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## Dissection of $\alpha 6 \beta 4$ Integrin-Dependent Signaling and Breast Carcinoma Invasion: A Dissertation

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**DISSECTION OF  $\alpha6\beta4$  INTEGRIN-  
DEPENDENT SIGNALING AND BREAST  
CARCINOMA INVASION: A DISSERTATION**

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**DISSECTION OF  $\alpha 6\beta 4$  INTEGRIN-  
DEPENDENT SIGNALING AND BREAST  
CARCINOMA INVASION**

A Dissertation Presented by

Xiaoqing Yang

Submitted to the Faculty of the  
University of Massachusetts Graduate School of Biomedical Sciences, Worcester  
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Department of Cancer Biology

# DISSECTION OF $\alpha6\beta4$ INTEGRIN-DEPENDENT SIGNALING AND BREAST CARCINOMA INVASION

A Dissertation Presented by

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## Abstract

Breast cancer is one of the most prevalent cancers in the world. Each year, over 400,000 women die from breast cancer world wide and metastasis is the main cause of their mortality. Tumor cell invasion into the adjacent tissue is the first step in the multistep process of cancer metastasis and it involves multiple protein changes. The  $\alpha6\beta4$  integrin, a transmembrane heterodimeric laminin receptor is associated with poor prognosis in many tumor types, including breast cancer. Src family kinase (SFK) activity is elevated in many cancers and this activity also correlates with invasive tumor behavior. The  $\alpha6\beta4$  integrin can stimulate SFK activation and promote cancer invasion, however the mechanism by which it does so is not known. In the current study, I provide novel mechanistic insight into how the  $\alpha6\beta4$  integrin selectively activates the Src family kinase member Fyn in response to receptor engagement. Specifically, the tyrosine phosphatase SHP2 is recruited to  $\alpha6\beta4$  and its catalytic activity is stimulated through a specific interaction of its N-terminal SH2 domain with pY1494 in the  $\beta4$  subunit. Importantly, both catalytic and non-catalytic functions of SHP2 are required for Fyn activation by  $\alpha6\beta4$ . Fyn is recruited to the  $\alpha6\beta4$ /SHP2 complex through an interaction with phospho-Y580 in the C-terminus of SHP2. In addition to activating Fyn, this interaction with Y580-SHP2 localizes Fyn to sites of receptor engagement, which is required for  $\alpha6\beta4$ -dependent invasion. Moreover, the selective activation of Fyn, but not Src, requires the palmitoylation modification of Fyn on its N-terminus. Of clinical relevance, phospho-Y580-SHP2 and phospho-Y418-SFK could be used as potential biomarkers of invasive breast cancer because their expression are elevated in high-grade breast tumors.

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## **CHAPTER I. Introduction**

Breast cancer is one of the most prevalent cancers in the world and it is the second leading cause of cancer related death in western women. Each year, over 400,000 women die from breast cancer [1-2]. With the development of early primary breast tumor detection technology, like mammographic screening, the mortality rate has decreased in recent years. However, if the malignant lesion has progressed from a localized primary tumor to metastatic disease, the survival rate and prognosis of breast cancer patient remains very poor. As with most malignant tumors, metastasis is the main cause of mortality in breast cancer patients [2].

Traditionally, cancer metastasis is viewed as a late acquired event in tumorigenesis. A series of steps happen from cancer cells breaking through the basement membrane, traveling through the circulatory system, surviving in a distant organ and forming a metastatic lesion. During this process, tumor cells go through constant genetic changes. Tumor cells with suitable genetic changes will be selected by different environments on their way to a distal organ and eventually get a chance to form a distal metastasis. In recent years, this model has been challenged by the studies of both mouse and human mammary tumors using gene expression profiling techniques [3-5]. The new findings indicate that a tumor's metastatic ability may be acquired at a much earlier stage of tumorigenesis. The metastatic capacity might be an inherent characteristic of breast cancer [6]. This new model will deepen our understanding of tumor metastasis mechanisms and give a better prognosis prediction for breast cancer patients.

Despite the argument of different mechanisms by which tumor cells metastasize, one thing that remains valid is that once a tumor cell becomes disseminated from its original organ, the detection, treatment and cure of cancer becomes more difficult. Invasion into the adjacent tissue is the first step in the multistep process of cancer metastasis. Migratory capacity coupled with extracellular matrix proteolysis and remodeling abilities are required for cancer cells to break through the basement membrane which under normal conditions holds the epithelial cells in place. For my thesis research project, I have been interested in deciphering the mechanisms by which breast cancer cells become invasive.

### **Integrin family molecules**

Since their first recognition about 3 decades ago, the integrin family of receptors have been the most studied and understood cell surface adhesion receptors [7]. Integrins mediate the transmembrane connections of the cytoskeleton to the extracellular matrix, such as collagen, laminin and fibronectin, as well as play some roles in certain cell-cell adhesion [7]. Integrins have multifaceted functions in development, immune responses and cancer development and they have been intensively studied over the past several decades [8].

Integrins exist only in metazoans. No integrin homologs have been found in prokaryotes or plants [9]. Integrins are heterodimeric cell surface receptors which are composed of one  $\alpha$  and one  $\beta$  subunit. Both of these subunits are one time transmembrane molecules and they interact with each other through non-covalent bonds. Only two  $\alpha$

subunits and one  $\beta$  subunit have been detected in *C. elegans* and five  $\alpha$  and two  $\beta$  subunits in *Drosophila* [10]. Researchers have identified a more complicated set of integrins in vertebrates [8]. In mammalian cells, 18  $\alpha$  and 8  $\beta$  integrin subunits assemble into 24 different integrins. Distinct combinations of  $\alpha$  and  $\beta$  subunits determine integrin ligand specificity (Figure 1-1). Specific integrin expression patterns determine which ECM molecules cells can bind and therefore what kind of mechanical or biochemical changes that will be transferred to the inside of the cell to affect cell behavior[11]. Data from integrin knockout mice suggest that each integrin has a distinct and nonredundant function since each integrin knockout phenotype is different from each other [8] (Table 1-1).

The main function of integrins is to mediate adhesion of the cells to the extracellular matrix (ECM) or to adjacent cells by connecting various components of the ECM to the actin cytoskeleton ( $\alpha6\beta4$  is an exception, which connects the ECM to the intermediate cytoskeleton). In response to ligand binding, integrins cluster and form a cell membrane structure called focal adhesions (FAs). Focal adhesions are dynamic protein complexes through which not only mechanical forces but also regulatory signals are transmitted. The assembly and disassembly of FAs are essential in cell migration. Small and unstable structures called focal complexes are initially formed in the leading edge of migratory cells. These focal complexes rarely have an opportunity to mature and they disassemble as the lamellipodium withdraws when the cell migrates on the ECM. Besides integrins, focal complexes also consist of adaptor proteins, such as talin and paxillin. As some focal complexes mature to FAs, more proteins, such as zyxin, will be recruited to

the complexes. Focal Adhesion Kinase (FAK) is a cytoplasmic tyrosine kinase which is implicated in transmitting signals from multiple cell surface receptors including integrins to regulate complex cell functions, such as cell survival and cell cycle progression [12-13]. FAK is localized to FA through directly interacting with  $\beta$ -integrins or indirectly interacting with integrin associated proteins, such as paxillin and talin [14-17]. Increased FAK expression and FAK activation have also been implicated in cancer progression [18-20]. An essential role of FAK is to regulate the cycle of formation and disassembly of FA for efficient cell migration and invasion [21]. Appropriately localized FAK can rescue the phenotype of motility deficient FAK-null cells [22]. FAK regulates FA turnover through at least three mechanisms. FAK regulates Rho-GTPases and Arf GTPases by physically interacting with GAP proteins Gaf or ASAP1, respectively. The inhibition of GTPase activity is associated with the re-arrangement of the actin cytoskeleton and the disassembly of focal adhesions [14, 23-24]. FAK can also inhibit Rho-GTPase activity by regulating the tyrosine phosphorylation of p190RhoGAP [25]. Thirdly, the calcium-dependent protease calpain might be involved in the dissociation of FAs since many FA components are known to be calpain substrates and inhibition of calpain results in defects in FA and ECM dissociation [26]. FAK functions as a scaffolding protein in binding calpain to target calpain proteolytic activity to focal adhesion sites [27].

Besides their roles in cell-ECM and cell-cell adhesion, integrins are also important in triggering complex signal transduction events. It has been well accepted that integrin initiated signaling pathways are essential for cells to survive and avoid apoptosis. Integrin triggered signaling pathways are similar to those stimulated by growth factor

receptors. Integrins can activate multiple signaling pathways by themselves. However, they also cooperate with growth factor receptors and growth factor stimulated signaling pathways also regulate integrin signals [28-29]. Moreover, integrin signaling is required for the full capacity of growth factor stimulated signaling events [30]. Such examples include integrin  $\alpha_V\beta_3$  cross-talk with the IGF-1 receptor [31], integrin  $\alpha_V\beta_3$  interaction with the TGF $\beta$  type II receptor [32], integrin  $\alpha_5\beta_1$  cooperation with the EGF receptor [30], and laminin binding integrin interaction with the c-Met receptor [33].

One of the most significant characteristics of integrin signaling is that the receptors signal bi-directionally, which means that integrins mediate both outside-in and inside-out signaling. Many integrins do not present on the cell surface in a constitutively active state. They exist in either a low-affinity conformation or a high-affinity conformation, which some researchers call an OFF or ON state, respectively. In an “OFF state”, the integrin neither binds its ligand nor does it signal [8]. When integrins bind to their extracellular ligands, they recruit adaptor proteins to their cytoplasmic tails to initiate downstream signaling cascades. Also, conformational changes modify their interaction with the actin cytoskeleton. This is termed outside-in signaling. On the other hand, binding with cellular adaptor proteins, such as talin and kindlin, or to the cytoskeleton will change the integrin conformation to a higher affinity state for extracellular matrix, which is termed as inside-out signaling [8, 34].

## **Integrin $\alpha6\beta4$**

The  $\alpha6\beta4$  integrin was first identified by two research groups independently more than 20 years ago [35-36]. It is expressed mainly on the basal surface of most epithelial cells. The  $\alpha6\beta4$  integrin has also been detected in a few other cell types, such as fibroblasts, Schwann cells, and thymocytes [37]. Unlike the  $\alpha6$  subunit, which can dimerize with either the  $\beta1$  or  $\beta4$  subunit,  $\beta4$  can only form a cell surface heterodimeric receptor with the  $\alpha6$  subunit [8]. This is the reason why some researchers in the field refer to  $\alpha6\beta4$  as  $\beta4$  for simplicity. Two characteristics make  $\alpha6\beta4$  different from all other integrins. Firstly, the  $\alpha6\beta4$  integrin links laminins in the basement membrane to the intermediate filament cytoskeleton instead of the actin cytoskeleton of the cell. Secondly, unlike other integrin subunits,  $\beta4$  has an unusually long cytoplasmic tail, which is about 1000 amino acids long instead of around 50 amino acids. This long cytoplasmic tail confers  $\alpha6\beta4$  its unique roles among integrin family molecules [8, 38]. The structure of the cytoplasmic tail of the  $\beta4$  subunit is characterized by two pairs of Type III fibronectin repeats separated by a connecting segment [39]. Multiple serine and tyrosine residues on the  $\beta4$  tail have been reported to be crucial for  $\alpha6\beta4$  functions [40-43].

$\alpha6\beta4$  has a central role in hemidesmosome structural organization in normal epithelial cells. It is essential in the dynamic process of assembly and disassembly of hemidesmosomes as well [44]. It is the intrinsic role of  $\alpha6\beta4$  in the formation and breakdown of hemidesmosomes that determines its main function in maintaining the integrity of epithelia, especially the outermost layer of the epidermis [38], as well as in

regulating cell migration under both physiological and pathological conditions [44]. Hemidesmosomes (HD) are multiprotein complexes on the basal surface of the epithelial cell. They visually look like half desmosomes by electron microscopy. HDs use integrins to link cells to the extracellular matrix.  $\alpha6\beta4$  knockout mice result in the loss of hemidesmosomes and hence the loss of stable adhesion of epithelia [45-47]. Without  $\alpha6\beta4$  expression, although the morphology of the skin looks normal, the epidermis is very easy to detach from the underlying basement membrane in response to mechanical stress due to the lack of hemidesmosome formation. This observation mimics the symptoms of human PA-JEB patients (Pyloric atresia associated with junctional epidermolysis bullosa: a rare inherited disorder characterized by pyloric stenosis and blistering of the skin) carrying either  $\alpha6$  or  $\beta4$  subunit mutations whose skins are fragile and form blisters easily [48-50].

Laminin is the major ECM component that interacts with the  $\alpha6\beta4$  integrin. Besides  $\alpha6\beta4$ , other integrins have also been reported to interact with laminin, such as  $\alpha6\beta1$ ,  $\alpha3\beta1$  and  $\alpha7\beta1$  [51-52]. Like  $\alpha6\beta1$ ,  $\alpha6\beta4$  interacts with multiple different laminin isoforms. Laminin-332 (previously called laminin-5) is the most important and preferred natural ligand of  $\alpha6\beta4$  [53-56]. Several studies suggest that the processing of laminin-332 is involved in HD formation. In normal epithelia,  $\alpha6\beta4$  interacts with the processed form of laminin-332 to secure the formation of stable HDs. Mature HD can only be formed on the processed laminin-332. When cells need to migrate, unprocessed laminin-332 is secreted by the cell to interact with  $\alpha3\beta1$  since it has a higher affinity for the unprocessed laminin-332 compared to  $\alpha6\beta4$  [51, 57]. Indeed, underneath the leading edge of

migratory cells, the unprocessed form of laminin-332 is present [58]. By using this strategy, cells adjust their turnover of the rigid HD structures to regulate their stable adhesion or migratory status. Several studies also suggest that HD formation may be independent of the laminin interaction with  $\alpha6\beta4$  integrin. They argue that HDs can be formed entirely through the interaction of the  $\beta4$  cytoplasmic tail with plectin, which connects  $\beta4$  to the intermediate filament cytoskeleton. One piece of evidence to support this possibility is that expressing a  $\beta4$  mutant that is unable to interact with laminin doesn't block the formation of structurally normal HDs containing all the essential HD components [59-62]. Consistent with the idea that the  $\beta4$ /plectin interaction is crucial for HD formation, patients with  $\beta4$  mutants which are deficient in binding plectin or plectin-deficient mice both show less robust HDs and fragile skin [63-66]. Therefore HD formation is likely to be driven from both outside laminin-332 and inside plectin interactions with  $\alpha6\beta4$ , respectively.

### **Integrin $\alpha6\beta4$ and cancer progression**

Besides its well established roles in maintaining epithelial integrity, many studies have revealed a strong correlation between the  $\alpha6\beta4$  integrin and solid tumor progression including tumor initiation, survival, and especially invasion and metastasis [38, 67-68]. Despite the loss of hemidesmosomes in most tumors,  $\alpha6\beta4$  integrin expression persists in many epithelial-derived tumors [38, 69]. The persistent and even higher expression level of  $\alpha6\beta4$  and its cell surface redistribution correlate with more malignant and aggressive cancers [70-71]. De novo expression of the  $\beta4$  subunit increases the invasive capacity of

$\beta$ 4-deficient rectal and breast carcinoma cells [72-73]. Moreover, abrogation of  $\beta$ 4 expression by siRNA results in decreased invasion in metastatic breast carcinoma cells [74]. The mechanisms that underlie  $\alpha$ 6 $\beta$ 4 integrin dependent cancer cell motility are likely the same as those employed by normal epithelial cells under some physiological conditions, like wound healing [38]. In transformed cells, engagement of the  $\alpha$ 6 $\beta$ 4 integrin stimulates the activation of several signaling molecules including phosphatidylinositol-3 kinase (PI3K), MAPK, NFAT, NF $\kappa$ B and Src family kinases (SFKs) [73, 75-78]. A core enzyme activated by the  $\alpha$ 6 $\beta$ 4 integrin to promote invasion is PI3K. In turn, PI3K activates downstream targets, such as the small GTP binding protein Rac, to promote cancer cell invasion [73]. A dual role hypothesis explains how an adhesion receptor switches to a signaling competent molecule to promote cancer progression [38]. When cancer cells undergo malignant transformation, factors in the tumor microenvironment mobilize the  $\alpha$ 6 $\beta$ 4 integrin from the rigid hemidesmosome structures to the more dynamic part of the cell surface. Upon release from HDs, the  $\alpha$ 6 $\beta$ 4 integrin switches from interacting with the intermediate filament cytoskeleton to the actin cytoskeleton in the leading edges of the cell, such as in lamellae and filopodia. Moreover, the released  $\alpha$ 6 $\beta$ 4 integrin will incorporate into certain cell surface microdomains where it will interact with growth factor receptors and other signaling molecules to promote cell motility as a signaling competent molecule [38, 68]. The mechanism by which  $\alpha$ 6 $\beta$ 4 is released from HDs involves the tumor microenvironment induced phosphorylation of a group of serine residues (S1356, S1360, and S1364) on the  $\beta$ 4 cytoplasmic tail [41, 79]. In contrast to the growth factor stimulated phosphorylation of this group of serine

residues, a constitutively phosphorylated serine residue (S1424) has also been reported to be involved in mobilizing  $\alpha6\beta4$  to the signaling platforms [80].

Integrins are constitutively endocytosed and then recycled or degraded [81-82]. Disruption of integrin endocytosis and recycling impairs cell spreading and migration, and remodeling of ECM components [83-84]. The ability of cells to redeploy integrins on their surface may be important for both normal epithelial cells and invasive cancer cells to migrate. This provides one potential mechanism by which  $\alpha6\beta4$  is mobilized from HDs to the more dynamic leading edges of invasive cancer cells to promote cancer invasion. Clathrin-mediated endocytosis has been associated with the turn-over of several integrins including  $\alpha6\beta1$ ,  $\alpha5\beta1$ ,  $\alpha v\beta3$  and  $\alpha v\beta6$  [85-86]. In contrast,  $\alpha6\beta4$  is associated with lipid rafts which suggests that caveolin might be involved in endocytosis of  $\alpha6\beta4$  [87-88]. A recent study has provided evidence that ARRDC3, an arrestin family member which is preferentially lost in a subset of breast cancers, is involved in regulating  $\alpha6\beta4$  internalization [89]. This study has shown that ARRDC3 interacts with  $\beta4$  to facilitate  $\alpha6\beta4$  internalization, ubiquitination and ultimate degradation. ARRDC3 controls breast cancer progression by negatively regulating  $\alpha6\beta4$  expression level [89].

Normal epithelial cells and endothelial cells require a solid substratum to grow [90-91]. Without growth factor and ECM proteins, cells tend to go through anoikis, a form of programmed cell death. However, cancer cells gain the ability to survive in the absence of growth factor and matrix attachment. This anchorage-independent growth of cells is one of the hallmarks of carcinogenesis. The  $\alpha6\beta4$  integrin has been reported to influence the survival of breast cancer cells through activation of the PI3K/AKT

signaling pathway in p53 mutant cell lines [92]. This result has been further confirmed by the observation that  $\alpha6\beta4$ -dependent survival was blocked in WT-p53 cancer cells since AKT was cleaved by the p53-dependent activation of caspase-3 [92-93]. Besides the intrinsic ability of activating PI3K/AKT to promote cancer survival,  $\alpha6\beta4$  also regulates cancer cell survival by providing ligands to either growth factor receptors or integrin receptors. Studies have shown that  $\alpha6\beta4$  mediated breast cancer survival is dependent on  $\alpha6\beta4$  induced VEGF production [94-95]. These studies also revealed that  $\alpha6\beta4$  regulates VEGF expression at the translational level through regulating the mTOR/eIF-4E pathway [94]. Like regulating growth factor VEGF secretion,  $\alpha6\beta4$  is also involved in autocrine stimulation of its own ligand laminin-332 to sustain anchorage-independent cell survival in 3-dimensional cultures [96]. The mechanism by which laminin-332 ligated  $\alpha6\beta4$  promotes cancer cell survival is through the activation of Rac GTPase /NF- $\kappa$ B signaling [97].

While  $\alpha6\beta4$  contributions to tumor progression, such as promoting tumor survival, migration, and invasion, have been known for some time, it is only recently that attention has been drawn to the fact that  $\alpha6\beta4$  is also involved in the initiation of tumors. Using antibodies to block either the  $\alpha6\beta4$  integrin or its ligand laminin-332 abolished the ability of genetically modified human keratinocytes to form tumors in immune-deficient mice. Moreover, keratinocytes isolated from patients with blistering skin were unable to form tumors since they are deficient in either the  $\beta4$  subunit or laminin-332. However, restoring the  $\beta4$  subunit or laminin-332 genes conferred the cells' tumorigenic ability

[98]. This finding is substantiated by multiple studies in the field. Depletion of  $\alpha 6\beta 4$  by siRNA reduces breast cancer growth both *in vitro* and *in vivo* [99-100]. Overexpression of the  $\beta 4$  subunit transforms rodent fibroblasts and induces tumorigenesis in nude mice [101]. Deletion of part of the  $\beta 4$  subunit cytoplasmic tail results in a decrease in mammary tumorigenesis [102]. Taken together,  $\alpha 6\beta 4$  not only promotes cancer progression but also is involved in a much earlier tumor initiation stage.

Lipid rafts and tetraspanin (4 times transmembrane superfamily protein) enriched microdomains are two kinds of cell surface microdomains that have been reported to be involved in  $\alpha 6\beta 4$  signaling functions. Lipid rafts are cell surface microdomains enriched with cholesterol, glycosphingolipids and signaling proteins. Lipid rafts are more tightly packed and organized than the surrounding lipid bilayer and they may float freely in the cell membrane [103]. With a concentration of signaling molecules, lipid rafts function as platforms in signal transduction. By recruiting regulatory molecules to or excluding them from lipid rafts, the cell is provided with a way to regulate its signaling events. Studies have shown that palmitoylation of the  $\beta 4$  proximal region (C732, C736, C738, C739, C742) is required for  $\alpha 6\beta 4$  to incorporate into lipid rafts and stimulate signaling pathways since mutation of these cysteine residues blocks both events [87]. Tetraspanin enriched microdomains (TEMs) are cell surface complexes consisting of multi-tetraspanins and non-tetraspanin proteins (including growth factor receptors) which interact with each other laterally. This distinct characteristic confers TEMs with another name, tetraspanin webs [104]. In contrast to the  $\alpha 6\beta 4$ /lipid raft model, studies from

another group have shown that multiple tetraspanins can be palmitoylated on their membrane proximal region and instead of incorporating into lipid rafts, palmitoylated  $\alpha 6\beta 4$  integrins integrate into tetraspanin webs to make them signaling competent [52].

It is natural to hypothesize that the signaling capacity of  $\alpha 6\beta 4$  is dependent on interaction with its ligand. Indeed, studies show that some breast cancer cells ligate  $\alpha 6\beta 4$  and activate the Rac/NF- $\kappa$ B signaling pathway by autocrine laminin-332 [97]. However, ligand-independent  $\alpha 6\beta 4$  stimulation of signaling and invasion in colorectal cancer cells has also been reported [72]. Moreover, an extracellular domain deleted  $\beta 4$  mutant retains its signaling capacity and cell migration and invasion promoting ability [105-106]. Similar results have been observed when antibodies are applied to block  $\alpha 6\beta 4$  dependent adhesion to its ligand [107]. Since the signaling functions of  $\alpha 6\beta 4$  are generally attributed to the long cytoplasmic tail of the  $\beta 4$  subunit and it doesn't have any intrinsic enzymatic activity, an adaptor model has been proposed to explain how  $\alpha 6\beta 4$  functions as a signaling molecule. This model suggests that upon redistribution into the leading edges of migratory cells,  $\alpha 6\beta 4$  interacts with growth factor receptors to mediate growth factor induced signaling events. Interactions between  $\alpha 6\beta 4$  and growth factor receptors, such as erbB2, Met, and Ron, have been reported [42, 105, 108].

As mentioned before, phosphorylation of serine residues in the  $\beta 4$  subunit cytoplasmic tail contributes to the regulation of  $\alpha 6\beta 4$ -mediated HD disassembly and cell adhesion. In contrast, phosphorylation of tyrosine residues is mainly responsible for  $\alpha 6\beta 4$  dependent signaling events to influence carcinogenesis and tumor progression [43]. At

least six tyrosine residues (Y1257, Y1422, Y1440, Y1494, Y1526 and Y1642) have been identified to participate in signaling in the  $\beta 4$  cytoplasmic tail [109-110]. Y1494 in the  $\beta 4$  subunit cytoplasmic domain is a key mediator of  $\alpha 6\beta 4$ -dependent signaling because mutation of Y1494 inhibits the ability of  $\alpha 6\beta 4$  to stimulate PI3K, MAPK and SFK activation [76]. Moreover, mutation of Y1494 significantly reduces  $\alpha 6\beta 4$ -dependent cancer invasion [111]. Y1494 is localized within an immune T cell inhibitory motif (ITIM) which has been characterized as a canonical binding site for Src-homology-2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP1) and SHP2 [112]. However, SHP tyrosine phosphatase involvement in  $\alpha 6\beta 4$ -dependent signaling and cancer cell invasion are not well understood.

### **SHP2 and Cancer Progression**

SHPs, the SH2 domain containing protein tyrosine phosphatases, are a family of non-transmembrane phosphatases. There are two types of SHPs in vertebrates which are SHP1 and SHP2. Only one SHP ortholog is present in *Drosophila* and *C. elegans*, Corkscrew and Ptp-2, respectively. The invertebrate SHPs seem to have primarily the SHP2 like roles suggesting that SHP2 is evolutionally more conserved and SHP1 evolved later in vertebrates [113]. Mammalian SHPs have two SH2 domains on their N-termini, which are called N-SH2 or C-SH2, reflecting their relative position to each other. A central classic PTP domain (protein tyrosine phosphatase domain) is followed by a short C-terminal tail containing two tyrosine residues that have been implicated in regulating SHP function. In between the two C-terminal tyrosine residues is a proline rich domain

whose functions are unknown [114]. (Figure 1-2a) SHP2 is expressed ubiquitously in mammalian cells whereas SHP1 expression is more restricted to lympho-hematopoietic cells [113-114]. Although they have highly homologous sequences and structures, the two mammalian SHPs have non-redundant functions. For cells which express high levels of both SHP1 and SHP2, losing either of the SHPs has dramatically different consequences to the cell [114]. SHP1 null mice die about 2-3 weeks after birth due to sterile inflammations affecting multiple viscera [115]. Homozygous deletion of Exon 2 or Exon 3 of SHP2 in mice results in embryonic lethality [116].

