, one key question is whether Liprin interaction with LAR determines what substrates LAR targets. Based on our data and previously published findings, we posit that Liprin- $\alpha$  increases LAR phosphatase activity promoting the disassembly of FAs and increase in cell motility.

LAR has been shown to dephosphorylate both pIR $\beta$  and pEphA2 at Y1150 and Y930, respectively. Ideally we would quantify the relative phosphorylation of EphA2 upon overexpression of 14-3-3 $\zeta$ , however no antibody exists for pEphA2 at Y930. Before making our own antibody, we validated our proposed method by observing the phosphorylation of pIR $\beta$ . While HEK 293 cells express pIR $\alpha$ , they do not express significant levels of pIR $\beta$  so the experiments described herein was conducted using HeLa cells.

Similar to the experiments described above, we carried out a Western blot analysis to measure LAR phosphatase activity. We used siRNA to knock down 14-3-3 $\zeta$ , and Liprin- $\beta$  in HeLa cells in parallel experiments. After a 12 hour incubation, each plate containing the transfected or untransfected (control) cells was split into three different plates. Each set of cells (consisting of one plate split from each of those initially transfected) will receive a different treatment prior to lysis and analysis. At 36 hours, one set of cells was treated with hypoxia (hypoxia is herein used to refer to conditions of low glucose and low oxygen, mimicking the conditions found in the interior of a tumor); at 46 hours, the second set was treated with serum starvation using EBSS media; the final set received no treatment, mimicking normoxic conditions. At 48 hours all three sets of cells were lysed and the proteins extracted. The proteins extracts were run on a Western blot and stained using an immunofluorescent anti-pIR $\beta$  Y1150/1151 antibody (Figure 13).



**Figure 13 Phosphorylation of IR $\beta$  in HeLa cells remains unchanged by knock down of Liprin- $\beta$  and 14-3-3 $\zeta$ .** HeLa cells transfected with siRNA to Liprin- $\beta$  or 14-3-3 $\zeta$  and analyzed for phosphorylation of IR $\beta$  in conditions of normoxia, hypoxia, and serum starvation showed no change. Gray arrow indicates nonspecific band from the anti-IR $\beta$  antibody.



The results of the analysis were inconclusive for a few different reasons: 1) The anti-pIR $\beta$  antibody bound nonspecifically to various proteins, and at the molecular weight corresponding to pIR $\beta$  it showed little to no signal. 2) We were unable to obtain a good knock down of Liprin- $\beta$ . 3) As this was only a preliminary experiment it lacked the proper controls, such as an antibody for pIR $\alpha$ . 4) While we used a lysis buffer shown previously to extract membrane-bound proteins, we cannot say for sure whether it was able to extract pIR $\beta$  from the phospholipid bilayer without causing it to precipitate with the cellular debris. We concluded that using siRNA knockdown of Liprins or 14-3-3 $\zeta$  in HeLa cells as described in this section and looking for an effect on phosphorylation is not the ideal method of evaluating the dephosphorylation of EphA2 by LAR PTPase.

## **2.8 Thoughts and Conclusions**

When synthesizing the data discussed in this chapter and writing out the results, or lack thereof, in the form of a thesis, it was very disheartening to realize that there is very little to actually include and even less that I can publish. It appears at first as though very little work was actually accomplished and in one sense of the word, that is true. Despite the work, thought, and effort put in by multiple researchers, I can offer no conclusive data. However, to me the work described in this chapter represents more than just data and conclusions. To me it represents two years of learning how to work in a lab, read primary literature, and most importantly think like a researcher. I have come to realize that the bulk of the work is not shown in the concluding research article, thesis, dissertation, or review, or other publication. Oftentimes the most important things are those not represented at all: the long hours spent rehashing data and literary searches when you should have been sleeping, the number of tears shed when your experiment didn't work yet again, and the elation of finally getting one tiny thing to work the way it should,

even if it isn't necessarily publishable. Like many others involved in research, it's these moments and the hunger for knowledge that keep me going despite the odds. I believe that this work with Liprins and 14-3-3 $\zeta$  sets forth the foundation for further research and actual conclusive data. In the following chapter I will discuss a few of the thoughts I have had about the future possibilities for research into Liprins and 14-3-3 $\zeta$  and their role in cancer cell metastasis.

### 3 FUTURE DIRECTION

#### 3.1 Identify the 14-3-3 $\zeta$ Binding Sites

Despite the work described in Chapter 2, we obtained no conclusive evidence as to where 14-3-3 $\zeta$  binds to Liprin- $\beta$ . We also were unable to elucidate whether it bound to Liprin- $\alpha$ , as the immunoprecipitation data seems to suggest. These results are most likely due to the fact that 14-3-3 family proteins normally dimerize and bind to two different phosphorylation sites within the same protein, though occasionally they have been found to bind one phospho-site each on two adjacent proteins. In order to determine the exact sites of binding on Liprins, we could approach the problem from one of two ways. The first is to use a proteomic study of one or both of the Liprins and analyze the data for sites of phosphorylation.