In non-stimulated cells, SHP2 assumes a closed conformation and its phosphatase activity is suppressed by an intramolecular inhibitory interaction between the N-SH2 domain (the backside of its p-Tyr binding pocket) and the PTP domain (the catalytic surface of PTP domain). In response to growth factor stimulation, SHP2 is recruited to phosphorylated tyrosine residues in RTKs or adaptor proteins through its N-SH2 domain. This releases the auto-inhibitory interactions of SHP2 to expose its PTP domain and activate its phosphatase activity [114, 117]. The C-SH2 may also be involved in the regulation of SHP2 phosphatase activity since bisphosphorylated ligands that engage both the N-SH2 and C-SH2 provide a higher stimulatory effect on SHP2 phosphatase activity [118]. The crystal structure of SHP2 reveals that the C-SH2 has minimal interactions with the PTP domain, which suggests the p-Tyr binding pocket of C-SH2 is ready to bind its ligand even when SHP2 is in a closed conformation [118]. It is suggested that SHP2 uses its C-SH2 to survey the cell for the bisphosphorylated binding ligand. Upon interaction between C-SH2 and p-Tyr, the increase of the local concentration of p-Tyr will release

SHP2 auto-inhibition by engaging the N-SH2 [114]. SHP2 PTP activity is regulated not only by its SH2 domains. It has been reported that the tyrosine residues Y542 and Y580 (human sequences) on the short C-terminal tail also have an effect on its PTP activity. Non-hydrolyzable p-Tyr 542 or p-Tyr 580 mimetics each stimulate SHP2 phosphatase activity by 2 to 3 fold. From mutagenesis and protease-resistance studies, it has been suggested that p-Tyr 542 engages the N-SH2 intramolecularly, whereas p-Tyr 580 interacts with C-SH2 [119]. Earlier studies, including the crystal structure studies, showed that C-SH2 has no physical interaction with the PTP domain or any effects on SHP2 phosphatase activity. Therefore, the functions of Y542, Y580, and the C-SH2 domain in regulating SHP2 phosphatase activity are still quite controversial.

Deregulated protein tyrosine phosphorylation is involved in tumor progression. Gain of function mutations of protein tyrosine kinases (PTKs) and/or PTK overexpression are responsible for their oncogenic functions. Therefore, PTPs have long been expected to be tumor suppressors. However, few, if any PTP have been reported to be a tumor suppressor [113]. As the first recognized proto-oncogene in the PTP family, SHP2 has been reported to be a positive (signaling enhancing) regulator downstream of multiple growth factor receptors and cytokine receptors. In contrast, SHP1 plays largely a negative regulatory role in signal transduction pathways to suppress cellular activation [120]. This notion has been substantiated by the finding that mutation of SHP1 partially rescues the haematopoietic defect caused by SHP2 mutation. This study suggests that SHP1 and SHP2 have opposite regulatory effects in the same signaling pathways [121].

Recently it has been reported that SHP2 may also have tumor suppressor functions in hepatocellular carcinogenesis through downregulation of inflammatory signaling [122].

SHP2 is encoded by PTPN11. Germ line mutations in PTPN11 cause about 50% of Noonan Syndrome (NS), which is an autosomal dominant disorder featuring abnormal facial structures, short stature and cardiac defects [123]. Somatic mutations of SHP2 cause many forms of childhood and adult leukemia (JMML, AML, ALL, etc) and solid tumors, including lung cancer, colon cancer, and melanoma [124]. In the majority of NS and tumors, the SHP2 mutations affect the N-SH2 and PTP autoinhibitory interaction faces resulting in “activated SHP2 mutants”. Low levels of SHP2 activity are associated with NS, whereas high levels are associated with neoplastic diseases [124]. SHP2 plays essential roles downstream of multiple RTK, cytokine and integrin receptors. The main function of SHP2 as a positive regulator is to activate and sustain the Erk MAPK signaling pathway. The oncogenic effects of SHP2 are thought to be mainly through activation of the Ras-Erk signaling pathway as some NS patients and cancer patients lacking PTPN11 mutations show gain of function mutations in Kras. It is known that SHP2 signals upstream of Ras [114, 125]. However the exact mechanisms by which SHP2 activates MAP kinases and the direct substrate(s) of SHP2 are still not completely understood [114]. SHP2 has also been implicated in PI3K, JNK, and NF- $\kappa$ B activation [126-129].

Another important role of SHP2 is to regulate Src Family Kinase (SFK) activation. In their short regulatory C-tails, SFKs have a critical negative regulatory residue Y529

(human Src as an example). pY529 mediates an intramolecular inhibitory interaction with its SH2 domain, which keeps 90-95% of SFKs in an inactive conformation under basal conditions [130]. For full SFK activation, Y529 must be dephosphorylated and Y418 must be autophosphorylated. Some cytoplasmic PTPs, including SHP2, PTP1B, and some transmembrane enzymes, including PTP $\alpha$ , PTP $\epsilon$  and PTP $\lambda$ , are candidate phosphatases for Y529 dephosphorylation [131]. It remains possible that SHP2 might activate SFKs by directly dephosphorylating p-Y529. However there is currently no evidence for a direct dephosphorylation mechanism. Rather, Neel et al have provided evidence that SHP2 regulates pY529 dephosphorylation and Src activation by an indirect dephosphorylation mechanism in response to multiple growth factor receptor activation and  $\beta$ 1 integrin clustering. Y529 of Src can be phosphorylated by Csk (C-terminal Src Kinase) [132]. To phosphorylate the inhibitory residue Y529, Csk is recruited through its SH2 domain to phosphorylated PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains), which is a transmembrane glycoprotein. Then the membrane-recruited Csk can phosphorylate the membrane-associated SFK and inhibit its activity. By dephosphorylating the Csk-binding tyrosine in PAG, SHP2 abolishes the recruitment of Csk to SFK and prevents Y529 phosphorylation [133]. Another group has proposed a similar mechanism. They demonstrated that SHP2 promotes Src activation by dephosphorylating another Csk-docking protein, Paxillin, which is a focal adhesion-associated adaptor protein [15, 134]. Although most of SHP2 functions require its PTP activity, studies have shown that SHP2 does have some PTP independent functions [135-136]. One study shows that SHP2 can activate Src independently of its catalytic function

by binding directly to the SH3 domain of Src and disrupting an intramolecular interaction that interferes with the catalytic domain [135].

### **SFKs and Cancer Progression**

The cytosolic Src Family Kinases (SFKs) are frequently activated in human cancers, and they play important roles in regulating cell proliferation, migration and survival [131, 137]. There are at least 9 SFKs: Src, Fyn, Yes, Lck, Blk, Fgr, Hck, Lyn and Yrk. All 9 SFKs are approximately 60 kd, with Src being 60 kd and Fyn being 59 kd [138]. All of the SFKs are highly homologous to each other and share the same structure and same mechanism of activation [139]. At their N-termini, SFKs contain a short membrane-targeting SH4 domain that can be differentially modified by lipids, followed by a unique domain which determines the specificity of each SFK member, followed by an SH3 domain, an SH2 domain, a linker region, a protein-tyrosine kinase domain and a short C-terminal tail that contains the critical negative regulatory residue Y529 (Figure 1-3) [139]. Two intramolecular interactions keep 90-95% of Src in an inactive conformation under basal conditions *in vivo*. One is the phospho-Y529 mediated intramolecular interaction with the SH2 domain. The other is the interaction between the SH3 domain and the proline rich motif in the linker region [130]. For full activation, Y529 must be dephosphorylated and Y418 located in the kinase domain must be autophosphorylated (Figure 1-2). Among all SFKs, Src is the most studied member and the involvement of Src in solid and hematologic malignancies has long been established [140-141]. Human breast cancer cells expressing constitutively active Src exhibit increased bone metastasis, while those expressing a kinase-dead mutant of Src show

decreased bone and lung metastasis in a mouse model [142]. The observation that Src expression increases with tumor progression suggests that Src is more involved in tumor invasion and metastasis than in tumor onset [143-146]. Like the  $\alpha6\beta4$  integrin, elevated Src activity can be used as a predictor for poor prognosis in cancer patients [147]. Taken together, these observations indicate a strong correlation between elevated Src kinase activity and breast cancer invasion and metastasis.

Although much progress has been made on the contributions of Src to tumor initiation and progression, less is known about other SFKs. Fyn was identified in 1986 and it is mainly associated with T-cell and neuronal signaling pathways. Therefore, much of the earlier work has focused on its roles in immune and neuronal systems [138, 148-149]. It has been established in recent years that Fyn, like Src, is also involved in tumorigenesis. Fyn is overexpressed in various solid tumors including squamous cell carcinoma and melanoma [138]. Overexpression of Fyn in NIH3T3 fibroblasts results in a prominent morphological change and increased anchorage-independent growth, which is a hallmark of carcinogenesis [150]. Studies have also shown that Fyn expression is upregulated in the progression from normal prostate epithelia to prostate cancer whereas other SFKs, like Src, Lyn, Fgr, and Hck, are either not significantly upregulated or only weakly upregulated [151]. Depending on the different model systems studied, specific SFK member(s) may play more important roles in carcinogenesis than other SFKs.

As with Src, Fyn also plays a multifaceted role in the process of tumorigenesis, including promoting cell growth, inhibiting cell apoptosis and promoting cell migration and invasion [151]. Inhibition of Fyn by expressing a kinase dead Fyn significantly

reduced the primary tumor size in invasive oral squamous cell carcinoma [152]. Knocking down Fyn, together with Src and Yes, results in a decrease in EGF stimulated AKT activation, which is known to be implicated in tumor cell growth and to have antiapoptotic effects [153].

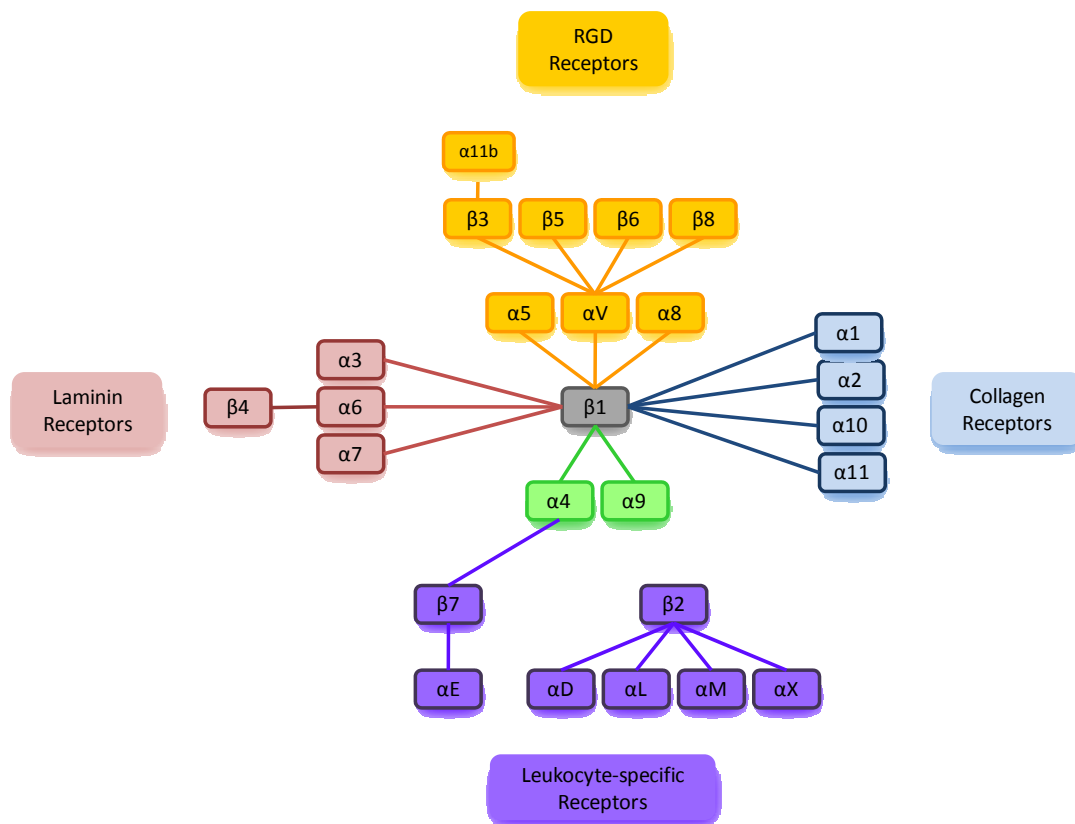
Besides functioning downstream of growth factor receptors, Fyn and other SFKs have also been implicated in mediating integrin initiated signals to regulate cell shape and motility. Both Fyn and Src have been shown to coimmunoprecipitate with FAK [154], and Fyn phosphorylates FAK at Y861 and Y925 [155]. FAK then autophosphorylates itself at Y397 [151]. The FAK and SFK complex is essential in regulating cell shape, motility and invasion by affecting a number of downstream signaling molecules including AKT, NF-KB and the Rho family of small GTPases [151]. Interactions between Fyn and Rho family GTPases control the morphological differentiation of oligodendrocytes [156]. Another study showed that Fyn promotes Stem Cell Factor stimulated Rac GTPase activity [157].

### **Rational for my Thesis Research Work**

Breast cancer is one of the most prevalent cancers in the world and it is the second leading cause of cancer related death in western women. Metastasis is the main cause of the mortality in breast cancer patients. Src family kinase activity is elevated in many cancers including breast cancer and this activity correlates with aggressive tumor behavior. The  $\alpha6\beta4$  integrin, which is also associated with poor prognosis in many tumor types, can stimulate SFK activation. Y1494 in the  $\beta4$  subunit is involved in  $\alpha6\beta4$ –

dependent activation of SFKs and tumor invasion. Y1494 is localized in the canonical SHP2 binding ITIM motif, suggesting that SHP2 may play a role in the  $\alpha6\beta4$ -dependent activation of SFKs and tumor invasion.

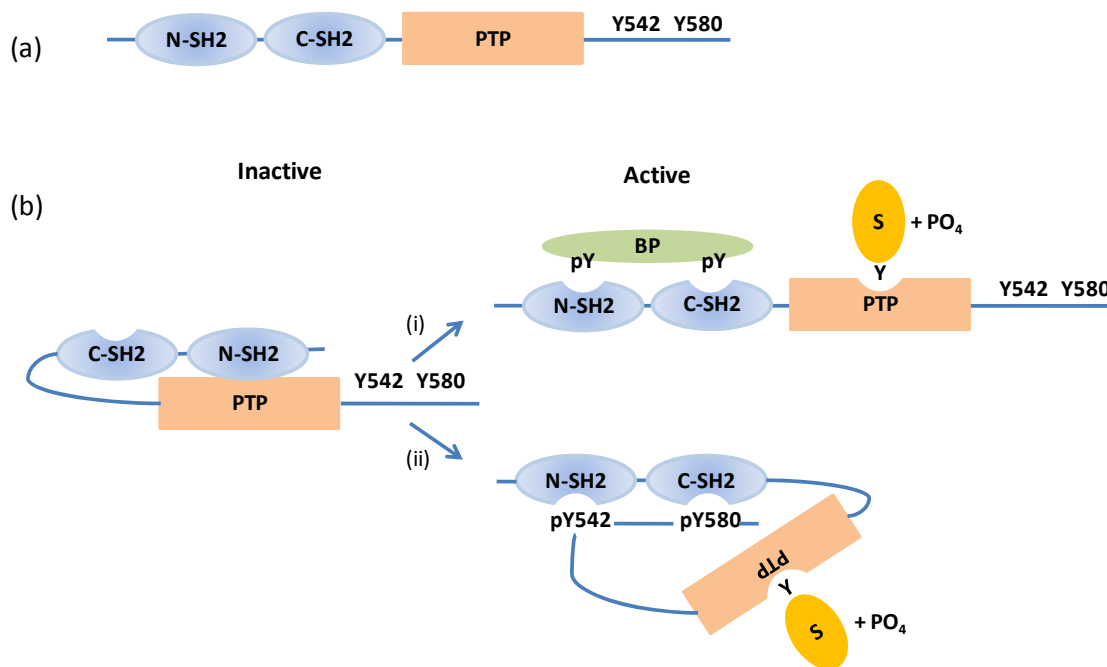
My thesis research focuses on deciphering the mechanisms by which  $\alpha6\beta4$  promotes cancer cell invasion. I am interested in the molecular mechanisms by which  $\beta4$  recruits and activates SHP2 and in turn SHP2 recruits and activates SFKs. I am also interested in the contributions of SHP2 and SFKs to breast cancer invasion.



**Figure 1-1. The mammalian integrin receptor family**

Integrins are cell surface heterodimeric receptors. 18 $\alpha$  and 8 $\beta$  subunits form 24 distinct integrins. The different combination of  $\alpha$  and  $\beta$  subunits determines the integrin ligand specificity. The expression of  $\beta2$  and  $\beta7$  integrins are restricted to white blood cells. The expression of  $\alpha4$  and  $\alpha9$  and  $\beta2$ -  $\beta8$  are restricted to chordates. In contrast, the receptors for laminins and RGD containing ligands are more ancient and expressed throughout metazoans.

Reference: Hynes, R.O., Integrins: bidirectional, allosteric signaling machines. Cell, 2002.110(6): p.673-687



**Figure 1-2. Structure and regulation of Src homology-2 (SH2) domain-containing phosphatases (SHPs)**

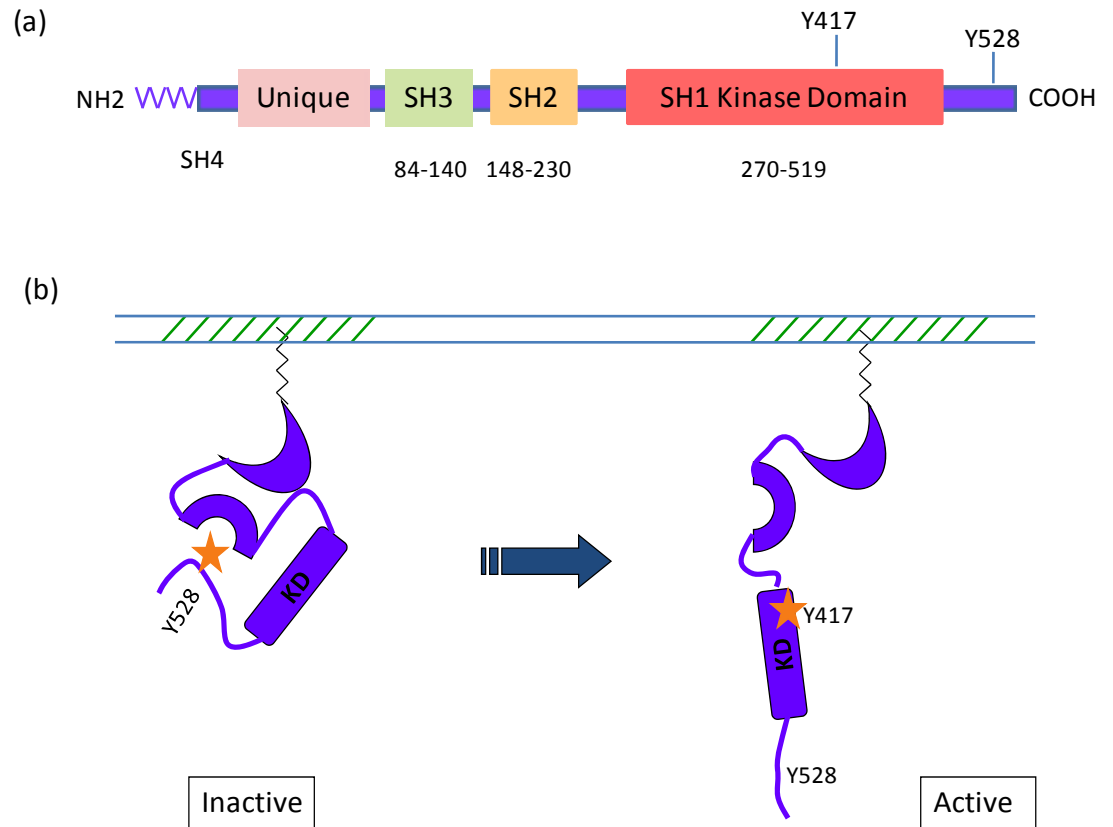
(a). Schematic of a typical member of the SHP subfamily, indicating the two SH2 domains (N-SH2 and C-SH2), catalytic protein-tyrosine phosphatase (PTP) domain and C-terminal tail.

(b). In the basal state, SHPs are largely inactive, because the ‘backside loop’ of the N-SH2 is inserted into the catalytic cleft. This results in mutual allosteric inhibition, with the N-SH2 inhibiting the PTP domain and the PTP domain contorting the Tyr–P peptide-binding pocket of the N-SH2 on the opposite surface. The C-SH2 is left unperturbed, with its Tyr–P peptide-binding pocket in a conformation suitable for binding an appropriate ligand. The C-SH2 probably has the primary targeting function to most SHP-binding proteins.

(i) In the first mechanism of SHP activation, a SHP-binding protein (BP) containing two Tyr–P sites (pY) – one that can bind to the C-SH2 and another that can bind the N-SH2 – comes into contact with the SHP. The C-SH2 is engaged first by its Tyr–P ligand. The resultant increase in local concentration of the ligand for the N-SH2 overcomes mutual allosteric inhibition, resulting in binding of the N-SH2 to its Tyr–P ligand, opening of the enzyme and activation.

(ii) A second mechanism for SHP activation via intramolecular binding of phosphorylated C-tail tyrosyl residues: phosphorylated Tyr542 (Tyr542–P) can bind to the N-SH2, whereas Tyr580–P can bind to the C-SH2.

Reference: Neel, B.G., The “SHP”ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. *Trends Biochem Sci*, 2003. 28(6): p. 284-293



**Figure 1-3. General linear protein structure and activation of Fyn and the Src Family Kinases**

(a). Schematic of a typical member of the SFKs, indicating the five domains and two important kinase activity regulatory tyrosine residues.

(b). Model of SFK activation mechanism. In the inactive state, SFKs are largely inactive, because of two intramolecular inhibitory interactions.

Table 1. Integrin Gene Knockout Phenotypes

$\alpha 1$	V, F	No immediately obvious developmental defects, reduced tumor vascularization	Gardner et al., 1996; Pozzi et al., 2000, 2002
$\alpha 2$	V, F	Few immediately obvious developmental defects, delayed platelet aggregation and reduced binding to monomeric collagen, reduced mammary gland branching	Holtkotter et al., 2002; Chen et al., 2002
$\alpha 3$	P	Kidney tubule defects, reduced branching morphogenesis in lungs, mild skin blistering, lamination defects in neocortex	Kriedberg et al., 1996; DiPersio et al., 1997; Anton et al., 1999
$\alpha 4$	E11/14	Defects in placenta (chorioallantoic fusion defect) and heart (epicardium, coronary vessels). Chimeras show defects in hematopoiesis.	Yang et al., 1995; Arroyo et al., 1996, 1999
$\alpha 5$	E10-11	Defects in mesoderm (posterior somites) and vascular development, neural crest apoptosis. Chimeras show muscular dystrophy	Yang et al., 1993; Goh et al., 1997; Taverna et al., 1998
$\alpha 6^a$	P	Severe skin blistering, other epithelial tissues also defective. Lamination defects in cortex and retina.	Georges-Labouesse et al., 1996, 1998
$\alpha 7$	V, F	Muscular dystrophy, defective myotendinous junctions	Mayer et al., 1997
$\alpha 8$	P	Small or absent kidneys, inner ear hair cell defects	Muller et al., 1997; Littlewood Evans et al., 2000
$\alpha 9$	V	Die within 10 days of birth, chylothorax due to lymphatic duct defect	Huang et al., 2000
$\alpha 10$		Not reported	
$\alpha 11$		Not reported	
$\alpha v$	E10/P	Two classes: embryonic lethality due to placental defects, perinatal lethality with cerebral vascular defects probably due to neuroepithelial defects, cleft palate. Most blood vessels develop normally	Bader et al., 1998; McCarty et al., 2002
$\alpha IIb^b$	V, F	Hemorrhage, no platelet aggregation	Tronik-Le Roux et al., 2000
$\alpha L$	V, F	Impaired leukocyte recruitment	Schmits et al., 1996
$\alpha M$	V, F	Defective phagocytosis and apoptosis of neutrophils, mast cell development defects, adipose accumulation.	Coxon et al., 1996; Tang et al., 1997; Dong et al., 1997
$\alpha X$		Not reported	
$\alpha D$		Not reported	
$\alpha E$	V, F	Greatly reduced numbers of intraepithelial lymphocytes.	Schon et al., 1999
$\beta 1$	E6.5	Peri-implantation lethality, ICM deteriorates, embryos fail to gastrulate. Extensive analyses of chimeras.	Fässler and Meyer, 1995; Stephens et al., 1995; Brakebusch et al., 1997
$\beta 2^c$	V, F	Leukocytosis, impaired inflammatory responses, skin infections, T cell proliferation defects	Scharffetter-Kochanek et al., 1998
$\beta 3^b$	V, F	Hemorrhage, no platelet aggregation, osteosclerosis, hypervascularisation of tumors	Hodivala-Dilke et al., 1999; McHugh et al., 2000; Reynolds et al., 2002
$\beta 4^a$	P	Severe skin blistering, other epithelial tissues also defective	van der Neut et al., 1996; Dowling et al., 1996
$\beta 5$	V, F	No immediately obvious developmental defects	Huang et al., 2000
$\beta 6$	V, F	Inflammation in skin and airways, impaired lung fibrosis—all probably due to failure to activate TGF $\beta$	Huang et al., 1996; Munger et al., 1999
$\beta 7$	V	Deficits in gut-associated lymphocytes—no Peyer's patches, reduced intraepithelial lymphocytes (IEL).	Wagner et al., 1996
$\beta 8$	E10/P	Two classes: embryonic lethality due to placental defects, perinatal lethality with cerebral vascular defects probably due to neuroepithelial defects. Most blood vessels develop normally.	Zhu et al., 2002

Reference citations are listed but not given in the reference list. They can be found in PubMed or in several extensive reviews, which also discuss the implications of the results as well as work with chimeric mice and recent work using conditional and tissue-specific ablation of integrins (Hynes, 1996; De Arcangelis and Georges-Labouesse, 2000; Sheppard, 2000; Bouvard et al., 2001).

Abbreviations: E, embryonic lethal (day of lethality); P, perinatal lethal; V, viable; F, fertile.

<sup>a,b,c</sup> Human mutations in these genes lead to disease (Hogg and Bates, 2000)

<sup>a</sup>  $\alpha 6\beta 4$  Epidermolysis bullosa (JEB-PA)—skin blistering (Pulkkinen and Uitto, 1999)

<sup>b</sup>  $\alpha IIb\beta 3$  Glanzmann thrombasthenia (GT)—bleeding (Kato, 1997)

<sup>c</sup>  $\beta 2$  Leukocyte adhesion deficiency (LAD)—failure in leukocyte recruitment (Etzioni et al., 1999)

### Table 1-1. Integrin gene knockout mice phenotypes

Reference: Hynes, R.O., Integrins: bidirectional, allosteric signaling machines. Cell, 2002.110(6): p.673-687

## **CHAPTER II. SHP2 Interacts with the $\beta 4$ Subunit to Mediate $\alpha 6\beta 4$ Integrin-Dependent Breast Carcinoma Cell Invasion**

Parts of this chapter represent work published as:

### **SHP2 mediates the localized activation of Fyn downstream of the $\alpha 6\beta 4$ integrin to promote carcinoma invasion**

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Mol. Cell Biol. Nov. 2010; 30 (22): 5306-17

### **Intrinsic signaling functions of the $\beta 4$ integrin intracellular domain**

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J. Biol. Chem. Oct. 2007; 282 (41):30322-30

Figure 2-4 D: The experiment was carried out by Udayan Dutta, Ph.D.

## Abstract

The  $\alpha6\beta4$  integrin, a cell surface laminin receptor, is associated with poor patient prognosis and reduced survival in many human cancers, including breast cancer. Considerable interest in understanding how this integrin is regulated and how it functions to promote tumor progression has been given to this unique integrin. In normal tissues, the  $\alpha6\beta4$  integrin plays a major role in maintaining the integrity of epithelia by binding to laminins in the basement membrane and regulating the assembly of hemidesmosomes on the basal epithelial cell surface. In pathophysiological conditions such as wound healing and invasive cancer, the stable adhesive interactions of the  $\alpha6\beta4$  receptor are disrupted. The  $\alpha6\beta4$  integrin is converted to a signaling competent receptor that promotes dynamic adhesion and invasion. However, the molecular mechanism by which  $\alpha6\beta4$  promotes cancer invasion is not fully understood. Both SFKs (cytosolic tyrosine kinases) and SHP2 (a cytosolic tyrosine phosphatase) have been implicated in  $\alpha6\beta4$ -dependent cancer invasion. In the current study, I investigated the mechanism by which SFKs are activated in a SHP2-dependent manner in response to  $\alpha6\beta4$  stimulation and determined the molecular mechanism by which  $\beta4$  recruits and activates SHP2.

## Introduction

Expression of the  $\alpha6\beta4$  integrin, a laminin receptor, is associated with poor patient prognosis and reduced survival in many human cancers [158]. For this reason, there is considerable interest in understanding how this integrin is regulated and how it functions to promote tumor progression. In normal tissues, the  $\alpha6\beta4$  integrin plays a major role in maintaining the integrity of epithelia by binding to laminins in the basement membrane and regulating the assembly of hemidesmosomes on the basal epithelial cell surface [159-160]. In pathophysiological conditions such as wound healing and cancer, the stable adhesive interactions of the  $\alpha6\beta4$  receptor are disrupted by phosphorylation of the  $\beta4$  cytoplasmic domain, converting  $\alpha6\beta4$  to a signaling competent receptor that promotes dynamic adhesion and invasion [38]. Phosphorylation of the  $\beta4$  subunit cytoplasmic domain on serine residues contributes to the dynamic adhesive functions of the receptor by disrupting interactions with hemidesmosomal proteins that regulate stable adhesion [41-42], whereas phosphorylation on tyrosine residues appears to mediate cooperation with growth factor signaling pathways and invasion in carcinoma cells [40].

In transformed cells, engagement of the  $\alpha6\beta4$  integrin stimulates the activation of several signaling molecules including phosphatidylinositol-3 kinase (PI3K), mitogen activated protein kinases (MAPK), NF $\kappa$ B and Src family kinases (SFKs) [76-78, 161]. In earlier studies, Y1494 in the  $\beta4$  subunit cytoplasmic domain was identified as an important mediator of  $\alpha6\beta4$ -dependent signaling by demonstrating that mutation of Y1494 inhibits the ability of  $\alpha6\beta4$  to stimulate PI3K, MAPK and SFK activation [76,

111]. Restoration of both PI3K and SFK signaling, but not MAPK signaling, rescues invasion in tumor cells expressing Y1494F- $\beta$ 4, indicating that PI3K and SFK signaling pathways cooperate downstream of Y1494 to promote  $\alpha$ 6 $\beta$ 4-dependent invasion [76]. Y1494 is localized within an immunoreceptor T cell inhibitory motif (ITIM), a canonical binding site for Src-homology-2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP1) and SHP2 [112]. Examination of a chimeric receptor containing the extracellular domain of TrkB and the transmembrane and cytoplasmic domains of the  $\beta$ 4 subunit demonstrated that SHP2 binds to and is activated by sequences in the  $\beta$ 4 cytoplasmic domain in response to dimerization [162]. Moreover, Y1494 is one of three tyrosine residues, along with Y1257 and Y1440, that mediate the interaction of SHP2 with the  $\beta$ 4 subunit cytoplasmic domain in response to c-Met signaling [163]. Importantly, SHP2 is essential for the activation of SFKs by both the chimeric TrkB/ $\beta$ 4 receptor and when the  $\beta$ 4 subunit functions as a signaling adaptor for c-Met [162-163]. However, the mechanism by which SHP2 activates SFKs in response to  $\alpha$ 6 $\beta$ 4 engagement has not been studied.

In the current study, I investigated the involvement of SHP2 in the  $\alpha$ 6 $\beta$ 4-dependent activation of SFKs and determined the molecular mechanism by which  $\beta$ 4 recruits and activates SHP2.

## Materials and Methods

**Cell lines, Antibodies and Reagents.** MDA-MB-435 cells expressing wild type and mutant  $\beta 4$  subunits were generated and maintained as described previously [111, 161]. MDA-MB-231 human breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository (Georgetown University) and maintained in RPMI medium containing 10% FBS.

The following antibodies were used: SHP2 (cat. #sc280), SFK (cat. #sc8056), from Santa Cruz Biotechnology, Inc; Phospho-Y418 Src (cat. #44660G) from Invitrogen;  $\beta 4$  subunit (439-9B) from R. Falcioni (Regina Elena Cancer Institute), cytoplasmic domain polyclonal antiserum from A. Mercurio (UMass Medical School); pY1494- $\beta 4$  from ECM Biosciences (cat.# IP1281);  $\alpha 6$  subunit (2B7) from A. Mercurio; actin (cat. #A2066) from Sigma; anti-rat (cat. #112-005-003) and anti-mouse (cat. #115-005-003) IgG from Jackson ImmunoResearch.

Calpeptin (cat. #0334 0051), Calpastatin Peptide (cat. #208902), ALLN (cat. #208719) and PP2 (Cat. # 529573) were obtained from Calbiochem. Matrigel (cat. #356237) was obtained from BD Biosciences and murine laminin-1 from Trevigen (cat. #3400-010-01) or Stemgent (cat. # 06-0002). WT and mutant SHP2 constructs and SHP2 shRNA in the pSUPER retroviral vector were gifts from B. Neel (Ontario Cancer Institute, Toronto). SHP2 SH2-GST fusion constructs were gifts from Dr. Eugene Chin (Brown University) [126].

**Integrin clustering.** Cells were serum starved overnight in medium containing 0.1% BSA. Cells were trypsinized and washed before being resuspended at a concentration of  $2 \times 10^6$  cells/ml and incubated for 30 minutes with or without integrin-specific antibodies (2  $\mu$ g/ml) in medium containing 0.1% BSA. The cells were washed once and added to plates which had been coated overnight with either anti-mouse or anti-rat IgG (100  $\mu$ g/10 cm plate), laminin (550  $\mu$ g/10 cm plate) or BSA (1%). Chemical inhibitors were added to the cells for 10 minutes prior to plating the cells in the coated plates. After incubation at 37°C for 30 minutes, the cells were washed once with PBS and lysed in a 20 mM Tris buffer, pH 7.4, containing 10% glycerol, 136 mM NaCl, 10% NP-40, 5mM EDTA, 1 mM sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ) and complete protease inhibitor cocktail (Roche) (Lysis Buffer A).

**Immunoprecipitations and immunoblots.** Cell extracts containing equivalent amounts of total protein were incubated for 3 hrs or overnight at 4°C with antibodies. Either protein A or protein G conjugated sepharose beads were added and incubated for an additional 1-2 hrs. Immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting [76].

**SHP2 *in vitro* tyrosine phosphatase assay.** SHP2 phosphatase activity was measured *in vitro* using the PTP Assay Kit-1 from Upstate Biotechnology. Cells were extracted in Lysis Buffer A without phosphatase inhibitors. Cell extracts containing equivalent amounts of total protein were incubated overnight with SHP2-specific Abs and protein G sepharose beads. The beads were washed four times with 10 mM Tris-HCl, pH 7.4 and

then resuspended in the same buffer with a tyrosine phosphopeptide (0.1mM) and incubated with gentle agitation for 1 hr at 37°C. The reaction was terminated by the addition of malachite green. SHP2 phosphatase activity was measured in a microtiter plate reader at 620nm following the manufacturer's instructions.

**2D invasion assay.** Matrigel invasion assays were performed using 6.5 mm Transwell chambers (8  $\mu$ m pore size; Costar) [76]. Matrigel, purified from the Englebreth-Holm-Swarm tumor, was diluted in cold distilled water, added to the Transwells (5 $\mu$ g/well), and dried in a sterile hood. The Matrigel was then reconstituted with medium for an hour at 37°C before the addition of cells. Cells ( $0.5 \times 10^5$ ) were resuspended in serum-free DMEM containing 0.1% BSA and added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers. After 5 hours, the cells that had invaded to the lower surface of the filters were fixed in methanol for 10 minutes. The fixed membranes were mounted on glass slides using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Invasion was quantified by counting the number of stained nuclei in five independent fields in each Transwell.

**3D Matrigel invasion assay.** A base layer of Matrigel (200  $\mu$ l/well) was overlaid in duplicate wells of a 24-well dish with  $1.0 \times 10^4$  cells suspended in 300  $\mu$ L of a 2:1 mixture of PBS and Matrigel. The Matrigel was overlaid with complete serum-containing medium (0.5 ml/well), which was changed every 3 days. Images were captured with SPOT image analysis software (Molecular Diagnostics).

**Statistics.** All data are represented as a mean +/- (standard error or standard deviation).

All statistical analyses were performed using the unpaired Student's *t*-test.

## Results

### **The $\alpha6\beta4$ integrin activates SFKs and this activation requires SHP2**

Research from other groups has shown that in HGF stimulated cells,  $\alpha6\beta4$  functions as an adaptor to activate Src [163]. A chimeric receptor containing the extracellular domain of TrkB and the transmembrane and cytoplasmic domains of the  $\beta4$  subunit demonstrated Src can be activated by sequences in the  $\beta4$  cytoplasmic domain in response to BDNF dimerization [162]. I sought to determine if ligation of  $\alpha6\beta4$  activates SFKs by engaging the intact  $\alpha6\beta4$  receptor. For this purpose, I examined SFK activation in response to Ab mediated ligation of either  $\alpha6\beta1$ , in mock transfected MDA-MB-435 cells, or  $\alpha6\beta1$  and  $\alpha6\beta4$  in MDA-MB-435 cells that had been transfected with the full-length  $\beta4$  integrin subunit. Although engagement of  $\alpha6\beta1$  with an  $\alpha6$  Ab stimulated SFK activation, the level of SFK activation in response to  $\alpha6\beta4$  ligation was markedly higher (Fig. 2-1, A). In the presence of calpeptin, a SHP2 phosphatase inhibitor,  $\alpha6\beta4$ -dependent activation of SFKs was inhibited, whereas  $\alpha6\beta1$ -dependent activation of SFKs was unaffected by the inhibition of SHP2 (Fig. 2-1, A). These results indicate that the mechanisms by which  $\alpha6\beta1$  and  $\alpha6\beta4$  activate SFKs are distinct. The  $\alpha6\beta4$ -dependent activation of SFKs was also examined in the presence of ALLN and a peptide derived from calpastatin that is a calpain-specific inhibitor [164]. Neither ALLN nor the calpastatin peptide inhibited SFK activation in response to ligation of the  $\alpha6\beta4$  integrin, demonstrating that the reduction in SFK activation observed using calpeptin resulted from SHP-2 inhibition (Fig. 2-1, B). Furthermore, stable expression of an shRNA against

SHP2 to reduce SHP2 protein levels also inhibited SFK activation significantly in response to antibody-mediated ligation of  $\alpha 6\beta 4$  (Fig. 2-1, C). I further confirmed that  $\alpha 6\beta 4$  engagement activates SFKs and this activation of SFKs is SHP2-dependent using MDA-MB-231 cells which endogenously express  $\alpha 6\beta 4$ . When compared with cells expressing vector alone (EV), SFK activation in response to  $\alpha 6\beta 4$  ligation in cells expressing DN-SHP2 (DN) was significantly impaired. For comparison, SFK activation was inhibited completely when SHP2 expression was suppressed by shRNA-mediated knockdown (KD) (Fig. 2-1, D), as I had observed in MDA-MB-435/WT- $\beta 4$  cells (Fig. 2-1, C). For cells that stably express the catalytically inactive DN-SHP2, the exogenously expressed SHP2 mutant was expressed at approximately equivalent levels as endogenous SHP2, as evidenced by the slower migrating band of the HA-tagged DN-SHP2 mutant (Fig. 2-1, D).

### **SHP-2 phosphatase activity is stimulated by $\alpha 6\beta 4$ engagement and required for SFK activation**

The fact that calpeptin, a SHP2 phosphatase activity inhibitor and the phosphatase-dead DN-SHP2 effectively diminished  $\alpha 6\beta 4$  dependent activation of SFKs suggested that SHP2 phosphatase activity is involved in SFK activation. (Fig. 2-1, A,B, and D). Structural analysis of SHP2 has revealed that the N-SH2 domain forms contacts with the catalytic domain and in doing so, blocks access of substrates to the active site [118]. This inactive state switches to an active state upon binding of a phosphopeptide to the N-SH2 domain [165]. To determine if ligation of  $\alpha 6\beta 4$  promotes SHP2 activity, SHP2 *in vitro* phosphatase assays were performed after  $\alpha 6\beta 4$  ligation. Engagement of

$\alpha 6\beta 4$  increased SHP2 phosphatase activity by approximately 65% in MDA-MB-435/WT- $\beta 4$  cells, but had no effect on Mock-transfected cells that do not express the  $\alpha 6\beta 4$  receptor (Fig. 2-2, A). A 2-fold induction of phosphatase activity was also observed upon ligation of endogenous  $\alpha 6\beta 4$  in MDA-MB-231 cells, and this increase was inhibited by expression of the catalytically inactive, substrate trapping SHP2 mutant (C459S-SHP2) that functions in a dominant negative manner (Fig. 2-2, B) [166].

### **$\beta 4$ and SHP2 interact with each other endogenously**

In response to c-Met signaling, SHP2 interacts with the  $\beta 4$  subunit as assayed by Far Western blotting in Cos7 cells, and by coimmunoprecipitation in response to HGF stimulation in both GTL16 and FG2 cells [163]. SHP2 also coimmunoprecipitates with a chimeric protein containing the  $\beta 4$  transmembrane and cytoplasmic domains [162]. To demonstrate an endogenous interaction between SHP2 and the  $\alpha 6\beta 4$  receptor in response to ligation, extracts from cells adherent to laminin were immunoprecipitated with either non-specific IgG (IgG) or  $\beta 4$ -specific Abs and immunoblotted for SHP2. Adhesion to laminin stimulated the association of SHP2 with  $\alpha 6\beta 4$ , which was not observed in the non-specific IgG control IP (Fig. 2-3). Total cell lysates were immunoblotted with either  $\beta 4$  or SHP2 Abs to show equal IP input. Total cell lysates were also immunoblotted with either p-SFK (Y418) or total SFK Abs as a positive control to confirm that laminin did stimulate SFK activation.

## **Phosphorylation of Y1494 is required for the interaction of $\beta$ 4 and SHP2**

To determine if Y1494 in the  $\beta$ 4 subunit cytoplasmic domain plays a role in the recruitment of SHP2 to the  $\alpha$ 6 $\beta$ 4 integrin in response to receptor ligation and if this binding stimulates SHP2 phosphatase activity, the phosphorylation of Y1494 was assessed. Ligation of  $\alpha$ 6 $\beta$ 4 with either laminin-1,  $\alpha$ 6-specific Abs or  $\beta$ 4-specific Abs stimulated phosphorylation of Y1494 (Fig. 2-4, A, B, C). To investigate if phosphorylation of Y1494 is required for the binding of SHP2 to the  $\beta$ 4 subunit, biotinylated peptides corresponding to 14 amino acids of the  $\beta$ 4 cytoplasmic domain surrounding Y1494 and the ITIM binding motif were synthesized (Fig. 2-4, D; ITIM underlined). Phosphotyrosyl was incorporated into the Y1494 site of one of the peptides to determine the importance of tyrosine phosphorylation of Y1494 for binding. These peptides were used to pull down SHP2 from cell extracts of MDA-MB-231 human breast carcinoma cells, which express the  $\alpha$ 6 $\beta$ 4 integrin and SHP2 endogenously. SHP2 was precipitated from the cell extracts by the phosphorylated  $\beta$ 4 peptide, but not by the non-phosphorylated peptide (Fig. 1B). Importantly, mutation of Y1494 inhibited the increase in SHP2 phosphatase activity in response to receptor ligation (Fig. 2-2, A). These results suggest that phosphorylation of Y1494 is required for the interaction of  $\beta$ 4 and SHP2 interaction and this interaction stimulates SHP2 phosphatase activity.

### **Intact Y1494 and Y1440 are required for stable $\beta$ 4-SHP2 interaction, activation of SFKs and $\alpha$ 6 $\beta$ 4-dependent invasion**

SHP2 contains two SH2 domains in the N-terminus of the protein that mediate its interactions with phospho-tyrosyl residues to recruit the phosphatase to signaling complexes and activate the catalytic activity [114]. To determine how SHP2 interacts with the  $\beta$ 4 subunit, SHP2 GST fusion proteins containing both SH2 domains (GST-N-C-SH2), the N-terminal SH2 domain (GST-N-SH2), or the C-terminal SH2 domain (GST-C-SH2) and GST alone were affinity purified with glutathione-Sepharose beads, and their expression was confirmed by immunoblotting (Fig. 2-5, A). The ability of SHP2-GST fusion proteins to pull-down the  $\beta$ 4 subunit from cell extracts of MDA-MB-435 cells that were transfected with the WT- $\beta$ 4 subunit (WT- $\beta$ 4) was examined. To increase the phosphorylation of the  $\beta$ 4 cytoplasmic domain, the cells were treated briefly with a sodium orthovanadate/hydrogen peroxidase ( $\text{Na}_3\text{VO}_4/\text{H}_2\text{O}_2$ ) mixture. Phosphorylation of the  $\beta$ 4 subunit is markedly increased in the presence of  $\text{Na}_3\text{VO}_4/\text{H}_2\text{O}_2$  [111]. All three SHP2/SH2-GST fusion proteins pulled down the full length WT- $\beta$ 4 subunit from cell extracts of treated cells (Fig. 2-5, B), indicating that both SH2 domains of SHP2 engage the  $\beta$ 4 subunit. To identify the specific tyrosine residues in the  $\beta$ 4 subunit that interact with SHP2, I next assessed the ability of the SHP2-GST fusion proteins to pull down  $\beta$ 4 subunits containing point mutations to phenylalanine at Y1494 (Y1494F- $\beta$ 4), Y1494/1257 (Y1494/1257F) or Y1440 (Y1440F- $\beta$ 4). These tyrosines had been previously implicated in the interaction of SHP2 with  $\alpha$ 6 $\beta$ 4 in response to c-Met stimulation using Far Western analysis [163]. Mutation of Y1494 reduced significantly

the binding of only the GST-N-SH2 fusion protein to the  $\beta 4$  subunit, suggesting that N-SH2 interacts with  $\beta 4$  subunit through phosphorylated Y1494. When Y1257 was mutated in combination with Y1494F, the pull-down pattern didn't change. This finding is consistent with our earlier observation that Y1257 plays a minimal role in  $\alpha 6\beta 4$ -dependent breast cancer cell invasion [111]. Mutation of Y1440 reduced significantly the binding of both individual SH2 domains (Fig. 2-5, B). Diminished binding of the N-SH2 domain to Y1440F- $\beta 4$  likely reflects the dependence of Y1494 phosphorylation on an intact Y1440 (Fig 2-5, C). Although the double N-C-SH2 domains were capable of pulling down the mutant Y1440F  $\beta 4$  subunit, the relative level of binding of this mutant subunit was significantly diminished compared with WT binding when total  $\beta 4$  expression levels were normalized (Fig. 2-5, D). These GST pull down data suggest that both tyrosine 1494 and tyrosine 1440 are required for the recruitment of SHP2 to the  $\beta 4$  subunit.

In support of the GST pull-down data (Fig. 2-5, B), mutation of either Y1494 or Y1440 alone inhibited the ability of SHP2 to co-immunoprecipitate with  $\alpha 6\beta 4$  after adhesion to laminin (Fig. 2-6, A). To assess further the importance of intact Y1494 and Y1440 in the  $\alpha 6\beta 4$ -dependent activation of SFKs, cells expressing WT- $\beta 4$ , Y1440F- $\beta 4$  and Y1494F- $\beta 4$  were evaluated for SFK activation after engagement of the  $\alpha 6\beta 4$  receptor by adhesion to laminin (Fig. 2-6, B) or clustering with  $\alpha 6$ -specific antibodies (Fig. 2-6, C). Mutation of either Y1440 or Y1494, which prevents recruitment and activation of SHP2, inhibited the ability of  $\alpha 6\beta 4$  to promote SFK activation. Furthermore, mutation of

either Y1440 or Y1494 in the  $\beta 4$  subunit cytoplasmic domain also impairs the ability of  $\alpha 6\beta 4$  to promote carcinoma invasion (Fig. 2-6, D).

## Discussion

In this study, I demonstrated that SHP2 is required for the  $\alpha6\beta4$ -dependent activation of SFKs. SHP2 interacts with  $\beta4$  endogenously in response to  $\alpha6\beta4$  integrin engagement and its phosphatase activity is stimulated upon recruitment to  $\alpha6\beta4$ . The  $\beta4$  subunit recruits and activates SHP2 through a double intermolecular interaction: pY1494 ( $\beta4$ ) interaction with N-SH2 (SHP2) and pY1440 ( $\beta4$ ) interaction with C-SH2 (SHP2). Both Y1494 and Y1440 in the  $\beta4$  subunit are essential for stable interactions between  $\beta4$  and SHP2, as well as SFK activation. Moreover, intact Y1494 and Y1440 in the  $\beta4$  subunit cytoplasmic tail are required for  $\alpha6\beta4$ -dependent invasion. Taken together, these results reveal that SHP2 is required for in the  $\alpha6\beta4$ -dependent activation of SFKs and identify the molecular mechanism by which  $\beta4$  recruits and activates SHP2 to promote breast cancer cell invasive ability.