The second is to make larger deletions within the Liprin protein(s) or to mutate two putative binding sites within the same plasmid. While each method could provide the necessary information, both have their drawbacks. Truncation or deletion mutations could cause the Liprin protein to fold in a manner that would disrupt interaction with the kinase and prevent phosphorylation, which would impede 14-3-3 $\zeta$  binding. If the kinase were able to gain access, the misfolding could also prevent 14-3-3 $\zeta$  itself from binding. Thus, large deletions are a useful but occasionally inaccurate tool for interaction studies. The problem with making double mutations of the identified sites is that it involves a large amount of work to obtain the desired results. For example, if we were to carry out double mutations on the Liprin-a plasmid, it would

require six new plasmids to be generated. Additionally, that doesn't account for the 14-3-3 $\zeta$  binding occurring between both subunits of a Liprin heterodimer. Despite the drawbacks, these methods could prove useful in obtaining the 14-3-3 $\zeta$ -binding-deficient Liprin mutants.

### **3.2 Mimicking Phosphorylation**

In our 14-3-3 $\zeta$  proteomics study where we identified Liprin- $\beta$  as a novel interacting partner, we saw that the interaction took place under normoxic conditions and that hypoxia treatment causes the dissociation of Liprin- $\beta$  and 14-3-3 $\zeta$ . This correlates with the LAR activity assays previously described in which serum starvation causes an increase in phosphorylation of LAR substrates, indicating that low glucose levels cause a decrease in LAR activity.<sup>135, 156</sup> Low glucose results in a decrease in kinase activity, causing a decrease in Liprin phosphorylation at the serine residues to which 14-3-3 $\zeta$  binds.

Once the location of 14-3-3 $\zeta$  binding has been identified by null mutations in the Liprin constructs, we can mimic phosphorylation by mutating the serine or threonine residue(s) to aspartic acid (D) or glutamic acid (E). The D or E mutant mimics phosphorylation by adding a permanent negative charge to the amino acid residue that it substitutes for, the residue at which phosphorylation normally takes place. This prevents the protein construct from becoming dephosphorylated. As the negative charge is necessary for the binding of 14-3-3 $\zeta$ , this mutation could act as a positive control for the effect of 14-3-3 $\zeta$  on the cellular mechanisms and complex formation described herein.

These phosphomimetic mutants should not require kinase activity in order to bind 14-3-3 $\zeta$  and thus these mutant Liprins should interact with 14-3-3 $\zeta$  without the need for high glucose concentrations (under hypoxic conditions and serum starvation). For further testing we could

utilize the phosphomimetic Liprin mutants to increase LAR activity even under conditions of hypoxia and serum starvation. In cells that overexpress the D/E Liprin mutants, we should see a decrease in LAR substrate phosphorylation indicating that 14-3-3 $\zeta$  binding to Liprins is sufficient and necessary to activate the LAR PTPase.

### **3.3 Liprin- $\alpha$ / $\beta$ and 14-3-3 $\zeta$ Complex Formation**

After identifying the binding sites through alanine mutations, we will use siRNA to determine the necessity of Liprin- $\beta$  in complex formation with Liprin- $\alpha$  and 14-3-3 $\zeta$ . Originally, only Liprin- $\beta$  was identified as a binding partner of 14-3-3 $\zeta$ , though Liprin- $\alpha$  cannot be ignored due to its ability to co-immunoprecipitate 14-3-3 $\zeta$ . However, the strength of the interaction between Liprin- $\alpha$  and 14-3-3 $\zeta$  might be weak enough that it was removed in the wash steps prior to mass spectrometry analysis. If so, Liprin- $\alpha$  could still bind to 14-3-3 $\zeta$  independently of Liprin- $\beta$ . By knocking out the endogenous Liprin- $\beta$  and immunoprecipitating Liprin- $\alpha$ , we can determine whether the beta subunit is necessary to facilitate the interaction between 14-3-3 $\zeta$  and Liprin- $\alpha$ . This will elucidate the function of Liprin- $\beta$  in complex formation and reveal if it is required for 14-3-3 $\zeta$  to carry out its role in Liprin-mediated metastasis. This theory could be tested by co-transfecting WT Liprin- $\alpha$  with a phosphorylation deficient Liprin- $\beta$  mutant, immunoprecipitating Liprin- $\alpha$ , and blotting for 14-3-3 $\zeta$ . Additionally, this could be validated by using siRNA to Liprin- $\beta$ , pulling down Liprin- $\alpha$ , and again blotting for 14-3-3 $\zeta$ .

As only Liprin- $\beta$  was found in the proteomics study, we can assume that if Liprin- $\alpha$  binds 14-3-3 $\zeta$  it does so through a weak interaction. With this knowledge, we would expect to see that the Liprin- $\beta$  knockdown causes a decrease in the binding between Liprin- $\alpha$  and 14-3-3 $\zeta$ . These results would indicate that, while 14-3-3 $\zeta$  may still bind to Liprin- $\alpha$ , the stronger and more

physiologically relevant interaction takes place on Liprin- $\beta$ . If not, and Liprin- $\alpha$  is capable of binding 14-3-3 $\zeta$  in the absence of Liprin- $\beta$ , then the binding of these two proteins is not dependent on the interaction between Liprin- $\beta$  and 14-3-3 $\zeta$ . From these experiments, the function of Liprin- $\beta$  in 14-3-3 $\zeta$ -Liprin binding will be more clearly defined and will assist in the experimental design and interpretation of results from the phenotypic characterization as described below.