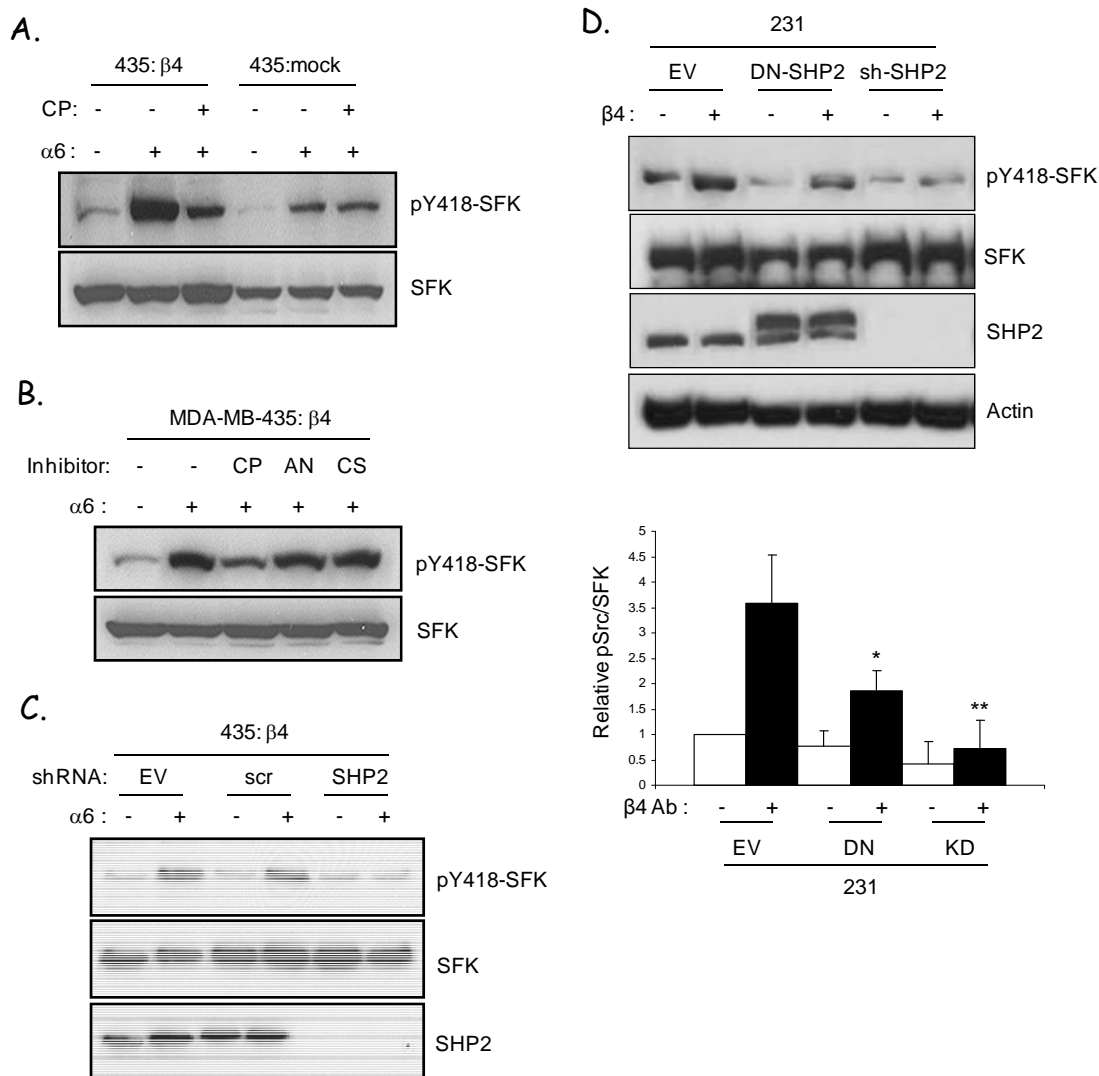
Y1494 in the  $\beta4$  subunit was originally identified as a potential regulator of signaling from the  $\alpha6\beta4$  integrin based upon its localization within a consensus-binding motif for the SH2-domain containing tyrosine phosphatases SHP1 and SHP2 [111-112]. Although it has been known for some time that mutation of Y1494 significantly impairs  $\alpha6\beta4$ -dependent invasion, the mechanism by which this tyrosine residue controls invasion had not been clearly elucidated [111]. Mutation of Y1494 diminishes the activation of both PI3K and SFK signaling pathways, which cooperate to promote invasion by  $\alpha6\beta4$  [76, 111]. Activation of PI3K is mediated through the insulin receptor substrate (IRS) adaptor proteins or through cooperation with growth factor receptors [38,

111]. In a previous study that investigated the function of the  $\beta 4$  subunit as a signaling adapter for the c-Met receptor, Y1494 was identified as one of three tyrosine residues that were required for SHP2 interaction with the  $\beta 4$  subunit in response to hepatocyte growth factor stimulation to promote anchorage-independent growth [163]. In this model system, Y1440 was identified as the major binding site, with minor contributions from Y1257 and Y1494 [163]. I have now demonstrated specific roles for Y1440 and Y1494 in the adhesion-dependent recruitment of SHP2 to the  $\alpha 6\beta 4$  receptor, and have also identified a unique role for Y1494 in the activation of SHP2 catalytic activity, through its selective binding to the N-SH2 domain of SHP2. When Y1494 is not phosphorylated, SHP2 phosphatase activity is not increased by  $\alpha 6\beta 4$  ligation, likely because the N-SH2 domain remains bound to the catalytic cleft and blocks substrate access. Binding of the N-SH2 domain to pY1494 is required to stimulate signaling downstream of SHP2 in response to  $\alpha 6\beta 4$  engagement.

There is relatively little information regarding the connection of SHP2 with solid tumor progression, although many of the upstream growth factor and integrin receptors that signal through SHP2 have been implicated in cancer [113]. Activating mutations in SHP2 have been identified in 35% of juvenile myelomonocytic leukemias (JMML), but the incidence of mutations in solid tumors is infrequent [167-168]. The majority of the JMML mutations disrupt the N-SH2 inhibitory interaction with the catalytic domain to increase basal phosphatase activity [169]. Interestingly, JMML patients lacking SHP2 mutations have either deletion of the NF-1 gene or activating Ras mutations, suggesting

an important role for SHP2 in the activation of Ras signaling in these tumors [168]. SHP2 regulates Ras signaling through the negative regulation of RasGAP recruitment to signaling complexes, thereby leading to sustained Ras activation and downstream MAPK signaling [170-171]. Activation of SHP2 in response to engagement of  $\alpha6\beta4$  through Y1494 could mimic activating SHP2 mutations to stimulate Ras signaling and promote tumor progression [119-120]. In support of this possibility, mutation of Y1494 diminishes  $\alpha6\beta4$ -dependent activation of MAPK signaling, which prevents anchorage-independent growth *in vitro* and tumor growth *in vivo* [76]. Therefore, in addition to regulating SFK activation to promote invasion, the  $\alpha6\beta4$ -dependent recruitment and activation of SHP2 is likely to contribute to multiple signaling pathways that promote tumor progression.

In summary, in nonstimulated cells, both SHP2 and SFKs are inactive because of their intramolecular inhibitory interactions. Upon engagement of the  $\alpha6\beta4$  integrin, the tyrosine residues in the  $\beta4$  cytoplasmic domain are phosphorylated. The  $\beta4$  tail recruits SHP2 through an interaction of its C-SH2 domain with pY1440 and its N-SH2 with pY1494, respectively.  $\alpha6\beta4$  activates SHP2 catalytic activity through the interaction of its N-SH2 domain with pY1494. The stable interaction between  $\beta4$  and SHP2 is essential for activating downstream signaling molecules, such as the SFKs (Fig. 2-7).

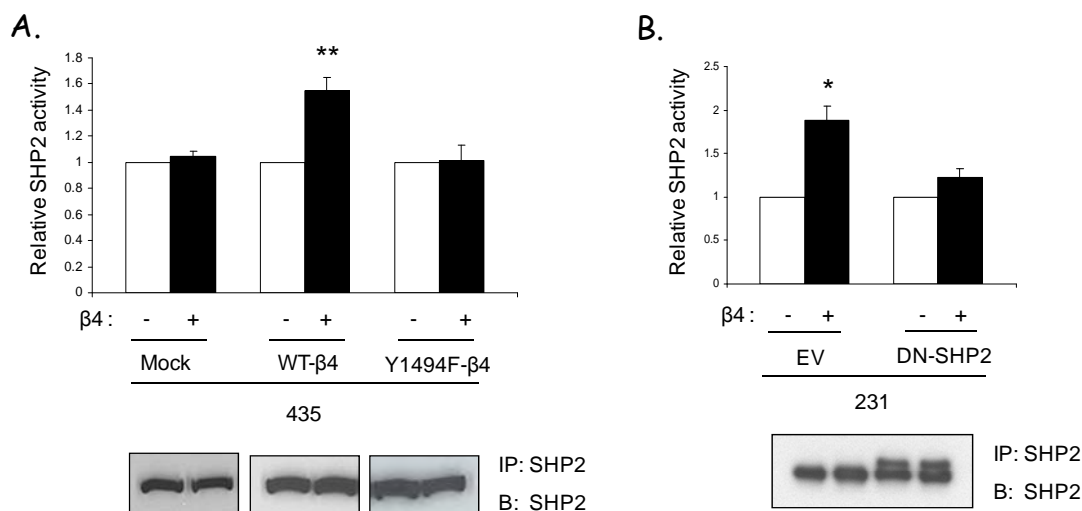


**Figure 2-1. The  $\alpha 6\beta 4$  activates SFK and this activation of SFKs requires SHP2**

(A) MDA-MB-435 cells that had been transfected with the  $\beta 4$ -integrin subunit ( $\beta 4$ ) or empty vector (mock) were incubated without or with an  $\alpha 6$ -specific antibody in either the absence or presence of 50  $\mu\text{g}/\text{ml}$  calpeptin (CP). Cells were allowed to adhere to anti-mouse IgG coated plates for 30 min. Cell extracts that contained equivalent amounts of total protein were immunoblotted for tyrosine 418-phosphorylated Src pSFK (Tyr-418) or total SFK. (B) MDA MB-435 cells transfected with the  $\beta 4$  subunit and either empty vector alone (pSUPER), a vector expressing a scrambled shRNA (scr), or a vector expressing an shRNA for SHP-2 (SHP-2) were incubated without or with an  $\alpha 6$ -specific antibody. Cells were allowed to adhere to anti-mouse IgG-coated plates for 30 min. Cell

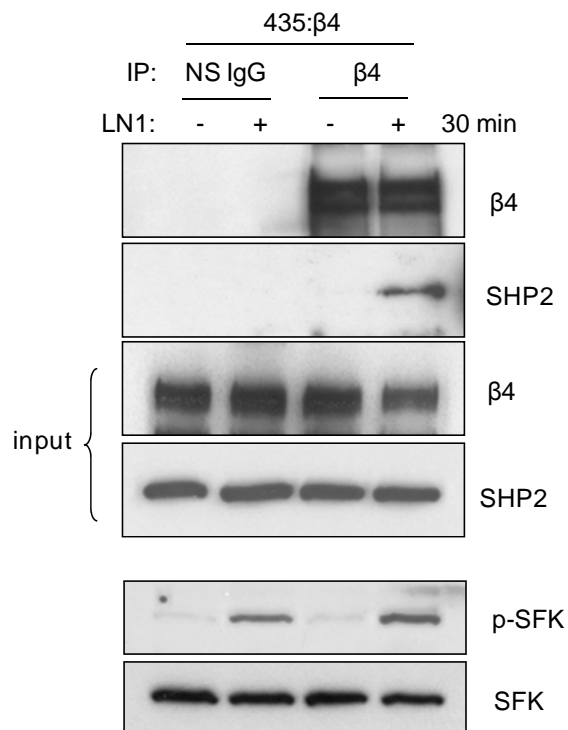
extracts that contained equivalent amounts of total protein were immunoblotted for tyrosine 418-phosphorylated SFK, total SFK, or total SHP-2. (C)

MDA-MB-231 cells stably expressing either empty vector (EV), HA tagged dominant negative SHP2 (DN) or SHP2-shRNA (KD) were maintained in suspension or incubated with  $\beta$ 4-specific antibodies and allowed to adhere to anti-mouse IgG-coated plates for 30 minutes. Aliquots of cell lysates containing equivalent amounts of total protein were immunoblotted with antibodies specific for phospho-Y418 of SFK, total SFK, SHP2 or actin (upper panel). The data shown in the graph represents the mean ( $\pm$  SD) of three independent experiments that were quantified by densitometry. (lower panel) \*,  $P \leq 0.04$ ; \*\*,  $P \leq 0.01$ . (D) MDA-MB-435 cells transfected with the  $\beta$ 4 subunit were incubated without (IgG) or with an  $\alpha$ 6-specific Ab in either the absence or presence of 50  $\mu$ g /ml calpeptin (CP), 50  $\mu$ M ALLN or 5  $\mu$ M calpastatin peptide (CS) and allowed to adhere to anti-mouse IgG coated plates for 30 min. Equal amounts of total protein from cell extracts were then immunoblotted for tyrosine 418 phosphorylated SFK, or total SFK.



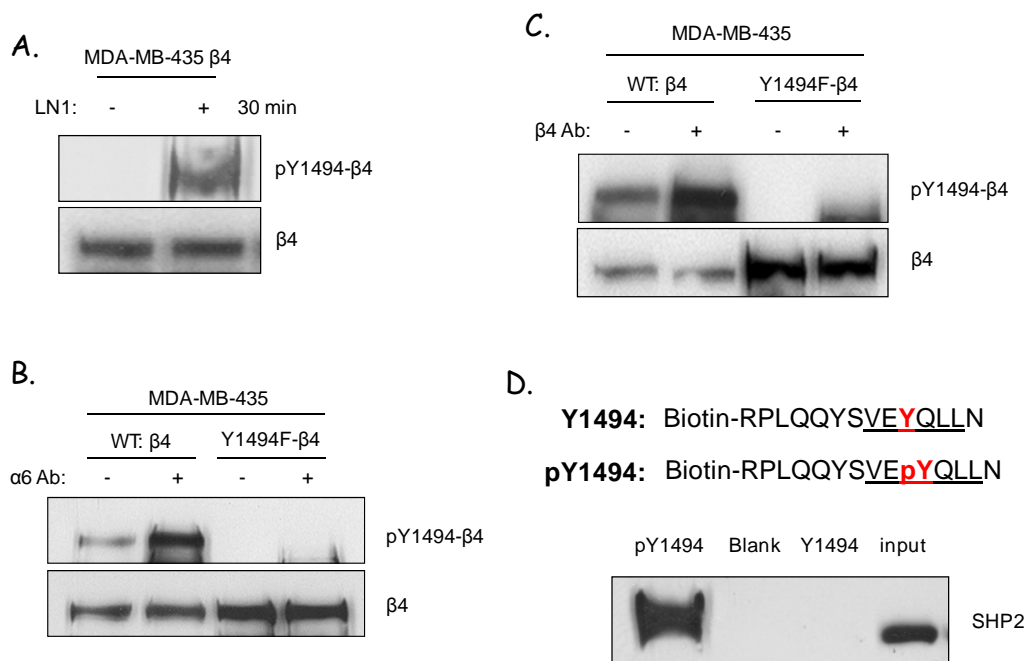
**Figure 2-2. SHP-2 phosphatase activity is stimulated in response to  $\alpha 6\beta 4$  ligation**

(A) MDA-MB-435 cells transfected with the vector alone (Mock), WT- $\beta 4$ , or Y1494F- $\beta 4$  were incubated with or without  $\beta 4$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with SHP2-specific Ab-conjugated agarose beads. The immune complexes were incubated with phosphotyrosyl peptides to assay phosphatase activity. One-tenth of the total immune complex was immunoblotted to determine the input. The data shown represent the mean ( $\pm$ the standard deviation) of three independent experiments. \*\*,  $P \leq 0.005$ . (B) MDA-MB-231 cells stably expressing either the empty vector (EV) or dominant negative SHP2 (DN-SHP2) were incubated with or without  $\beta 4$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with SHP2-specific Ab-conjugated agarose beads. The immune complexes were incubated with phosphotyrosyl peptides to assay phosphatase activity. One-tenth of the total immune complex was immunoblotted to determine the input. The data shown represent the mean ( $\pm$ the standard deviation) of three independent experiments. \*,  $P \leq 0.004$ .



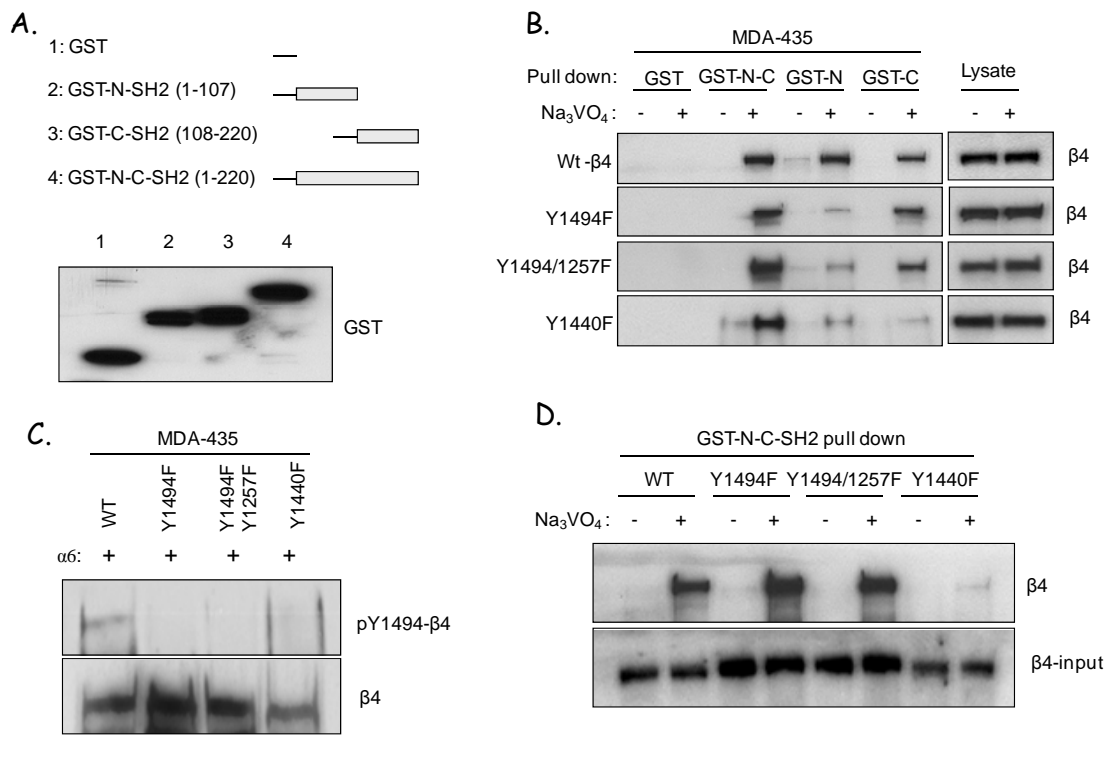
### Figure 2-3. Endogenous interaction of β4 and SHP2

MDA-MB-435/WT-β4 cells were allowed to adhere to BSA (-) or laminin 1 (+)-coated plates for 30 min. Aliquots of cell lysates were immunoprecipitated with nonspecific IgG or β4-specific Abs and immunoblotted with Abs specific for SHP2 and β4. Total cell lysates were immunoblotted with SHP2- and β4-specific Abs (input). Total cell lysates were also immunoblotted with p-SFK(Y418) and total SFK Abs



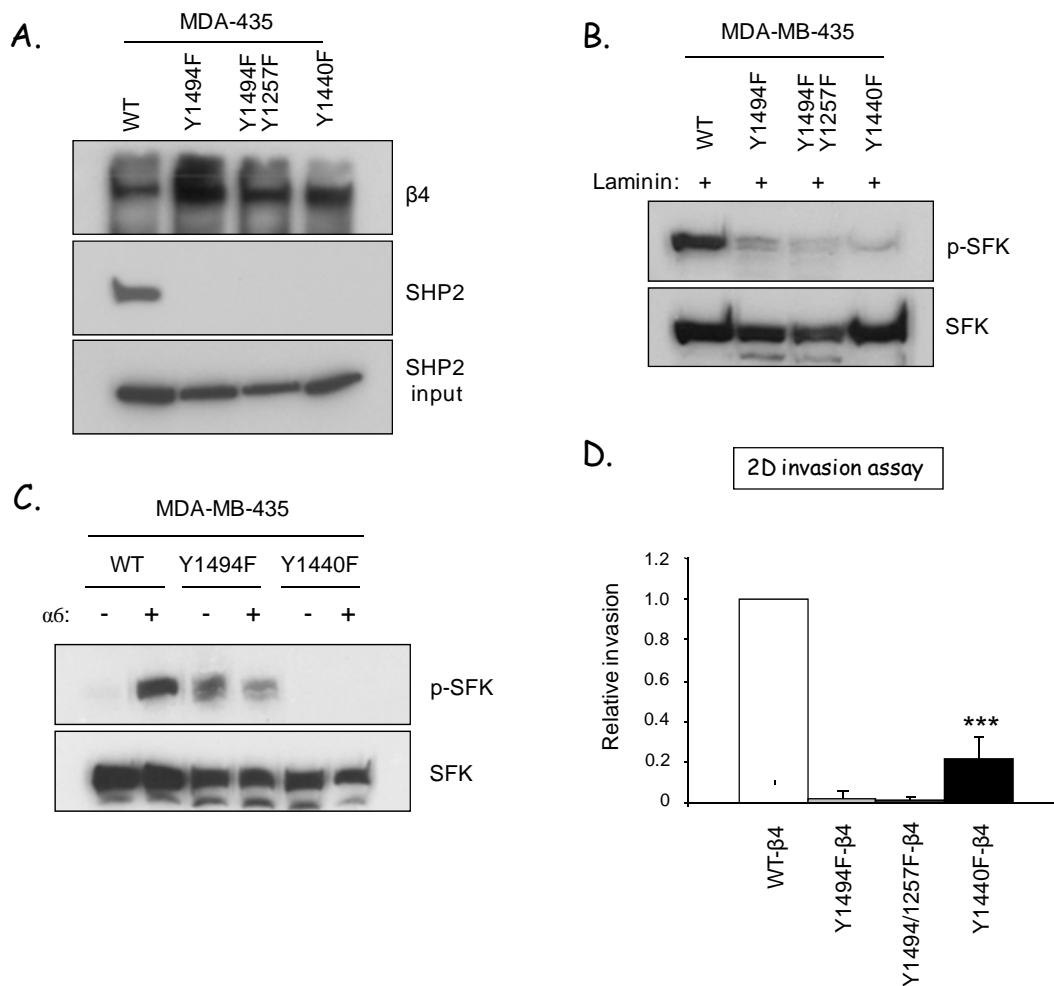
**Figure 2-4. phosphorylation of Y1494 is required for  $\beta 4$  and SHP2 interaction**

(A) MDA-MB-435 cells transfected with WT- $\beta 4$  were serum starved overnight and allowed to adhere to either BSA or lminin coated plates for 30 minutes. Aliquots of cell lysates containing equivalent amounts of total protein were immunoblotted with antibodies specific for phospho-Y1494 of the  $\beta 4$  subunit and total  $\beta 4$  expression levels. (B) MDA-MB-435 cells transfected with either WT- $\beta 4$  or Y1494F- $\beta 4$  were maintained in suspension or incubated with  $\alpha 6$ -specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 minutes. Aliquots of cell lysates containing equivalent amounts of total protein were immunoblotted with antibodies specific for phospho-Y1494 of the  $\beta 4$  subunit. The phospho-immunoblot was stripped and reprobed for total  $\beta 4$  expression levels. (C) MDA-MB-435 cells transfected with either WT- $\beta 4$  or Y1494F- $\beta 4$  were maintained in suspension or incubated with  $\beta 4$ -specific antibodies and allowed to adhere to anti-Rat IgG coated plates for 30 minutes. Aliquots of cell lysates containing equivalent amounts of total protein were immunoblotted with antibodies specific for phospho-Y1494 of the  $\beta 4$  subunit. The phospho-immunoblot was stripped and reprobed for total  $\beta 4$  expression levels. (D) MDA-MB-231 cell lysates containing equivalent amounts of total protein were incubated with biotinylated peptides bound to streptavidin-coated beads and immunoblotted with SHP2-specific antibodies.



**Figure 2-5. SHP2 interacts with Y1494 through its N-SH2 domain and Y1440 through C-SH2 domain**

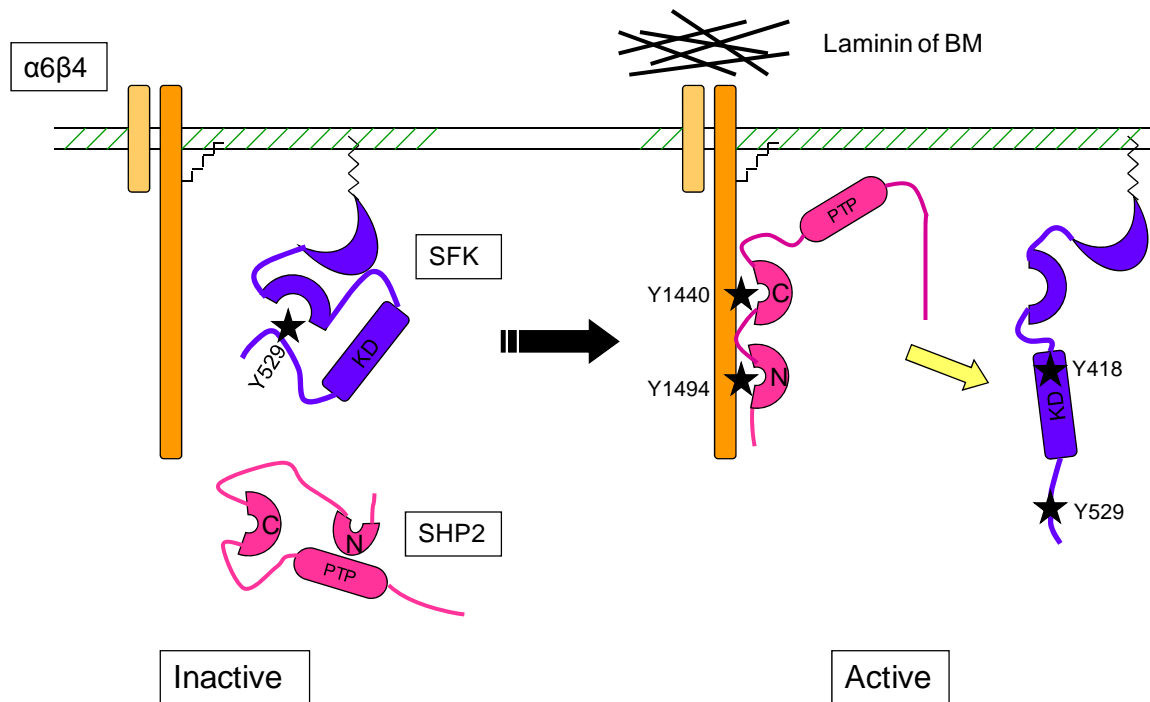
(A) SHP2 GST fusion proteins containing both SH2 domains (GST-N-C-SH2), the N-terminal SH2 domain (GST-N-SH2), or the C-terminal SH2 domain (GST-C-SH2) and GST alone were affinity purified with glutathione-Sepharose beads, and their expression was confirmed by western blotting. (B) Cell extracts from MDA-MB-435/WT-β4, MDA-MB 435/Y1494F-β4, MDA-MB 435/Y1494/1257F-β4, and MDA-MB-435/Y1440F-β4 transfectants left untreated or treated with Na<sub>3</sub>VO<sub>4</sub>-H<sub>2</sub>O<sub>2</sub> to increase tyrosine phosphorylation were incubated with the SHP2-GST fusion proteins and then immunoblotted with a β4-specific antiserum. WT-β4, MDA-MB-435 cells transfected with the WT β4 subunit; Y1494F-β4, MDA-MB-435 cells transfected with the Y1494F mutant β4 subunit; Y1494/1257F-β4, MDA-MB-435 cells transfected with the Y1494/1257F mutant β4 subunit; Y1440F-β4, MDA-MB-435 cells transfected with the Y1440F mutant β4 subunit. (C) MDA-MB-435 cells transfected with either WT-β4, Y1494F-β4, Y1494/1257F-β4 or Y1440F-β4 were incubated with α6-specific antibodies and allowed to adhere to anti-mouse IgG coated plates for 30 minutes. Aliquots of cell lysates containing equivalent amounts of total protein were immunoblotted with antibodies specific for phospho-Y1494 of the β4 subunit. The phospho-immunoblot was then stripped and reprobed for total β4 expression levels. (D) Cell extracts from MDA-MB-435/WT-β4, MDA-MB 435/Y1494F-β4, MDA-MB 435/Y1494/1257F-β4, and MDA-MB-435/Y1440F-β4 transfectants left untreated or treated with Na<sub>3</sub>VO<sub>4</sub>-H<sub>2</sub>O<sub>2</sub> to increase tyrosine phosphorylation were incubated with the GST-N-S-SH2 fusion proteins and then immunoblotted with a β4-specific antiserum.



**Figure 2-6. Intact Y1494 and Y1440 are required for stable  $\beta 4$ -SHP2 interaction, activation of SFKs and  $\alpha 6\beta 4$ -dependent invasion**

(A) MDA-MB-435 cells transfected with either WT- $\beta 4$ , Y1494F- $\beta 4$ , Y1494/1257F- $\beta 4$  or Y1440F- $\beta 4$  were serum starved overnight and then allowed to adhere to laminin 1-coated plates for 30 min. Equal aliquots of cell lysates were immunoprecipitated with  $\beta 4$ -specific Abs and immunoblotted with Abs specific for SHP2 and  $\beta 4$ . Total cell lysates were also immunoblotted with SHP2 Abs. (B) MDA-MB-435 cells transfected with WT- $\beta 4$ , Y1494F- $\beta 4$ , Y1257F, Y1494F- $\beta 4$ , or Y1440F- $\beta 4$  were serum starved overnight and then allowed to adhere to laminin-coated plates for 30 minutes. Aliquots of cell lysates were immunoblotted with Abs specific for pY418-SFK. The pY418-SFK immunoblots were stripped and reprobed for total SFK expression. (C) MDA-MB-435 cells transfected with WT- $\beta 4$ , Y1494F- $\beta 4$ , or Y1440F- $\beta 4$  were incubated with or without  $\alpha 6$ -specific Abs and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were

immunoblotted with Abs specific for pY418-SFK and total SFK. (D) MDA-MB-435 cells transfected with WT- $\beta$ 4, Y1494F- $\beta$ 4, Y1257/1494F- $\beta$ 4, or Y1440F- $\beta$ 4 were assayed for the ability to invade Matrigel using a Transwell assay chamber. The data shown represent the mean ( $\pm$  the standard deviation) of five independent invasion assays performed in duplicate. \*\*\*,  $P \leq 0.0001$ .



**Figure 2-7. Schematic of SHP2 interaction with  $\beta4$  subunit**

In the inactive state, both SHP2 and SFK are inactive because of intramolecular inhibitory interactions. Upon engagement of the  $\alpha6\beta4$  integrin with its ligand laminin (active state), the tyrosine residues in the  $\beta4$  cytoplasmic domain are phosphorylated. The  $\beta4$  tail recruits SHP2 through an interaction of its C-SH2 domain with pY1440 and activates SHP2 catalytic activity through the interaction of its N-SH2 domain with pY1494.

### **CHAPTER III. SHP2 Recruits and Activates Fyn to Mediate $\alpha6\beta4$ - Dependent Breast Carcinoma Cell Invasion**

Parts of this chapter represent work published as:

**SHP2 mediates the localized activation of Fyn downstream of the  $\alpha6\beta4$  integrin to promote carcinoma invasion**

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## Abstract

The cytoplasmic tyrosine phosphatase SHP2 plays critical roles in regulating a variety of cellular processes including cell growth, differentiation, cell spreading, and oncogenic transformation. Gain of function mutations of SHP2 are a cause of Noonan syndrome as well as some forms of leukemia. An important role of SHP2 is to regulate Src Family Kinase (SFK) activation. Several mechanisms have been proposed to explain how SHP2 mediates SFK activation, including mechanisms involving direct or indirect SHP2 phosphatase activity and phosphatase-independent activity. In the current study, I show that both catalytic and non-catalytic functions of SHP2 are required for SHP2-dependent activation of the SFK, Fyn by the  $\alpha6\beta4$  integrin. Moreover, I identify p-Y580-SHP2 and p-Y418-SFK as novel molecular markers for invasive breast cancer and demonstrate the importance of this  $\alpha6\beta4$ /SHP2/Fyn signaling pathway for carcinoma invasion.

## Introduction

The  $\alpha6\beta4$  integrin is unique amongst the integrin family of adhesion molecules in that it has a very long cytoplasmic tail, which is responsible for  $\alpha6\beta4$ -dependent signaling events [38]. In addition to its roles in promoting cell survival, promoting cell cycle progression, regulating gene transcription and regulating protein translation, the  $\alpha6\beta4$  integrin has been recognized to be an important regulator of migration and invasion [38, 109]. It is likely that  $\alpha6\beta4$  uses similar signaling mechanisms to promote both normal epithelial cell and invasive cancer cell migration and invasion. A core enzyme which is important for  $\alpha6\beta4$ -dependent cancer invasion is PI3K [73]. PI3K further activates downstream effectors such as Akt, Rac and mTOR to promote cancer cell migration, invasion and survival [38]. The  $\alpha6\beta4$  integrin has also been reported to function as an adaptor to cooperate with the c-MET receptor to activate SFKs [163]. Data from our own lab has shown that  $\alpha6\beta4$ -dependent cancer invasion occurs through combined activation of both PI3K and SFKs. In previous work, I established that the tyrosine phosphatase SHP2 is recruited to the  $\beta4$  cytoplasmic tail and that the  $\alpha6\beta4$ -dependent activation of SFKs requires SHP2.

The cytoplasmic tyrosine phosphatase SHP2 plays critical roles in regulating a variety of cellular processes including cell growth, differentiation, cell cycle, cell spreading, and oncogenic transformation. SHP2 contains two tandem SH2 domains, which function as phospho-tyrosine binding domains and mediate the interaction of SHP2 with its binding partners or substrates. Following the two SH2 domains are a classic PTP domain and a short C-terminal tail. Gain of function mutations of SHP2 are a

cause of Noonan syndrome as well as some forms of leukemia [114]. The greatest sequence divergence between SHP1 and SHP2 occurs in their C-terminal tails. Various truncation experiments have shown that the C-tail of the SHPs may regulate their phosphatase activity. However, this concept is still quite controversial. Truncation of the last 35 amino acids markedly enhance SHP1 phosphatase activity, whereas truncation of the last 60 amino acids has no effect on its phosphatase activity in an *in vitro* assay [172-173]. Located on the C-tail of SHPs are tyrosine residues, serine residues, and a proline-rich domain which may play some role in the function of SHPs [120]. Both SHP1 and SHP2 have been reported to undergo tyrosine phosphorylation on their C-tail, however the significance of this tyrosine phosphorylation is unclear. Two models have been proposed for the function of tyrosine modification on SHPs C-terminal tail. Phosphorylated tyrosine residues may serve as binding sites to recruit SH2 domain containing proteins, such as Grb-2 and SHIP. Tyrosine phosphorylation may also directly regulate SHP phosphatase activity [120].

Src Family Kinases are nonreceptor tyrosine kinases which are present in all metazoan cells. SFKs are prototypical modular signaling proteins comprised of a lipid modified N-terminus followed by SH3, SH2, and tyrosine kinase domains, and a short C-terminal regulatory tail. SFKs are involved in many receptor tyrosine kinase and integrin signaling pathways in a variety of tumor types including breast cancer [140]. Elevated SFK activity correlates strongly with breast cancer invasion and metastasis and these kinases are frequently activated in human cancers [174]. Given the parallels between  $\alpha 6\beta 4$  expression and SFK activation in cancer and my previous data demonstrating that

$\alpha6\beta4$  activates SFKs in a SHP2-dependent manner, further investigation of the mechanism by which  $\alpha6\beta4$  activates this pathway is warranted. In this study, I sought to elucidate the molecular mechanism by which engagement of  $\alpha6\beta4$  activates SFKs, and the significance of the  $\beta4$ /SHP2/SFK signaling axis for tumor progression.

## Materials and Methods

**Cell lines, Antibodies and Reagents.** MDA-MB-435 cells expressing wild type and mutant  $\beta 4$  subunits were generated and maintained as described previously [111, 161]. MDA-MB-231 human breast carcinoma cells were obtained from the Lombardi Breast Cancer Depository (Georgetown University) and maintained in RPMI medium containing 10% FBS.

The following antibodies were used: phospho-Y542 SHP2 (cat. #3751) and phospho-Y580 SHP2 (cat. #3754) from Cell Signaling; SHP2 (cat. #sc280), SFK (cat. #sc8056), Src (cat. #sc-19), Fyn (cat. #sc-16) and Yes (cat. #sc-14) from Santa Cruz Biotechnology, Inc; Phospho-Y418 Src (cat. #44660G) from Invitrogen;  $\beta 4$  subunit (439-9B) from R. Falcioni (Regina Elena Cancer Institute), cytoplasmic domain polyclonal antiserum from A. Mercurio (UMass Medical School); pY1494- $\beta 4$  from ECM Biosciences (cat.# IP1281);  $\alpha 6$  subunit (2B7) from A. Mercurio; HA (cat.# 11867423001) from Roche; actin (cat. #A2066) from Sigma; anti-rat (cat. #112-005-003) and anti-mouse (cat. #115-005-003) IgG from Jackson ImmunoResearch.

Calpeptin (cat. #0334 0051), Calpastatin Peptide (cat. #208902), ALLN (cat. #208719) and PP2 (Cat. # 529573) were obtained from Calbiochem. Matrigel (cat. #356237) was obtained from BD Biosciences and murine laminin-1 from Trevigen (cat. #3400-010-01) or Stemgent (cat. # 06-0002). WT and mutant SHP2 constructs in the pSUPER retroviral vector were gifts from B. Neel (Ontario Cancer Institute, Toronto). Human Fyn siRNA was obtained from Qiagen (cat. # SI02659545).

**Integrin clustering.** Cells were serum starved overnight in medium containing 0.1% BSA. Cells were trypsinized and washed before being resuspended at a concentration of  $10^6$  cells/ml and incubated for 30 minutes with or without integrin-specific antibodies (2 $\mu$ g/ml) in medium containing 0.1% BSA. The cells were washed once and added to plates which had been coated overnight with either anti-mouse or anti-rat IgG (100  $\mu$ g/10 cm plate), laminin (550  $\mu$ g/10 cm plate) or BSA (1%). Chemical inhibitors were added to the cells for 10 minutes prior to plating the cells in the coated plates. After incubation at 37°C for 30 minutes, the cells were washed once with PBS and lysed in a 20 mM Tris buffer, pH 7.4, containing 10% glycerol, 136 mM NaCl, 10% NP-40, 5mM EDTA, 1 mM sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ) and complete protease inhibitor cocktail (Roche) (Lysis Buffer A).

**Immunoprecipitations and immunoblots.** Cell extracts containing equivalent amounts of total protein were incubated for 3 hrs or overnight at 4°C with antibodies. Either protein A or protein G conjugated sepharose beads were added and incubated for an additional 1-2 hrs. Immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting [76].

**SHP2 *in vitro* tyrosine phosphatase assay.** SHP2 phosphatase activity was measured *in vitro* using the PTP Assay Kit-1 from Upstate Biotechnology. Cells were extracted in Lysis Buffer A without phosphatase inhibitors. Cell extracts containing equivalent amounts of total protein were incubated overnight with SHP2-specific Abs and protein G sepharose beads. The beads were washed four times with 10 mM Tris-HCl, pH 7.4 and

then resuspended in the same buffer with a tyrosine phosphopeptide (0.1mM) and incubated with gentle agitation for 1 hr at 37°C. The reaction was terminated by the addition of malachite green. SHP2 phosphatase activity was measured in a microtiter plate reader at 620nm following the manufacturer's instructions.

**2D invasion and adhesion assays.** Matrigel invasion assays were performed using 6.5 mm Transwell chambers (8 µm pore size; Costar) [76]. Matrigel, purified from the Englebreth-Holm-Swarm tumor, was diluted in cold distilled water, added to the Transwells (5ug/well), and dried in a sterile hood. The Matrigel was then reconstituted with medium for an hour at 37°C before the addition of cells. Cells ( $0.5 \times 10^5$ ) were resuspended in serum-free DMEM containing 0.1% BSA and added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers. After 5 hours, the cells that had invaded to the lower surface of the filters were fixed in methanol for 10 minutes. The fixed membranes were mounted on glass slides using Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Invasion was quantified by counting the number of stained nuclei in five independent fields in each Transwell.

Laminin adhesion assays were performed in multiwell tissue culture plates (11.3 mm diameter). The plates were coated overnight at 4°C with 0.2 ml of PBS containing murine laminin-1 (20ug/ml). The wells were then washed with PBS and blocked with RPMI containing 0.1% BSA. Cells ( $10^5$ ) were resuspended in blocking buffer and added to the protein coated wells. After a 60 minute incubation at 37°C, the wells were washed

three times, fixed for 15 minutes with methanol, and stained with a 0.2% solution of crystal violet in 2% ethanol. After washing, the crystal violet stain was solubilized with a 1% solution of SDS and adhesion was quantitated by measuring the absorbance at 595nm [111].

**3D Matrigel invasion assay.** A base layer of Matrigel (200  $\mu$ l/well) was overlaid in duplicate wells of a 24-well dish with  $1.0 \times 10^4$  cells suspended in 300  $\mu$ L of a 2:1 mixture of PBS and Matrigel. The Matrigel was overlaid with complete serum-containing medium (0.5 ml/well), which was changed every 3 days. Images were captured with SPOT image analysis software (Molecular Diagnostics).

**Tumor extraction.** Frozen tumors were homogenized at 4 °C in T-PER tissue protein extraction reagent (Pierce Biotechnology, Inc.), containing 1 mM sodium orthovanadate, 10 mM NaF, and protease inhibitors (Complete mini; Roche Applied Science).

**Statistics.** All data are represented as a mean +/- (standard error or standard deviation). All statistical analyses were performed using the unpaired Student's *t*-test.

## Results

### **Y542 of SHP2 is phosphorylated in response to $\alpha6\beta4$ engagement, while Y580 is constitutively phosphorylated**

There are two tyrosines in the C-terminal tail of SHP2 that can regulate SHP2 catalytic activity and can also serve in a non-catalytic capacity as binding sites for intermolecular interactions [120]. Although some progress has been made in recent years in understanding how phosphorylation of these tyrosines contributes to SHP2 function, the detailed molecular mechanisms by which phosphorylation of these residues impacts SHP2 catalytic activity and function remain controversial and are likely to be determined by the specific upstream stimulus [119, 175]. To determine if either Y542 or Y580 are phosphorylated in response to  $\alpha6\beta4$  ligation, the phosphorylation status of these tyrosine residues was examined in two metastatic carcinoma cell lines, MDA-MB-231 and MDA-MB-435. Phosphorylation of Y542 increased in response to  $\alpha6\beta4$  ligation using either  $\alpha6$ - or  $\beta4$ -specific Abs or its physiological ligand laminin to engage the receptor (Fig. 3-1, A-C). In contrast, Y580 was constitutively phosphorylated in both cell lines and the phosphorylation level did not increase in response to  $\alpha6\beta4$  ligation.

### **SFK phosphorylates Y542, but not Y580 of SHP2, in a positive feedback loop**

Y564 in the C-terminal tail of SHP1, which is equivalent to Y580 in SHP2, is phosphorylated by the SFK member Lck [176]. Moreover, Src is capable of phosphorylating SHP1 in an *in vitro* kinase assay [177]. Therefore, I sought to determine if SFKs participate in a feedback loop to regulate SHP2 function by phosphorylating

either Y542 or Y580 in the C-terminal tail. The phosphorylation of Y542 in response to  $\alpha 6\beta 4$  ligation was completely blocked by the SFK inhibitor PP2 in MDA-MB-435/WT- $\beta 4$  cells, but the constitutive phosphorylation of Y580 was not altered (Fig. 3-2, A). Inhibition of SHP2 phosphatase activity by calpeptin diminished SFK activation, similar to expression of DN-SHP2 (Fig. 2-1, D), and caused a corresponding decrease in the phosphorylation of Y542 (Fig. 3-2, A). As controls for the specificity of calpeptin's inhibition of SHP2, calpain-specific inhibitors did not diminish the phosphorylation of Y542 (Fig. 3-2, B).

Since Y1494 and Y1440 in the  $\beta 4$  subunit are both essential for  $\beta 4$ -SHP2 interaction and activation of SFKs (Fig. 2-6, A-C), I next evaluated the contribution of Y1494 and Y1440 to the phosphorylation of Y542 and Y580 in the SHP2 C-terminal tail. Mutation of either Y1494 or Y1440 blocked the phosphorylation of Y542 in response to  $\alpha 6\beta 4$  ligation with  $\alpha 6$  Abs (Fig. 3-3, A) or adhesion to Laminin-1 (Fig. 3-3, B). The diminished phosphorylation of Y542 correlated with the decreased activation of SFKs, which further supports that SFKs phosphorylate Y542 in SHP2 in a feed-back loop (Fig. 3-2, A).

### **Y580 in SHP2 is required for SFK activation by the $\alpha 6\beta 4$ integrin**

To understand the functional significance of phosphorylation of Y542 and Y580 in the C-terminus of SHP2 with regard to SFK activation by the  $\alpha 6\beta 4$  integrin, MDA-MB-231 cells that stably express HA-tagged Y542F-SHP2, Y580F-SHP2 and Y542F/Y580F-SHP2 mutants were generated (Fig. 3-4, A). All of the mutants were

expressed at a similar level to that of HA-tagged WT-SHP2 (Fig. 3-4, B). As was observed for endogenous SHP2, WT-SHP2 and the Y542F-SHP2 mutant were constitutively phosphorylated on Y580 (Fig. 3-4, B). Moreover, ligation of  $\alpha 6\beta 4$  stimulated the phosphorylation of Y542 in exogenously expressed WT-SHP2 and in the Y580F-SHP2 mutant (Fig. 3-4, C). However, the level of Y542 phosphorylation was significantly diminished when Y580 was mutated, suggesting that phosphorylation of Y580 contributes to the SFK-dependent phosphorylation of Y542 (Fig. 3-4, C). Next I evaluated the contribution of the SHP2 C-terminal tyrosines to  $\alpha 6\beta 4$ -dependent SFK activation. Mutation of Y542 resulted in a modest reduction in activation in response to  $\alpha 6\beta 4$  ligation (Fig. 3-4, D). In contrast, mutation of Y580 either alone or in combination with Y542 significantly diminished SFK activation (Fig. 3-4, D) as quantified by densitometry (Fig. 3-4, E).

Y542 and Y580 in the SHP2 C-terminal tail have been reported to be involved in the regulation of SHP2 phosphatase activity [120]. To determine if these tyrosine residues play a role in regulating  $\alpha 6\beta 4$ -dependent SHP2 phosphatase activity and if SHP2 phosphatase activity correlates with SFK activation, *in vitro* phosphatase assays were performed after ligation of  $\alpha 6\beta 4$  by  $\beta 4$ -specific Abs. Mutation of Y542 and Y580 individually reduced the phosphatase activity of SHP2, whereas the activity of the double Y542F/Y580F mutant was equivalent to that observed for WT-SHP2 (Fig. 3-5). This finding mimics a previous report that deletion of the SHP1 C-terminal tail activates the catalytic activity of the phosphatase [172]. The inability of the double mutant, which retains phosphatase activity, to activate SFKs suggests that Y580 may contribute to  $\alpha 6\beta 4$ -

dependent SFK activation by a mechanism that is independent of its regulation of SHP2 catalytic activity.

### **Y580 is required for the physical interaction of SHP2 and Fyn**

Y580 in SHP2 is localized within a binding motif that is recognized by the SH2-domains of several SFK members (Scansite). Therefore, we sought to determine if SHP2 and SFKs physically interact with each other and if this interaction is regulated by  $\alpha6\beta4$  engagement. In MDA-MB-231 cells, pan-SFK Abs co-immunoprecipitated SHP2 in the absence of  $\alpha6\beta4$  ligation and the SFK-SHP2 interaction increased upon ligation of the receptor (Fig. 3-6 A). MDA-MB-231 cells express only the SFK member Fyn, whereas MDA-MB-435 cells express both Src and Fyn. Neither cell line expresses the SFK member Yes (Fig. 3-6, B). To determine if there is specificity in the binding of SFKs to SHP2, antibodies that selectively recognize individual family members were used for the immunoprecipitations. SHP2 co-immunoprecipitated with Fyn from both cell lines, but no interaction was observed with Src in the MDA-MB-435/WT- $\beta4$  cells, indicating that Fyn, but not Src, is recruited to SHP2 downstream of  $\alpha6\beta4$  (Fig. 3-6, C-E). To investigate further the contribution of Y542 and Y580 to the interaction of SHP2 with Fyn, HA-specific antibodies were used to immunoprecipitate exogenously expressed SHP2 proteins after ligation of  $\alpha6\beta4$ . Fyn co-immunoprecipitated with WT-SHP2 and also with the Y542F-SHP2 mutant. However, mutation of Y580 prevented the interaction of SHP2 with Fyn (Fig. 3-6, F, upper panel). The reverse immunoprecipitation using Fyn antibodies revealed a similar requirement for an intact Y580 to pull down

SHP2 (Fig. 3-6, F, lower panel). In contrast, a GST fusion protein containing the tandem SH2 domains of SHP2 failed to pull down Fyn, indicating that the interaction of Fyn with SHP2 is independent of these domains (data not shown). Therefore, Y580 is required for the physical interaction of SHP2 and Fyn in response to  $\alpha 6\beta 4$  engagement. This observation is consistent with the finding that mutation of Y580 either alone or in combination with Y542 significantly diminished SFK activation (Fig. 3-4, D, E).

### **Fyn is the SFK that phosphorylates Y542 in SHP2**

The specific involvement of Fyn in  $\alpha 6\beta 4$ -dependent signaling was investigated using siRNA to suppress Fyn expression. Cells transfected with Fyn-specific siRNA showed diminished phosphorylation of Y542-SHP2 in response to  $\alpha 6\beta 4$  engagement (Fig. 3-7, A). To investigate further, MDA-MB-231 cells that stably over-express either HA-tagged WT-Fyn or DN-Fyn were generated. HA-tagged Fyn is evidenced by the slower migrating bands in the Western blots (Fig. 3-7, B). Both HA-tagged WT-Fyn and DN-Fyn were expressed at approximately two to three fold higher levels compared to endogenous Fyn. In response to  $\alpha 6\beta 4$  ligation, HA-tagged WT-Fyn was activated as evidenced by phosphorylation of Y418, and Y542-SHP2 phosphorylation levels increased correspondingly. In contrast, the activation of Fyn, and in turn the phosphorylation of Y542-SHP2, were completely blocked in DN-Fyn transfectants suggesting that Fyn is indeed the SFK which is involved in these  $\alpha 6\beta 4$  signaling events (Fig. 3-7, B).

### **Palmitoylation is required for $\alpha 6\beta 4$ -dependent SHP2-Fyn interaction**

Covalent attachment of long saturated fatty acids to proteins influences protein localization and function. The most common fatty acid modifications of proteins is myristoylation and palmitoylation, which make proteins more hydrophobic and increase membrane localization. Palmitoylation of the  $\beta 4$  subunit localizes  $\alpha 6\beta 4$  to a tetraspanin-rich cell surface microdomain [52]. Src can be modified on its N-terminus by myristoylation, whereas Fyn can be both myristoylated and palmitoylated [178-180]. Different lipid modifications may localize membrane bound molecules to distinct cell surface microdomains therefore conferring on the cell a way to selectively activate target molecules. To test the hypothesis that palmitoylation of Fyn is required for activation by  $\alpha 6\beta 4$ , HA-tagged palmitoylation deficient Fyn (C3, 6S) was overexpressed in MDA-MB-231 cells. The C3,6S mutant Fyn is not activated by  $\alpha 6\beta 4$  ligation as evidenced by the lack of an increase of in Y418 phosphorylation and a markedly decreased induction of Y542-SHP2 phosphorylation. The small increase in SHP2 phosphorylation observed in the palmitoylation deficient Fyn mutant cells most likely is due to the activation of endogenous Fyn. In both  $\alpha 6\beta 4$  ligated and non-ligated cells, Fyn and SHP2 are phosphorylated at a higher level compared to WT-Fyn transfectants in the cells expressing C3,6S-Fyn, which may be explained by the much higher expression level of C3, 6S-Fyn than WT-Fyn (Fig. 3-8, A). To further investigate the hypothesis that palmitoylation of Fyn is required for its activation by  $\alpha 6\beta 4$ , reverse co-immunoprecipitations were performed. HA tagged WT-Fyn, but not palmitoylation

deficient Fyn, co-immunoprecipitated with SHP2 in response to  $\alpha 6\beta 4$  engagement (Fig. 3-8, B, C).