### **3.4 Effect on LAR Localization**

Liprin- $\alpha$  overexpression has been shown to increase the amount of LAR localized to the cell periphery. In COS-7 cells under normal conditions and without treatment, LAR is evenly distributed throughout the cytoplasm, however, upon overexpression of Liprin- $\alpha$  LAR begins to have a punctate expression pattern within the cell, localized at the periphery.<sup>117</sup> Additionally, truncated LAR constructs that are missing the D2 PTPase domain where Liprin- $\alpha$  binding occurs also spread evenly throughout the cell and do not congregate at the cell periphery. We posit that Liprin- $\alpha$ -LAR binding is increased upon overexpression of 14-3-3 $\zeta$  which facilitates the complex formation with Liprin- $\alpha$  and LAR and recruitment to the cell periphery.

This could be tested using immunofluorescent staining and microscopy to visualize the effect of 14-3-3 $\zeta$  overexpression or knock down on LAR localization. We would expect to observe a correlation between the level of 14-3-3 $\zeta$  expression and the localization of LAR. By imaging 14-3-3 $\zeta$  and Liprins, we would also expect to observe increased fluorescence correlating to 14-3-3 $\zeta$  and Liprin recruitment to the cell edge where they can recruit LAR and initiate its PTPase activity.

### 3.5 Focal Adhesion Turnover

Tumor cells develop the ability to spread throughout an organism and avoid chemotherapy by internalizing FAs, thus releasing from the primary tumor, and subverting anoikis in order to metastasize. Previous work in non-cancerous cells has shown that full length Liprin- $\alpha$  interacts with LAR to cause the disassembly of focal adhesions, particularly at the leading edge of moving cells. Cells use FAs and AJs to maintain contact with the surrounding cells and ECM in order to facilitate signal transduction, increase stability, and prevent cancerous cells from metastasizing and forming secondary tumors. We postulate that cancer cells use 14-3-3 $\zeta$ -mediated interactions to utilize the inherent cellular mechanism of LAR-mediated disassembly of FAs to release from the primary tumor and metastasize.

This could be tested using GFP-tagged Paxillin, a component of FAs, and imaging their turnover at the cell surface through immunofluorescent microscopy. Transfection of GFP-Paxillin could be done in MDA-MB-231 cells, a breast cancer cell line known to have high levels of cell motility, in the presence and absence of 14-3-3 $\zeta$ . Live cell fluorescence microscopy could be utilized to visualize where in the cell the FAs congregate. We anticipate that 14-3-3 $\zeta$  overexpression will increase the GFP-Paxillin, and thus FA, turnover in cells. Conversely, the cells in which 14-3-3 $\zeta$  is knocked down will show more stable levels of GFP-Paxillin at the cell surface. In the MDA-MB-231 breast cancer cell line that has increased cell motility, we anticipate that 14-3-3 $\zeta$  will cause the GFP-Paxillin to localize to the leading edge and show increased turnover rates.

### 3.6 Liprin-Mediated Metastasis in Breast Cancer Cells

The experiments described above elucidate how 14-3-3 $\zeta$  affects some of the hallmarks of cell motility, such as FA turnover and LAR localization to the cell periphery. However, the results of these tests do not provide conclusive evidence that the Liprin-14-3-3 $\zeta$  interaction causes an increase in cell motility or, in the case of breast cancer cells, metastasis. In order to more clearly define the role of this interaction in metastasis, the 14-3-3 $\zeta$  and Liprin binding could be disrupted in the highly metastatic breast cancer cell line MDA-MB-231 by overexpressing Liprin SA or TA mutants. In a similar fashion, HEK 293 cells that do not normally metastasize could be transfected with 14-3-3 $\zeta$  and WT Liprins. The live cell imaging of lamellipodia formation as well as motility and movement could be done in both cell lines following a procedure similar to that described in Astro *et al*, 2011.<sup>144</sup>

14-3-3 $\zeta$  has been shown to correlate with increased metastasis in cancer cells and is used by some as a marker for patient prognosis and likelihood of cancer recurrence. We expect that the untreated MDA-MB-231 cells will show a higher level of lamellipodia formation as well as motility. When treated with Liprin SA or TA mutants, the amount of movement as well as the lamellipodia formation should be decreased due to the lack of antagonizing effect of 14-3-3 $\zeta$  via the Liprin pathway. By overexpressing 14-3-3 $\zeta$  and Liprins in HEK 293 cells and plating them on a surface that allows for movement, we expect to see an increase in motility, even in these cells known to be non-invasive. This would indicate that 14-3-3 $\zeta$  and Liprin overexpression is sufficient to induce metastasis.



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