### **A positive role for the $\alpha 6\beta 4$ -SHP2-SFK pathway in tumor invasion**

The  $\alpha 6\beta 4$  integrin promotes tumor cell invasion and SFK activation is required for this  $\alpha 6\beta 4$ -dependent function [76, 161]. Mutation of either Y1440 or Y1494 in the  $\beta 4$  subunit cytoplasmic domain inhibits SHP2 recruitment, SFK activation and also impairs the ability of  $\alpha 6\beta 4$  to promote carcinoma invasion (Fig. 2-6). To evaluate the overall importance of SHP2 and the contributions of the C-terminal Tyrosine residues for carcinoma invasion, MDA-MB-231 cells expressing WT-SHP2 and SHP2 mutants were assayed for their invasive potential using Transwell invasion chambers. Expression of WT-SHP2 increased invasion, and expression of DN-SHP2 decreased invasion, when compared with cells expressing empty vector (Fig. 3-9, A). Expression of Y542F-SHP2, Y580F-SHP2 and Y542F/Y580F-SHP2 mutants also increased invasion above the level observed for the vector control cells. However, the invasion was significantly lower for all three of the mutant cell lines when compared with WT-SHP2 expressing cells. Previous studies have implicated SHP2 in the regulation of cell adhesion and spreading, which could influence invasive potential [181]. To determine if changes in cell adhesion could explain the differences in cell invasion that were observed for SHP2 mutant expressing cells, the cells were also assayed for their adhesion to laminin-1 substrates. As shown in Fig. 3-9 B, all of the cell lines adhered to laminin at equivalent levels.

To investigate the contribution of SHP2 to invasion in a 3D-assay that more accurately reflects the tumor microenvironment, cells were embedded within a Matrigel matrix. When grown in a 3D matrix, non-invasive cells form round, compact colonies, whereas invasive cells exhibit a stellate, invasive morphology. Cells expressing WT-SHP2 formed very diffuse, invasive colonies, as we had observed previously for parental MDA-MB-231 and MDA-MB-435/WT- $\beta$ 4 cells (Fig. 3-9, C) [76]. In contrast, cells expressing Y542F-SHP2, Y580F-SHP2 and Y542F/Y580F-SHP2 formed progressively less invasive colonies, with the double Y542F/Y580F-SHP2 expressing cells being the least invasive of the SHP2 C-terminal tyrosine-mutant cell lines. Moreover, expression of DN-SHP2 completely inhibited invasion in the 3D-Matrigel matrix and the colonies formed by cells expressing this catalytically inactive SHP2 mutant were similar in morphology to parental MDA-MB-231 cells that were grown in the presence of the SFK inhibitor PP2 (Fig. 3-9, C) and to MDA-MB-435/Y1494F- $\beta$ 4 cells [76]. Taken together, the 2D and 3D invasion assays demonstrate that the ability of  $\alpha$ 6 $\beta$ 4 to recruit and activate SHP2 and to activate Fyn are essential for the  $\alpha$ 6 $\beta$ 4 integrin to optimally promote invasion.

### **Palmitoylation of Fyn is required for invasion**

Fyn plays a multifaceted role in the process of tumorigenesis, including promoting cell growth, inhibiting cell apoptosis and promoting cell migration and invasion [151]. To evaluate the contribution of palmitoylation of Fyn to tumor cell invasion, MDA-MB-231 cells transfected with either empty vector or the indicated HA-tagged Fyn constructs were assayed for their ability to invade Matrigel using both 2-D and 3-D Matrigel invasion

assays. WT-Fyn significantly increased cell invasion in both assays. WT-Fyn transfected cells showed a more invasive phenotype as they formed invasive colonies much earlier compared to empty vector control cells (Fig. 3-10, B). However this increase in invasion was not observed in the palmitoylation deficient Fyn (C3, 6S) transfectants (Fig. 3-10, A, B). Therefore, palmitoylation of Fyn is required for appropriate localization of Fyn to the cell surface microdomain to promote cancer cell invasion.

### **A positive correlation between p-Y580-SHP2, p-SFK and tumor grade in human breast cancer**

My *in vitro* studies implicate Y580-SHP2 as an important regulator of Fyn activation and carcinoma invasion downstream of the  $\alpha6\beta4$  integrin. To determine if this  $\alpha6\beta4$ /SHP2/Fyn signaling pathway is active *in vivo*, I assessed the phosphorylation status of Y580-SHP2 and Y418-SFK in tumors that were generated using MDA-MB-435 cells expressing empty vector, WT- $\beta4$  or Y1494F- $\beta4$  [76]. Phosphorylation of Y418-SFK is enhanced significantly in tumors that express  $\alpha6\beta4$  and this activation is dependent upon Y1494, which is required for SHP2 recruitment and activation (Fig. 3-11, A, B) [76]. Y580-SHP2 phosphorylation correlates with pY418-SFK, as it is also enhanced by expression of  $\alpha6\beta4$  and suppressed by mutation of Y1494 (Fig. 3-11, A, B). Additionally, I compared the phosphorylation status of Y580-SHP2 in human Grade I and Grade III breast tumors. Grade III tumors are poorly differentiated and generally have a higher risk of metastasis than well-differentiated, Grade I tumors [182]. Tumor extracts were immunoblotted for pY580-SHP2 and pY418-SFK and phosphoprotein levels were normalized to total protein expression. The levels of Y580-SHP2 phosphorylation and

pY418-SFK were markedly higher in Grade III tumors than in Grade I tumors (Fig. 3-11, C, D), supporting a potential role for this signaling pathway in the more aggressive behavior of these tumors. Taken together, these results support a potential role for this SHP2/Fyn signaling pathway in the more aggressive behavior of tumors that express  $\alpha6\beta4$ .

## Discussion

In this study, I identified a novel mechanism by which SHP2 mediates the selective activation of Fyn by the  $\alpha6\beta4$  integrin and demonstrate the importance of this  $\alpha6\beta4$ /SHP2/Fyn signaling pathway for carcinoma invasion. Engagement of the  $\alpha6\beta4$  integrin promotes the interaction of SHP2 with the  $\beta4$  subunit cytoplasmic domain. Fyn, but not Src, is recruited to SHP2 through phospho-Y580 in the C-terminus of SHP2, and this interaction requires palmitoylation of Fyn, and is necessary for the activation of Fyn downstream of  $\alpha6\beta4$ . Upon activation, Fyn phosphorylates SHP2 on Y542, creating a positive feedback loop that contributes to sustained SHP2 signaling. Both  $\alpha6\beta4$  mutants that cannot recruit and activate SHP2 and SHP2 mutants that cannot recruit and activate Fyn have diminished abilities to promote breast carcinoma invasion. *In vivo*, pY580-SHP2 and pY418-SFK levels are increased in tumors that express the  $\alpha6\beta4$  integrin, and this activation is dependent upon Y1494. Taken together, these results reveal how the  $\alpha6\beta4$  integrin localizes Fyn activation to promote breast carcinoma invasion and identify pY580-SHP2 and pY1494- $\beta4$  as potential indicators of invasive potential.

I have identified that the SHP2-dependent activation of Fyn by  $\alpha6\beta4$  requires the recruitment of Fyn to pY580 in the SHP2 C-terminal tail. SFK activation is regulated through intramolecular interactions that are controlled by phosphorylation at inhibitory and stimulatory sites [131]. Phosphorylation of the C-terminal tail (Y528 in human Fyn) inhibits activation by promoting an interaction of the N-terminal SH2 domain with this site. Dephosphorylation of the inhibitory tyrosine can disrupt this interaction and

promote auto-phosphorylation in the activation loop (Y417 in human Fyn) to enhance catalytic activity. A number of studies have investigated the role of SHP2 in SFK activation and both direct and indirect mechanisms have been reported. In response to growth factor stimulation, phosphorylation of the inhibitory Y527 in the C-terminus of c-Src diminishes through a SHP2-dependent decrease in the recruitment of Csk kinase to the membrane domains where Src is localized. The transmembrane glycoprotein PAG and the focal adhesion protein paxillin, which are substrates of SHP2, have been implicated in this recruitment of Csk [133-134]. SHP2 has also been reported to activate Src independently of its catalytic function by binding directly to the SH3 domain of Src and disrupting an intramolecular interaction that interferes with the catalytic domain [135]. I did not observe any changes in phosphorylation of the inhibitory C-terminal tyrosine of Fyn in response to  $\alpha 6\beta 4$  engagement (data not shown). However, recruitment of Fyn to Y580 in the C-terminus of SHP2 via its SH2 domain would disrupt the intramolecular inhibition to allow autophosphorylation of the activation loop, even in the presence of persistent phosphorylation of the Fyn C-terminus [183]. Although my data support that the catalytic activity of SHP2 contributes to Fyn activation in response to  $\alpha 6\beta 4$  engagement, the reduced ability of the C-terminal double Y542F/Y580F mutant that retains full phosphatase activity to stimulate Fyn activation demonstrates that the physical interaction of SHP2 and Fyn is also essential for optimal activation of this pathway in response to  $\alpha 6\beta 4$  engagement.

In previous studies, the ability of the  $\alpha 6\beta 4$  integrin to activate Fyn was shown to be dependent upon palmitoylation of the membrane proximal region of the  $\beta 4$  subunit [52,

87]. However, the direct link between Fyn and the  $\alpha6\beta4$  receptor remained an open question. Fyn is also palmitoylated, which provides a mechanism to localize Fyn in the plasma membrane in proximity to the  $\alpha6\beta4$  integrin, where it can mediate signals at sites of adhesive contacts to promote motility and invasion [178]. The potential importance of this localization is underscored by the fact that Src is not palmitoylated, and therefore does not localize to the membrane domains containing  $\alpha6\beta4$ , and it is not activated by this integrin receptor [178]. My current data confirms that different lipid modifications on the N-terminus of SFKs are responsible for selective activation of certain SFK over others. My data also demonstrate that localization to membrane domains alone is not sufficient for Fyn activation because the recruitment of Fyn to pY580-SHP2 is required to activate Fyn in response to  $\alpha6\beta4$  engagement. My data also support a positive Fyn feedback loop that mediates the phosphorylation of Y542 in the C-terminus of SHP2 to increase SHP2 catalytic activity.

The fact that mutation of Y542 did not significantly reduce Fyn activation by  $\alpha6\beta4$ , but did diminish invasion, provides evidence that additional SHP2 substrates are likely to cooperate with Fyn to enhance  $\alpha6\beta4$ -dependent invasion. Interestingly, Fyn and SHP2 inversely regulate the activity of some signaling molecules that contribute to tumor invasion. For example, Fyn phosphorylates and activates p190RhoGAP to inactivate the small GTP-binding protein RhoA, whereas SHP2 dephosphorylates p190RhoGAP, thereby maintaining RhoA in an active GTP-bound state to stimulate downstream effectors [166, 184-185]. RhoA can directly influence cell motility and invasion through

its regulation of the actin cytoskeleton, and the ability of RhoGTPases to cycle between active and inactive states is essential for this function [186]. Engagement of the  $\alpha6\beta4$  integrin activates RhoA to promote lamellae formation and migration [187]. The formation of an active SHP2/Fyn complex would allow for the dynamic regulation of RhoA downstream of  $\alpha6\beta4$ . Additional common downstream targets of Fyn and SHP2 that are likely to be important for promoting invasion include the focal adhesion components p130Cas and paxillin [188].

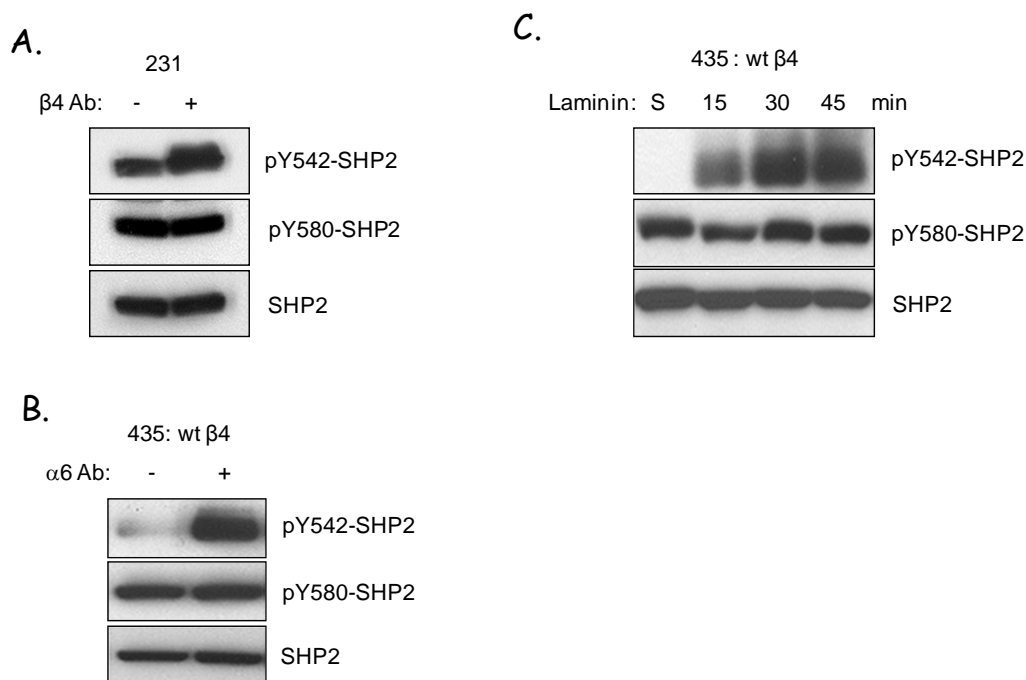
Phosphatase-dead, dominant-negative SHP2, Y542 and Y580 mutant SHP2 all significantly decreased tumor cell invasion (Fig. 3-9). The phosphatase dead SHP2 mutant completely blocked tumor cell invasion as shown in both 2D and 3D invasion assays. Y542 and Y580 mutants decreased tumor invasion, but to a lesser extent when compared with the phosphatase dead SHP2 mutant. These findings suggest that SHP2 phosphatase activity may contribute more to tumor invasion than the tyrosine residues on the SHP2 C-terminal tail. The distinct morphology of the SHP2 mutant cell colonies in 3D could be explained by different tumor cell survival. However, based on the similar size of the colonies, it is likely that the SHP2 mutant cells are alive. Therefore, we believe that the differences in morphology amongst the SHP2 transfectants are due to the different invasive ability of the cells (Fig. 3-9 C).

In the two invasive breast cancer cell lines I used for my thesis research, Y542 is phosphorylated by Fyn in response to  $\alpha6\beta4$  engagement, while Y580 phosphorylation level is not affected by  $\alpha6\beta4$  involvement, and is not a target of SFKs. However, in the

orthotopic mouse tumors, Y580 phosphorylation seems to be affected by the presence of WT  $\alpha6\beta4$  (Fig. 3-11 A). This finding can be explained by the fact that the *in vivo* microenvironment of the tumor cells is much more complicated than *in vitro* stimulations, such as laminin clustering and Ab-mediated  $\alpha6\beta4$  engagement. The same set of tumor samples have shown that WT- $\beta4$  tumors are associated with a more aggressive and malignant phenotype [76]. This is consistent with our model that p-Y580-SHP2 and p-Y418-SFK positively correlate with worse patient prognosis and could be used as diagnostic markers. Moreover blots from human breast tumor lysates confirmed that this hypothesis is valid in the set of samples we tested (Fig. 3-11 C).

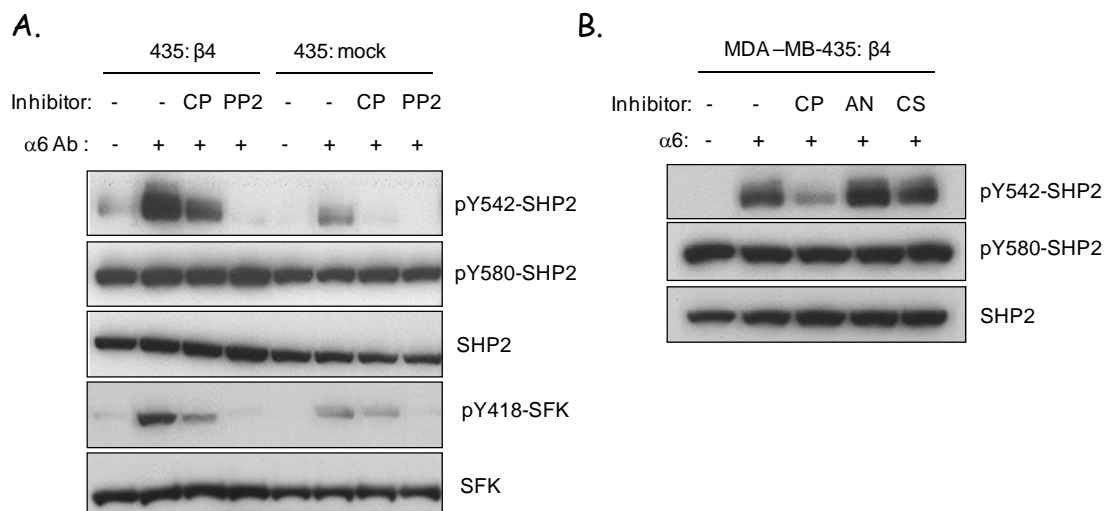
SFKs are expressed and activated in many tumor types and numerous studies have demonstrated that SFK activity is associated with poor patient outcomes [174]. In breast cancer, a Src-responsive gene signature that reflects active Src signaling has recently been found to be tightly associated with latent bone metastasis, and Src promotes growth and survival in the bone microenvironment [189]. Breast tumors that express  $\alpha6\beta4$  have increased angiogenesis and enhanced metastasis when compared with tumors that either lack expression of this integrin receptor or express a mutant Y1494F- $\beta4$  subunit [76, 105, 190]. The  $\alpha6\beta4$  integrin, which is also associated with poor prognosis in many tumor types, can stimulate SFK activation, however the mechanism by which it does so was not known. In the current study, I provide novel mechanistic insight into how the  $\alpha6\beta4$  integrin selectively activates the Src family member Fyn in response to receptor engagement. Both catalytic and non-catalytic functions of SHP2 are required for Fyn

activation by  $\alpha 6\beta 4$ . Specifically, the tyrosine phosphatase SHP2 is recruited to  $\alpha 6\beta 4$  and its catalytic activity is stimulated through a specific interaction of its N-terminal SH2 domain with pY1494 in the  $\beta 4$  subunit. Fyn is recruited to the  $\alpha 6\beta 4$ /SHP2 complex through an interaction with phospho-Y580 in the C-terminus of SHP2. In addition to activating Fyn, this interaction with Y580-SHP2 localizes Fyn to sites of receptor engagement, which is required for  $\alpha 6\beta 4$ -dependent invasion. Palmitoylation modification of Fyn on its N-terminus confers on Fyn the ability to be selectively activated by  $\alpha 6\beta 4$  engagement over other SFKs. The enhanced phosphorylation of Y580-SHP2 and Y418-SFK in human orthotopic breast tumors that express WT- $\beta 4$ , and the dependence of this SFK activation on Y1494, supports the involvement of an  $\alpha 6\beta 4$ /SHP2/Fyn signaling pathway in carcinoma invasion and tumor progression.



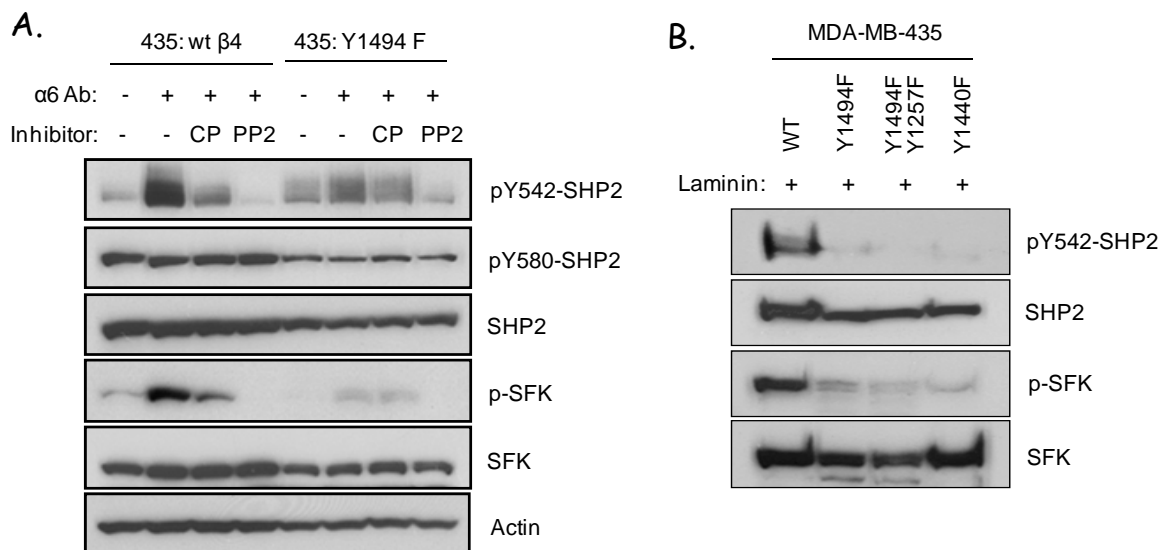
**Figure 3-1. Y542 of SHP2 is phosphorylated in response to  $\alpha 6\beta 4$  engagement, while Y580 is constitutively phosphorylated**

Cells were incubated with or without integrin-specific antibodies and allowed to adhere to anti-mouse IgG-coated plates or laminin-1 coated plates. Aliquots of cell lysates were immunoblotted with antibodies specific for the indicated phospho-proteins. The phospho-immunoblots were stripped and re-probed for total protein expression levels. (A) MDA-MB-231 cells were ligated with (+) or without (-)  $\beta 4$ -specific Abs. (B) MDA-MB-435/WT- $\beta 4$  cells were ligated with (+) or without (-)  $\alpha 6$ -specific Abs. (C) MDA-MB-435/WT- $\beta 4$  cells were allowed to adhere to laminin-1 coated plates for the indicated time periods.



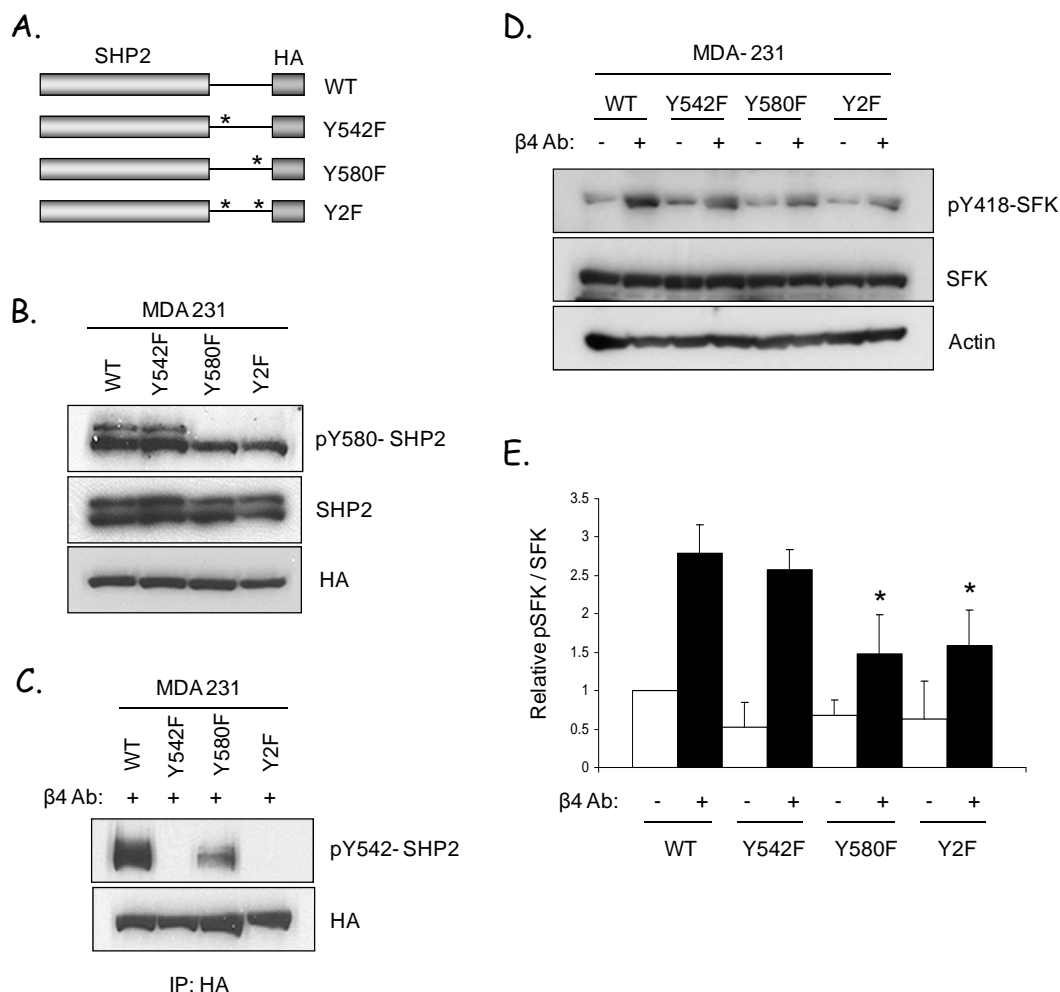
**Figure 3-2. SFKs phosphorylate Y542, but not Y580, of SHP2 in a positive feed-back loop**

(A) MDA-MB-435 cells that were transfected with WT- $\beta$ 4 or empty vector (Mock) were ligated with  $\alpha$ 6-specific antibodies in the absence or presence of the SHP2 inhibitor calpeptin (CP; 50 $\mu$ g/ml) or the SFK inhibitor PP2 (10 $\mu$ M). (B) MDA-MB-435/WT- $\beta$ 4 cells were ligated with  $\alpha$ 6-specific antibodies in the absence or presence of calpeptin (CP; 50 $\mu$ g/ml), ALLN (50 $\mu$ M) or calpastatin peptide (CS; 5 $\mu$ M).



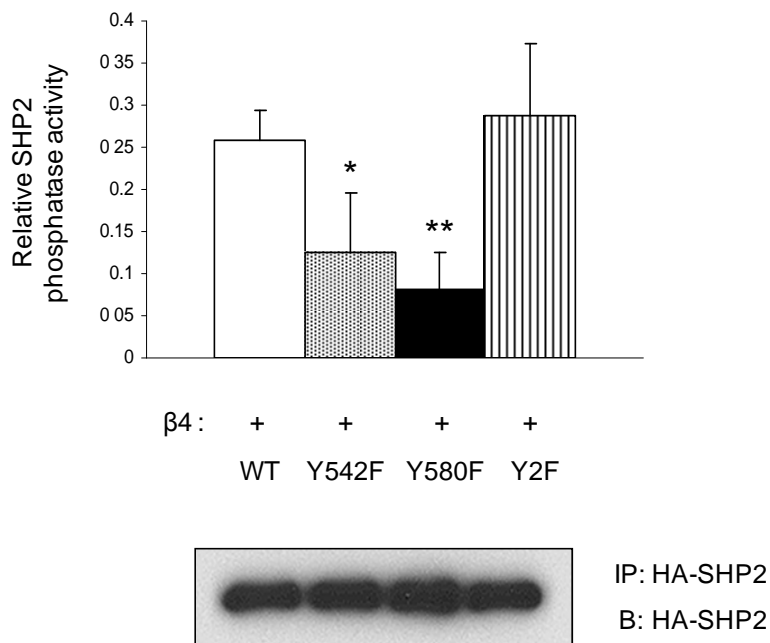
**Figure 3-3. Intact Y1494 and Y1440 of  $\beta 4$  subunit are essential for  $\alpha 6\beta 4$ -dependent phosphorylation of Y542-SHP2 by SFKs**

(A) MDA-MB-435 cells that were transfected with WT- $\beta 4$  or Y1494F- $\beta 4$  were ligated with  $\alpha 6$ -specific antibodies in the absence or presence of the SHP2 inhibitor calpeptin (CP; 50 $\mu$ g/ml) or the SFK inhibitor PP2 (10 $\mu$ M). (B) MDA-MB-435 cells transfected with either WT- $\beta 4$ , Y1494F- $\beta 4$ , Y1257/1494F- $\beta 4$  or Y1440F- $\beta 4$  were serum starved overnight and then allowed to adhere to laminin coated plates. Aliquots of cell lysates were immunoblotted with antibodies specific for pY542-SHP2 or pY418-SFK. The pY542-SHP2 and pY418-SFK immunoblots were stripped and re probed for total SHP2 or SFK expression, respectively.



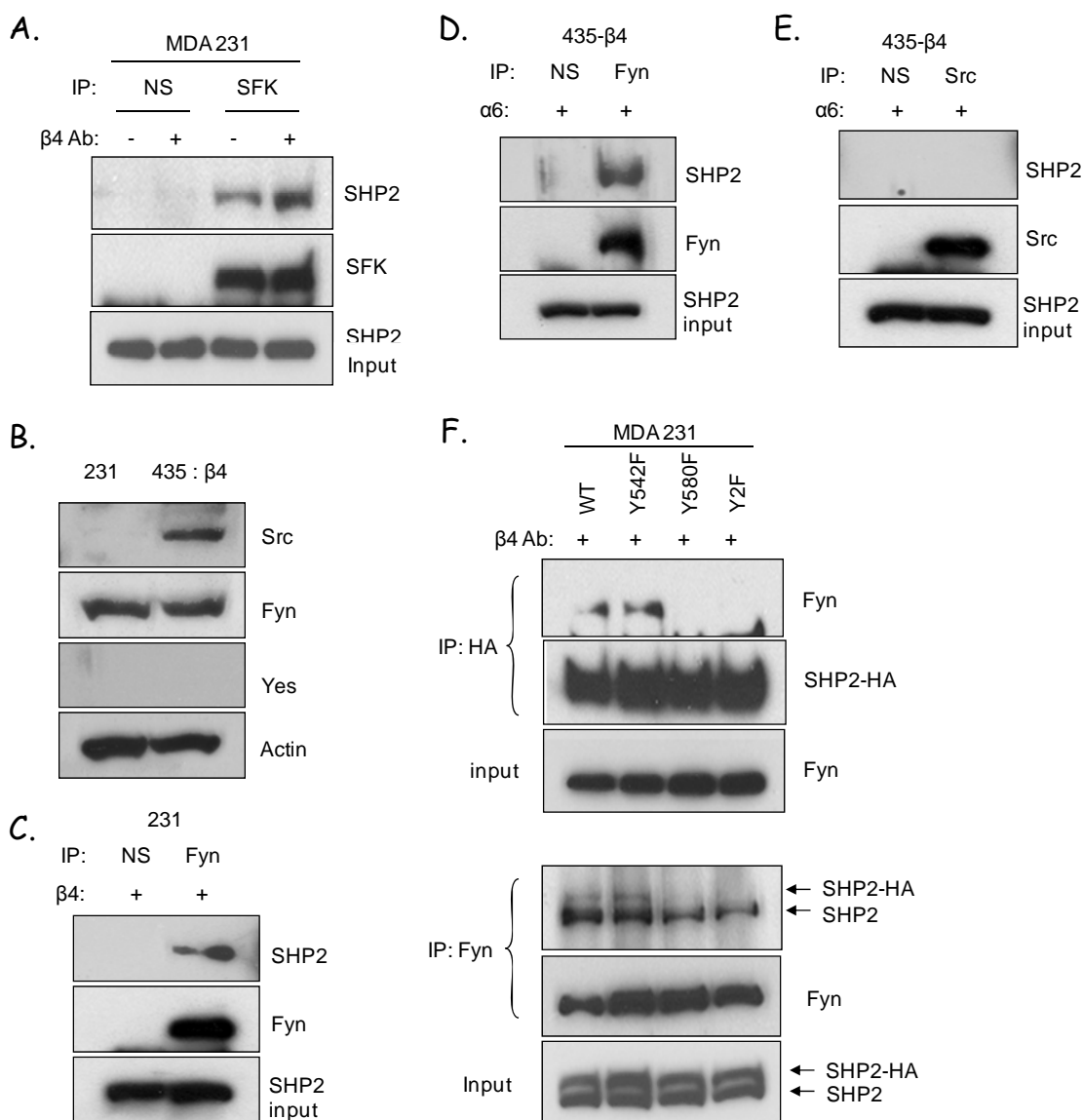
**Figure 3-4 Y580 in SHP2 is required for SFK activation by  $\alpha 6\beta 4$  signaling pathway**

(A) Schematic representation of HA tagged SHP2 Y542/Y580 mutants. (B) Aliquots of cell extracts from MDA-MB-231 cells stably expressing the HA-tagged SHP2 constructs were immunoblotted with antibodies specific for pY580-SHP2 and HA. The phospho-immunoblot was stripped and reprobed for total SHP2 levels. (C) MDA-MB-231 cells stably expressing HA-tagged SHP2 constructs were incubated with  $\beta 4$ -specific antibodies and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with HA-specific antibodies and immunoblotted with antibodies specific for pY542-SHP2. The phospho-immunoblot was stripped and reprobed for total SHP2 expression levels. (D) MDA-MB-231 cells stably expressing the HA-tagged SHP2 constructs were incubated with (+) or without (-)  $\beta 4$ -specific antibodies and allowed to adhere to anti-Rat IgG-coated plates. Aliquots of cell lysates were immunoblotted with antibodies specific for pY418-SFK or Actin. The phospho-immunoblot was stripped and reprobed for total SFK expression levels. The data shown in the graph represent the mean ( $\pm$  SD) of three independent experiments that were quantified by densitometry. \*,  $p \leq 0.03$ .



**Figure 3-5. Y580-SHP2 contributes to SFK activation independent of its regulation of SHP2 phosphatase activity**

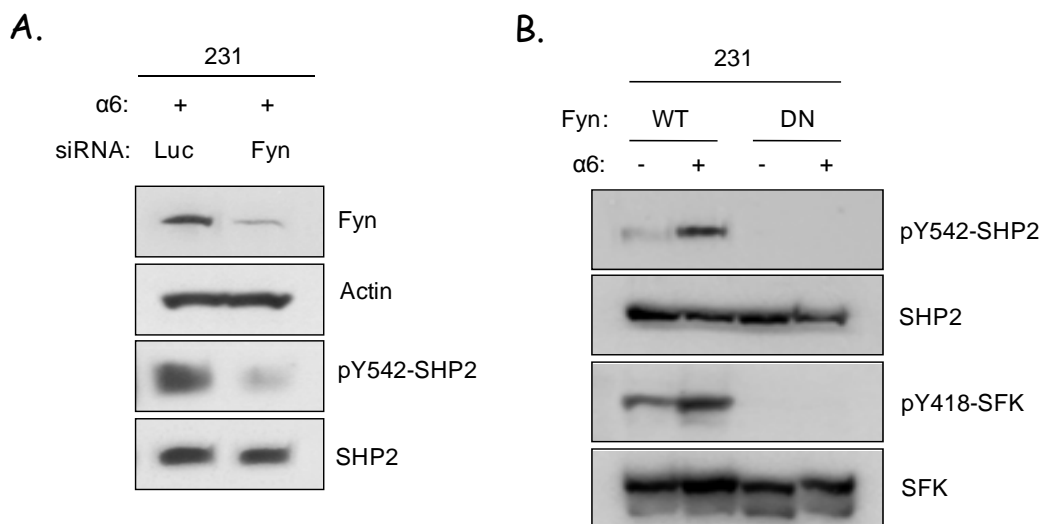
MDA-MB-231 cells stably expressing HA-tagged SHP2 constructs were incubated with  $\beta$ 4-specific antibodies and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with HA-specific antibodies and the immune complexes were incubated with phosphotyrosyl peptides to assay phosphatase activity. 1/10 of the total immune complex was immunoblotted to determine input. The data shown represent the mean ( $\pm$  SD) of 3 independent experiments. \*,  $p \leq 0.04$ ; \*\*,  $p \leq 0.005$ .



**Figure 3-6. Y580-SHP2 is required for the physical interaction of SHP2 and Fyn**

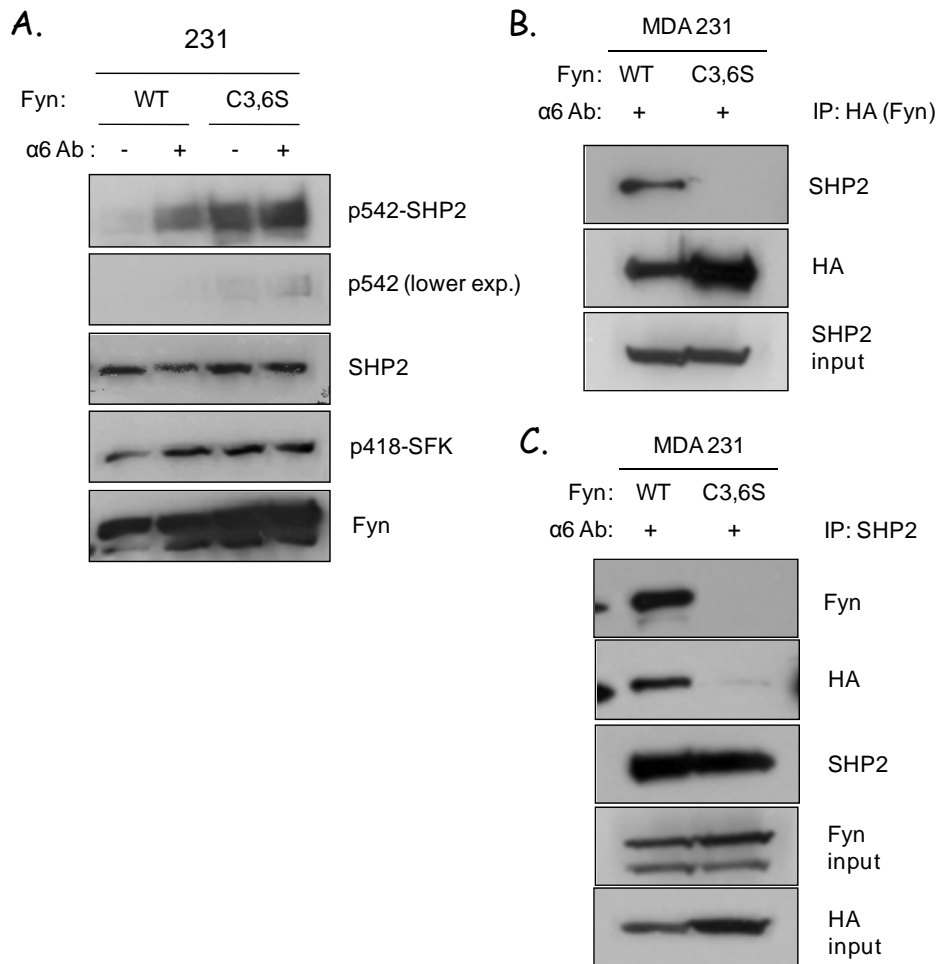
(A) MDA-MB-231 cells were incubated with or without  $\beta$ 4-specific antibodies and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with non-specific IgG (IgG) or SFK antibodies and immunoblotted with antibodies specific for SHP2. The immunoblot was stripped and re-probed for total SFK expression levels. Total cell lysates were also immunoblotted with SHP2-specific antibodies (SHP2 input). (B) Aliquots of cell lysates were immunoblotted with antibodies

specific for Src, Fyn, Yes and Actin. 231, MDA-MB-231 cells; 435- $\beta$ 4, MDA-MB-435 cells transfected with WT- $\beta$ 4. (C, D and E) MDA-MB-231 and MDA-MB-435/WT- $\beta$ 4 cells were incubated with integrin-specific antibodies and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with either non-specific IgG (IgG) or antibodies that specifically recognize Fyn or Src and immunoblotted with antibodies specific for SHP2. The immunoblot was stripped and reprobed for total Fyn or Src expression levels, respectively. Total cell lysates were also immunoblotted with SHP2-specific antibodies (SHP2 input). (F) MDA-MB-231 cells stably expressing HA-tagged SHP2 constructs were incubated with  $\beta$ 4-specific antibodies and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with Abs that recognize either HA- or Fyn and immunoblotted with antibodies that recognize either Fyn (HA-IP) or SHP2 (Fyn-IP). The immunoblots were stripped and reprobed for total HA or Fyn expression levels, respectively. Total cell lysates were also immunoblotted with SHP2- and Fyn- antibodies (Input).



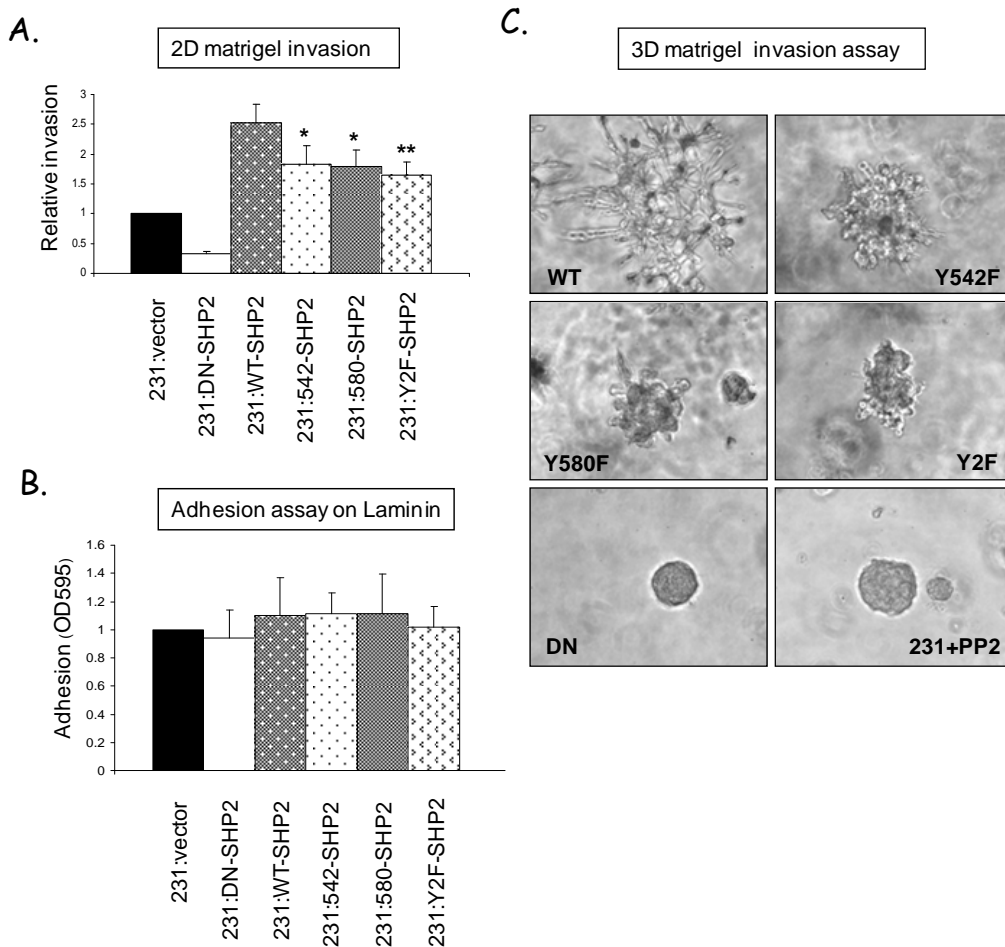
**Figure 3-7. Fyn is the SFK that phosphorylates Y542 in SHP2 in response to  $\alpha 6\beta 4$  engagement**

(A) MDA-MB-231 cells were transfected with siRNA specific for either luciferase (Luc) or human Fyn for 48 hrs. Cells were incubated with or without  $\alpha 6$ -specific antibodies and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoblotted with antibodies specific for Fyn, pY542-SHP2 or Actin. The phospho-immunoblot was stripped and reprobed for total SHP2 expression. (B) MDA-MB-231 cells stably expressing the HA-tagged Fyn constructs were incubated with (+) or without (-)  $\alpha 6$ -specific antibodies and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoblotted with antibodies specific for pY542-SHP2 or pY418-SFK. The phospho-immunoblot was stripped and reprobed for total SHP2 or SFK expression levels, respectively.



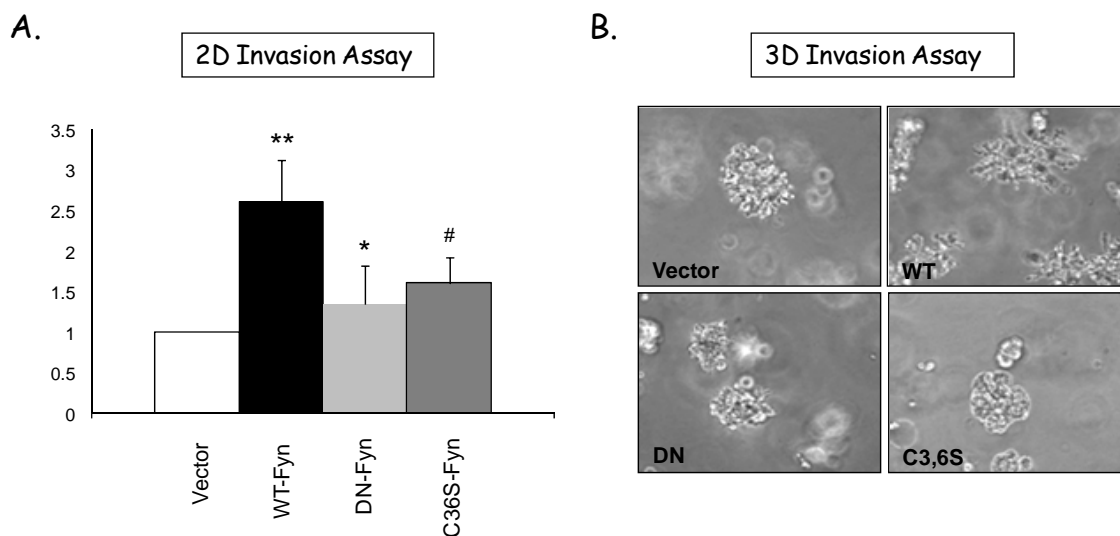
**Figure 3-8. Palmitoylation is required for the  $\alpha 6\beta 4$ -dependent SHP2-Fyn interaction**

(A) MDA-MB-231 cells stably expressing HA-tagged Fyn constructs were incubated with (+) or without (-)  $\alpha 6$ -specific antibodies and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoblotted with antibodies specific for pY542-SHP2 or pY418-SFK. The phospho-immunoblots were stripped and reprobred for total SHP2 or SFK expression levels, respectively. (B, C) MDA-MB-231 cells stably expressing HA-tagged Fyn constructs were incubated with  $\alpha 6$ -specific antibodies and allowed to adhere to anti-mouse IgG-coated plates. Aliquots of cell lysates were immunoprecipitated with Abs that recognize either HA or SHP2 and immunoblotted with antibodies that recognize either SHP2 (HA-IP) or Fyn (SHP2-IP). The immunoblots were stripped and reprobred for total HA or SHP2 expression levels, respectively. Total cell lysates were also immunoblotted with SHP2, Fyn and HA antibodies (Input).



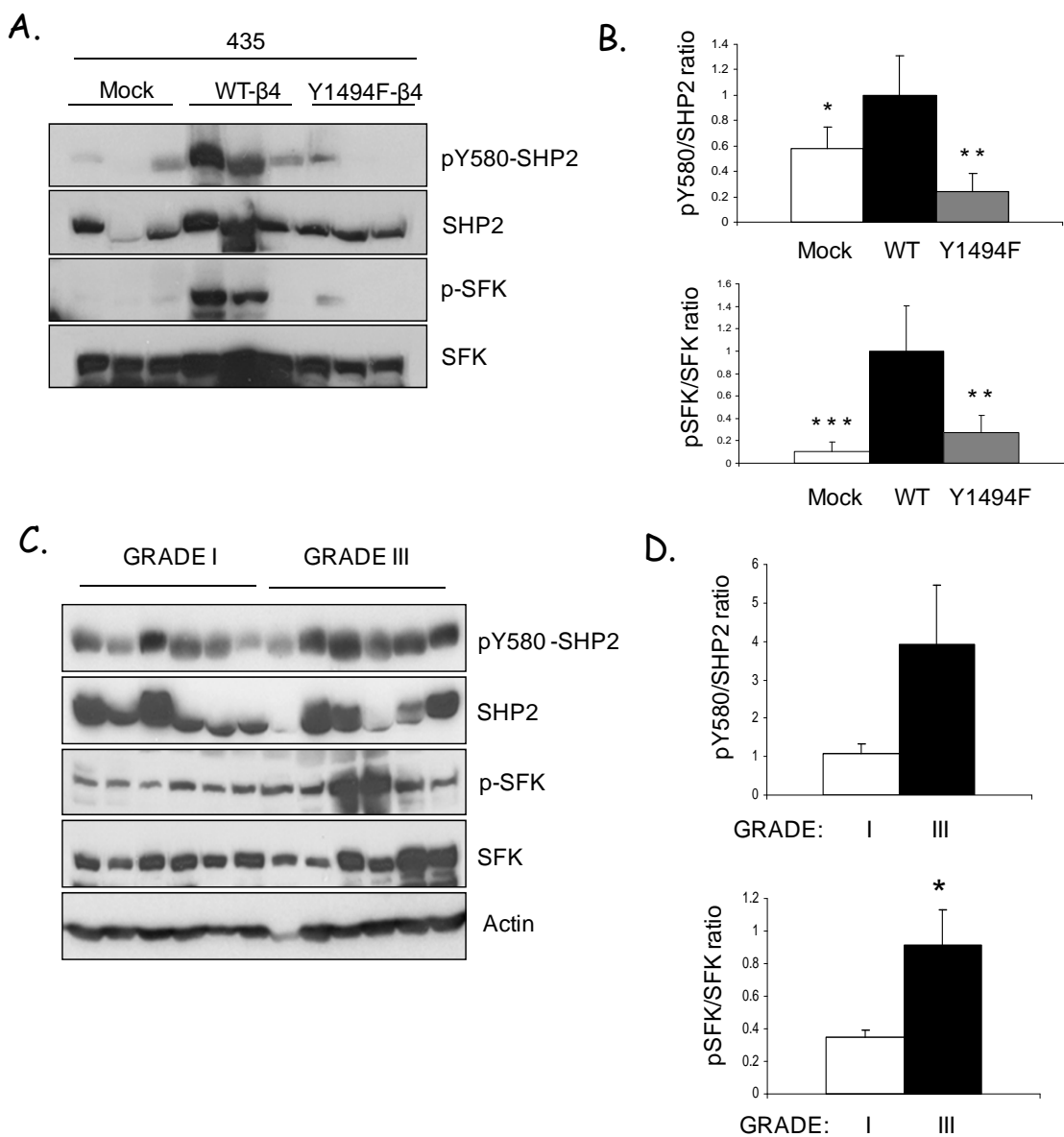
**Figure 3-9. A positive role for the  $\alpha 6\beta 4$ -SHP2-SFK pathway in tumor invasion**

(A) MDA-MB-231 cells transfected with either empty vector or the indicated HA-tagged SHP2 constructs were allowed to adhere to laminin-1 coated wells for 1 h at 37°C. After washing, cells were fixed, stained with crystal violet, and quantified by measuring absorbance at 595nm. The data shown represent the mean (+/- SD) of three independent assays performed in triplicate. (B) MDA-MB-231 cells transfected with either empty vector or the indicated HA-tagged SHP2 constructs were assayed for their ability to invade Matrigel using a Transwell assay chamber. The data shown represent the mean (+/- SD) of three independent invasion assays performed in duplicate. \*,  $p \leq 0.02$ ; \*\*,  $p \leq 0.008$ . (C) Representative images captured at 20X magnification of MDA-MB-231 cells transfected with the indicated HA-tagged SHP2 constructs or incubated in the presence of PP2 and grown for 10 days in 3D Matrigel culture.



**Figure 3-10. Palmitoylation of Fyn is required for invasion**

(A) MDA-MB-231 cells transfected with either empty vector or the indicated HA-tagged Fyn constructs were assayed for their ability to invade Matrigel using a Transwell assay chamber. The data shown represent the mean ( $\pm$  SD) of three independent invasion assays performed in duplicate. \*,  $p \leq 0.04$ . \*\*,  $p \leq 0.006$ ; #,  $p \leq 0.05$ . (B) Representative images captured at 20X magnification of MDA-MB-231 cells transfected with the indicated HA-tagged Fyn constructs after 7 days in 3D Matrigel culture.



**Figure 3-11. A positive correlation between p-Y580-SHP2, p-SFK and tumor grade in human breast cancer**

(A) Aliquots of tumor extracts from Mock, WT-β4 and Y1494F-β4 derived tumors were immunoblotted with antibodies specific for pY580-SHP2 and pY418-SFK. The immunoblots were stripped and reprobbed for total SHP2 and SFK expression levels. (B) The data shown in the graphs represent the mean levels of pY580-SHP2/SHP2 and pY418-SFK/SFK that were quantified by densitometry for each tumor type (n=6 for each tumor type). \*,  $p \leq 0.003$ ; \*\*,  $p \leq 0.0004$ ; \*\*\*,  $p \leq 0.02$ . (C) Aliquots of tumor extracts from Grade I (n = 6) and Grade III (n = 6) human breast tumors containing equivalent amounts of total protein were immunoblotted with

antibodies specific for pY580-SHP2, pY418-Src and actin. The phospho-immunoblots were stripped and reprobed for total SHP2 and Src expression levels, respectively. (D) The data in figure (C) were quantified by densitometry for each tumor grade. \*,  $p \leq 0.03$ .

## **CHAPTER IV. Final Thoughts and Future Directions**

Besides its well established roles in maintaining the integrity of epithelia, the  $\alpha6\beta4$  integrin has been recognized for its important functions in tumorigenesis, such as promotion of tumor cell migration, invasion, survival, and stimulation of angiogenesis [38, 191-192].  $\alpha6\beta4$ -dependent tumor initiation and progression occurs through activation of multiple signaling pathways, such as the  $\alpha6\beta4$ -dependent activation of PI3K [73]. Another example is the SFK-mediated phosphorylation of the large cytoplasmic tail of  $\beta4$ , recruitment of Shc and activation of Ras and MAPK [87, 193-194]. The  $\alpha6\beta4$  integrin may play its multifaceted role in tumor progression through regulating different signaling pathways. A recent study by our lab has shown that  $\alpha6\beta4$  regulates anchorage-independent growth through activation of the extracellular signal-regulated kinase signaling pathway, and it regulates invasion through combined activation of PI3K and a SFK [76]. As a core enzyme activated by the  $\alpha6\beta4$  integrin, PI3K has been extensively studied. PI3K is a lipid kinase that phosphorylates the D3 position of inositol lipids to form the products PI-3-P, PI-3,4-P2, and PI-3,4,5-P3 in response to many different stimuli. Upon generation, these second messengers bind and recruit signaling molecules to the plasma membrane to interact with other regulatory and effector molecules [195]. The  $\alpha6\beta4$ -dependent activation of PI3K is likely to be through an indirect manner because the lack of a p85 (PI3K regulatory subunit) binding motif in the  $\beta4$  cytoplasmic tail [73]. Several indirect molecular mechanisms have been proposed including the involvement of Insulin Receptor Substrate proteins (IRS1 and IRS2), the localization of  $\alpha6\beta4$  into lipid rafts and association of  $\alpha6\beta4$  with specific growth factor receptors which are known to activate PI3K [87, 111]. A recent study has shown that the  $\alpha6\beta4$  integrin

regulates ErbB3 expression at the translational level. The increased ErbB2/ErbB3 heterodimer formation stimulates the PI3K/AKT signaling axis to promote tumor survival [191]. In contrast, although it has been known that SFKs are involved in  $\alpha6\beta4$  signaling events for some time, less is known about how SFKs are activated by  $\alpha6\beta4$  at the molecular level. Y1494 in the  $\beta4$  cytoplasmic tail plays essential roles in  $\alpha6\beta4$  tumorigenesis functions. Mutation of this tyrosine residue to phenylalanine inhibited the  $\alpha6\beta4$ -dependent activation of PI3K, as well as tumor invasion and survival [111]. The importance of Y1494 in the  $\beta4$  cytoplasmic domain has also been demonstrated by the fact that mutation of Y1494 significantly decreased the overall tyrosine phosphorylation level in the  $\beta4$  subunit upon  $\alpha6\beta4$  engagement implying that Y1494 might function as a master tyrosine residue in  $\alpha6\beta4$  signaling events [111]. The importance of Y1494 has also been highlighted by its involvement in binding a crucial cytosolic tyrosine phosphatase SHP2 [112, 162-163].

In the current study, I identified a novel molecular mechanism by which SHP2 mediates the selective activation of a Src Family Kinase, Fyn, in response to  $\alpha6\beta4$  integrin engagement and demonstrated the importance of this  $\alpha6\beta4$ /SHP2/Fyn signaling pathway for carcinoma invasion. Upon engagement of the  $\alpha6\beta4$  integrin, Y1494 and Y1440 in the  $\beta4$  subunit cytoplasmic domain interact with the N-SH2 and C-SH2 domains of SHP2, respectively. The Y1494 and N-SH2 interaction stimulates the catalytic activity of SHP2. Constitutively phosphorylated Y580 in the C-terminus of SHP2 is required for recruitment of Fyn, but not Src, to the  $\alpha6\beta4$ /SHP2 signaling

complex, and this interaction is necessary for the activation of Fyn downstream of  $\alpha 6\beta 4$ . Moreover, activated Fyn phosphorylates SHP2 on Y542 in a positive feedback manner. Palmitoylation modification on the N-terminus of Fyn, but not Src, localizes Fyn to the same cell surface microdomain as  $\alpha 6\beta 4$  and is responsible for the selective activation of Fyn instead of other SFKs. The intact  $\alpha 6\beta 4$ -SHP2-Fyn signaling axis is required for  $\alpha 6\beta 4$ -dependent breast carcinoma invasion since blocking any step results in diminished cancer cell invasive abilities (Fig. 4-1). More importantly, my *in vivo* data suggest that pY580-SHP2 and pY418-SFK levels are increased in tumors that express the  $\alpha 6\beta 4$  integrin, and this activation is dependent upon Y1494. Also in a subset of human breast tumor samples, pY580-SHP2 and pY418-SFK levels positively correlate with tumor grades suggesting that both of these markers may be potential indicators of breast cancer invasive potential.

Six tyrosine residues (Y1257, Y1422, Y1440, Y1494, Y1526 and Y1642) in the  $\beta 4$  cytoplasmic tail have been identified to participate in signaling [109-110, 163]. SFKs, such as Fyn and Yes, or other receptor tyrosine kinases, phosphorylate five major tyrosine residues (Y1422, Y1440, Y1494, Y1526 and Y1642) in the  $\beta 4$  signaling domain [87, 111, 193]. The phosphorylated tyrosine residues mediate recruitment and activation of downstream signaling events. For example, phospho-Y1526 mediates recruitment of Shc and activation of Ras-ERK signaling [193]. Y1257 and Y1494 have been identified to be located within canonical SHP2 binding ITIM motifs [111]. Y1440 is embedded within a degenerate consensus SHP2 binding motif [163]. My GST pull-down data

suggests that Y1494 and Y1440 in the  $\beta 4$  subunit interact with N-SH2 and C-SH2 of SHP2, respectively, whereas Y1257 plays a minimal role, if any, in  $\beta 4$ -SHP2 interaction (Fig. 2-5, B). Y1440F mutant  $\beta 4$  loses not only interaction with C-SH2, but also N-SH2 (Fig. 2-5, B, lowest panel). This could be explained by the fact that Y1494 phosphorylation and interaction with N-SH2-SHP2 requires intact Y1440 (Fig. 2-5, C). A two-step phosphorylation event might be the case. Y1440 may first get phosphorylated in response to  $\alpha 6\beta 4$  ligation and then some conformational changes happen in the  $\beta 4$  subunit to expose Y1494 for further phosphorylation, which may or may not be by the same tyrosine kinase as phosphorylates Y1440. This two step phosphorylation is supported by the mechanism by which SHP2 is recruited and activated. SHP2 uses its C-SH2 to survey the cell for a bisphosphorylated binding ligand. Upon interaction between C-SH2 and p-Tyr, the increase of the local concentration of p-Tyr will release SHP2 auto-inhibition between its N-SH2 and PTP domain [114]. Therefore, Y1440F  $\beta 4$  can't recruit SHP2 to the  $\alpha 6\beta 4$  integrin in the first place as well as a loss in phosphorylation in Y1494. This hypothesis has been further supported by co-immunoprecipitation assays in response to laminin ligation. Loss of either intact Y1440 or Y1494 impairs the stable interaction between  $\beta 4$  and SHP2 (Fig. 2-6, B). Moreover, the Y1494F mutant  $\beta 4$  can't activate SHP2 phosphatase activity (Fig. 2-2, A), suggesting that upon recruitment to phospho-Y1440 by its C-SH2, SHP2 still needs to engage its N-SH2 with phospho-Y1494 to disrupt its intramolecular interaction and get activated. To further confirm this model, SHP2 *in vitro* phosphatase assays should be performed using Y1440F cells. No significant increase of SHP2 phosphatase activity should be expected in these cells.

Furthermore, if a phospho-Y1440 antibody becomes commercially available, western blotting can be performed to show that Y1440 indeed gets phosphorylated in response to  $\alpha 6\beta 4$  engagement as does Y1494 (Fig. 2-4, A,B,C). In response to HGF stimulation, the Trusolino group has reported that Y1257, Y1440 and Y1494 are necessary for efficient  $\beta 4$  and SHP2 coimmunoprecipitation, with Y1440 being the major binding site for SHP2 [163]. These data confirm the importance of Y1494 and Y1440 in the  $\beta 4$ /SHP2 physical interaction. However, in this paper they didn't explain how three tyrosine residues interact with two SH2 domains in SHP2. The difference between my data and theirs may be explained by the distinct cell system and different stimulation to the cells. The insignificance of Y1257 in  $\alpha 6\beta 4$ -mediated signaling pathways is further confirmed by the fact that mutation of this tyrosine residue didn't affect  $\alpha 6\beta 4$  dependent breast cancer cell invasion or total cellular tyrosine phosphorylation levels [111].

Intact Y1440 and Y1494 in the  $\beta 4$  subunit are not only essential for stable  $\beta 4$ -SHP2 interaction and  $\alpha 6\beta 4$ -dependent signaling events (Fig. 2-6, A,B,C; Fig. 3-3, B), but also for  $\alpha 6\beta 4$ -dependent invasion (Fig. 2-6, D). In response to laminin clustering and antibody-mediated  $\alpha 6\beta 4$  ligation, the activation level of Fyn was significantly reduced in both Y1494F- $\beta 4$  and Y1440F- $\beta 4$  transfected cells. In fact, the reduction of Fyn activation in Y1440F cells is even higher than that in Y1494F cells. However, 2-D invasion assays showed that the invasive ability of Y1440F- $\beta 4$  mutant cells is significantly higher than that of both Y1494F and Y1494/1257F- $\beta 4$  cells (1440 vs 1494:  $P < 0.006$ ; 1440 vs 1494/1257:  $P < 0.003$ ). In our earlier studies, we found that in contrast to Y1494F- $\beta 4$

mutant cells, which are deficient in activating PI3K/AKT signaling pathway, Y1440F cells maintain PI3K activation to some extent compared to WT- $\beta$ 4 cells in response to  $\alpha$ 6 $\beta$ 4 stimulation (data not shown), which could explain their higher invasive potential. Singly mutating Y1494 affects  $\alpha$ 6 $\beta$ 4-dependent invasion more than Y1440 does, suggesting that Y1494 is the most important tyrosine residue in the  $\beta$ 4 subunit to promote cell invasion. This notion is substantiated by 3-D invasion assays showing that Y1494F- $\beta$ 4 cells form round, very compact colonies when embedded in Matrigel, whereas Y1440F cells form roundish colonies, however the edges of those colonies are not as smooth as those formed by Y1494F cells (Fig 4-2) [76]. It has been reported that phospho-Y1440 and phospho-Y1442 are essential in SH2-mediated interaction with Shc to activate the MAPK signaling pathway. In this study, phospho-Y1440 was identified to be the primary binding motif for Shc [193]. This result implies that besides involvement in activating Fyn to regulate cell invasion, Y1440 might be important in regulating other cell signaling events, such as proliferation and cell cycle progression.

Y542 in the SHP2 C-terminal tail is a substrate of Fyn, which was demonstrated by the reduction of phospho-Y542 in  $\beta$ 4 mutant transfected cells (Fig. 3-3, B), by both SHP2 and SFK chemical inhibitors and Fyn siRNA treated cells (Fig. 3-2; Fig. 3-7, A), as well as in DN-Fyn stably transfected cells (Fig. 3-7, B). Phospho-Y542 and phospho-Y580 have been reported to function as adaptors to recruit SH2 domain containing molecules, such as Grb2 and SHIP [196-201]. I examined the potential interactions between SHP2 and either Grb2 or SHIP2 by coimmunoprecipitation. No physical

interaction was detected in the coIP assay (data not shown). However, these results don't exclude the possibility that phospho-Y542 may function as an adaptor to recruit other unknown SH2 domain containing signaling molecules. Upon phosphorylation, Y542 in the SHP2 C-terminal tail has also been reported to regulate SHP2 phosphatase activity. Using non-hydrolysable phospho-tyrosine mimics to substitute both Y542 and Y580 in the SHP2 C-terminal tail, studies have shown that the non-hydrolysable tyrosine mimics stimulated SHP2 phosphatase activity through a mechanism involving phospho-Y542/N-SH2 and phospho-Y580/C-SH2 intramolecular interactions. They also showed that a single Y542 substitution is enough to stimulate SHP2 activity and activate MAPK signaling in living cells [119]. Another group reported a similar result that non-hydrolysable phospho-Y536 (Y542-SHP2 equivalent) and phospho-Y564 (Y580-SHP2 equivalent) in the SHP1 C-terminal tail stimulates SHP1 phosphatase activity with Y536 being more potent than Y564 [202]. In my study, *in vitro* phosphatase assays were performed to determine if tyrosine residues in the SHP2 C-terminal tail play a role in regulating  $\alpha\beta\gamma\delta$ -dependent SHP2 phosphatase activity. Mutation of Y542 and Y580 individually reduced the phosphatase activity of SHP2, implying that both Y542 and Y580 are involved in regulating SHP2 phosphatase activity. However, the activity of the double Y542F/Y580F mutant was equivalent to that observed for WT-SHP2 (Fig. 3-5). This finding mimics a previous report that deletion of the SHP1 C-terminal tail activates the catalytic activity of the phosphatase [172]. Moreover, a possible explanation for the maintenance of high phosphatase activity in the double Y542F/Y580F mutant SHP2 may involve the phosphorylation of serine or threonine residues in the SHP2 C-terminal tail.

Phosphorylation on both serine and threonine residues have been detected and the phosphorylation levels increase in response to EGF stimulation in PC12 phaeochromocytoma cells [203]. Another group has shown that upon phosphorylation on threonine residues, but not on serine residues, SHP2 phosphatase activity decreased [204]. This raises the possibility that the double Y542F/Y580F mutant SHP2 may have lost its phosphorylation on serine and/or threonine residues, which keep SHP2 phosphatase activity in check upon phosphorylation.

Upon recruitment to the  $\alpha 6\beta 4$  integrin, SHP2 phosphatase activity is stimulated (Fig. 2-2). However, SHP2 phosphatase activity is not directly involved in the activation of Fyn in response to  $\alpha 6\beta 4$  ligation because the double Y542F/Y580F mutant which retains almost the same level of phosphatase activity compared to WT-SHP2 was unable to activate Fyn (Fig. 3-4, D, E; Fig.3-5). Similarly, mutation of Y542 significantly diminished SHP2 phosphatase activity but didn't reduce Fyn activation by  $\alpha 6\beta 4$  (Fig. 3-4, D, E; Fig.3-5). Moreover, although Y542-SHP2 mutant cells maintained a high level of Fyn activation, they showed a low SHP2 phosphatase activity and defective invasion suggesting that additional SHP2 substrates are likely to cooperate with Fyn to enhance  $\alpha 6\beta 4$ -dependent invasion (Fig. 3-4, D, E; Fig.3-5; Fig. 3-9). Therefore, both SHP2 catalytic and non-catalytic functions play a role in  $\alpha 6\beta 4$  signaling pathways. This notion is supported by the fact that SHP2 functions in both catalytic-dependent and independent manners in interleukin-3 stimulated hematopoietic cells [136]. One potential SHP2 substrate that may be important for invasion is the Rho GTPase [73, 166]. To discover

SHP2 substrate/substrates in response to  $\alpha 6\beta 4$  engagement, both HA-tagged WT-SHP2 and substrate trapping SHP2 (C459S-SHP2) can be expressed and assayed for their ability to pull down substrates. The substrate trapping SHP2 should interact with, but not dephosphorylate, its substrates in response to  $\alpha 6\beta 4$  engagement, and therefore maintain interactions when compared with WT-SHP2. SHP2 substrates can then be identified by Mass-spectrometry. If the C459S-SHP2 doesn't trap its substrate, more substrate-trapping SHP2 mutants have been reported that might be more potent candidates, such as T466A-SHP2 and D425A/Q506A-SHP2 [166, 205].

Two mechanisms by which SHP2 activates SFKs have been reported. One mechanism involves SHP2 directly or indirectly dephosphorylating SFKs on their negative regulatory residue Y529 (human Src as an example). SHP2 phosphatase activity is required in this mechanism. A second mechanism involves SHP2 activating Src independently of its catalytic function by binding directly to the SH3 domain of Src and disrupting the intramolecular inhibitory interaction that interferes with the catalytic domain [135]. In my study, no decrease on phospho-Y528 Fyn was detected by Western Blotting (data not shown). This negative result could be due to the fact that only a small pool of Fyn is dephosphorylated and this decrease is not strong enough to show on Western Blotting. To test this hypothesis, coimmunoprecipitation using SHP2 Antibodies can be performed. Phospho-Y529-Src antibody would be used to detect any phosphorylation change in this SFK inhibitory tyrosine site.

My data suggests that intact Y580 in the SHP2 C-terminal tail is essential for the  $\alpha6\beta4$ -dependent activation of Fyn (Fig. 3-6, F). The potential mechanism by which SHP2 activates Fyn is that phospho-Y580 of SHP2 is used to interact with the SH2 domain of Fyn. This interaction releases the intramolecular inhibitory interaction between the SH2 domain of Fyn and phospho-Y527 and activates Fyn. However, it is also possible that SHP2 doesn't directly interact with Fyn. There may be intermediate molecule/molecules between SHP2 and Fyn and phospho-Y580 is actually required for recruiting these intermediate molecules. To test this hypothesis, GST tagged Fyn could be generated *in vitro* and a GST pull-down assay could be performed using  $\alpha6\beta4$  stimulated cell lysates. If GST-Fyn pulls down SHP2, a direct interaction between SHP2 and Fyn could be further confirmed by using a more definitive technique-FRET (Fluorescence Resonance Energy Transfer). The strength of FRET microscopy is that it shows whether two proteins interact with each other directly in response to certain stimulation in live cells. If Fyn and SHP2 don't interact directly, a whole SHP2 coimmunoprecipitation sample could be sent for Mass-Spectrometry analysis followed by protein domain analysis (using website, such as <http://scansite.mit.edu/>) to find a potential protein which might interact with SHP2 in a phospho-Y580 dependent manner.

The  $\alpha6\beta4$  integrin promotes breast cancer invasion through combined activation of PI3K and Fyn [43, 73, 76, 162]. Both Src and Fyn have been reported to be involved in tumor migration and invasion. The selective activation of Fyn in response to  $\alpha6\beta4$  ligation is through differential N-terminal lipid modification of the SFKs and differential cell surface microdomain localization. The importance of this localization is underscored

by the fact that Src is not palmitoylated, and therefore does not localize to the membrane domains containing both  $\alpha6\beta4$  and Fyn, and it is not activated by this integrin receptor [178]. My data confirms this hypothesis by demonstrating that in palmitoylation deficient Fyn overexpressing cells, not only the signaling events induced by ligating  $\alpha6\beta4$  integrin were blocked, but also the cells' invasive ability (Fig. 3-8, 3-10). All of the SFKs are highly homologous to each other and share the same structure and same mechanism of activation [139]. To further confirm that differential lipid modification on the N-terminus is responsible for  $\alpha6\beta4$ -dependent selectivity of Fyn activation, a mutant Src can be generated that is palmitoylated on its N-terminus. This mutant Src would be expected to be able to be recruited to the  $\beta4$  signaling complex as we have observed for Fyn. Another strategy to test this hypothesis would be to perform an N-terminus domain-swapping experiment. A chimeric protein with the Fyn N-terminus and Src body and C-terminal tail should be expected to be recruited to the  $\alpha6\beta4$ /SHP2 signaling complex in response to  $\alpha6\beta4$  engagement. Similar experiments have been carried out by the Giancotti lab showing that in 293T cells,  $\alpha6\beta4$  coimmunoprecipitates with Fyn, but not Src. In contrast, mutation of the two cysteine residues required for palmitoylation (Fyn-C3, 6S) reduced the association of Fyn with  $\alpha6\beta4$  significantly. Moreover, when a chimeric protein comprising the N-terminus of Fyn with Src (Fyn1-13/ Src14-533) were expressed, the Fyn-Src chimera interaction with  $\alpha6\beta4$  increased greatly compared to Src [206]. Although these data were generated in 293T fibroblasts, which do not normally express  $\alpha6\beta4$  integrin endogenously, the data support the hypothesis that palmitoylation on the

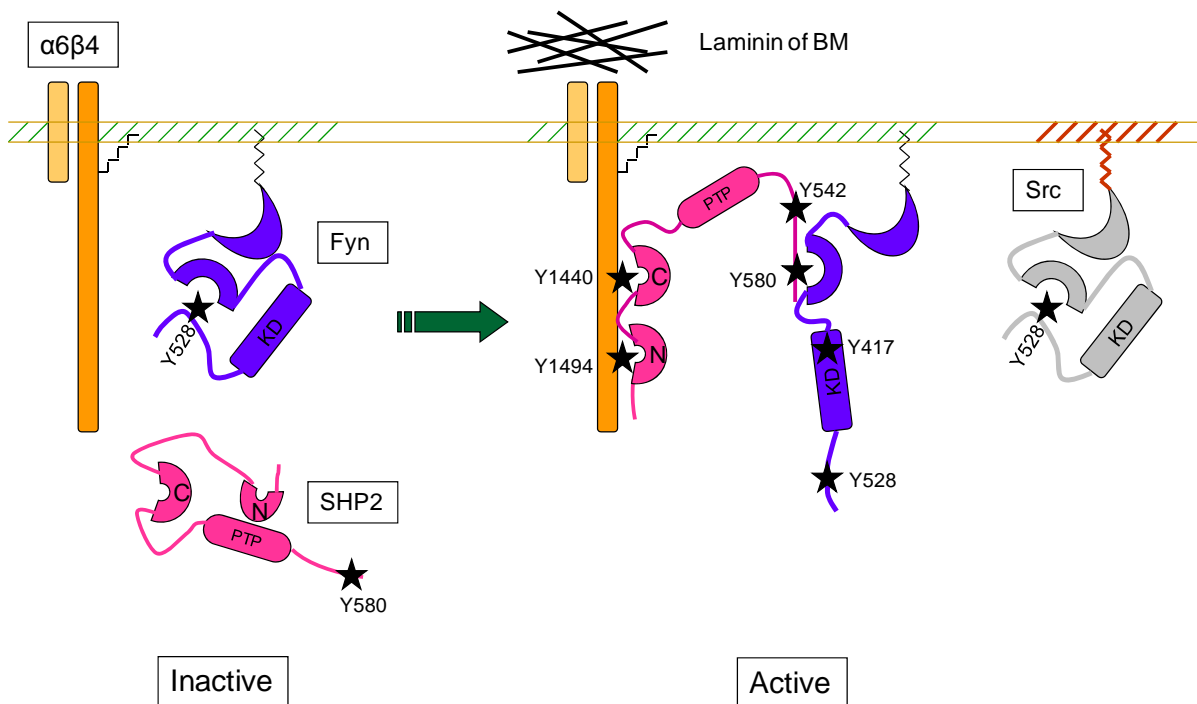
Fyn N-terminus determines its colocalization with  $\alpha6\beta4$  integrin and its selective activation. In the same 293T system, they also reported that only the membrane proximal portion of the cytoplasmic domain of  $\beta4$  (aa854–aa1183) is required for association with Fyn [206]. This is not what I have observed in my study. Firstly, in epithelial derived breast carcinoma cells, which endogenously express the  $\alpha6\beta4$  integrin, SHP2 functions to recruit Fyn to  $\alpha6\beta4$  (Fig. 2-1). Secondly, mutation of Y1440 and Y1494 in the  $\beta4$  subunit diminishes the activation of Fyn suggesting that the membrane proximal portion of the cytoplasmic domain of  $\beta4$  is not the only region essential in binding/activating Fyn (Fig 2-6, B, C).

To specify the exact contributions of distinct SFKs has been extremely difficult because all of the commercially available p-Y418 Src antibodies crossreact with at least activated Fyn and Yes. Some researchers even believe that these antibodies crossreact with all active SFKs [138]. This is the reason that some earlier work by our lab and other groups reported that Src is activated by the  $\alpha6\beta4$  integrin to promote cancer invasion [76, 162]. When both constitutively active PI3K and Src are expressed in the Y1494F- $\beta4$  mutant transfected cells, the invasive ability of these cells increased significantly [76]. This result is consistent with the fact that both Src and Fyn have a pro-invasion capacity [138, 140]. Treating WT- $\beta4$  expressing cells with both the PI3K inhibitor Ly294002, and the SFK inhibitor PP2, greatly reduced the cells' invasive ability. PP2 is known to inhibit almost all SFKs including Src and Fyn [76]. Therefore, the  $\alpha6\beta4$ -dependent breast cancer invasion is through combined activation of PI3K and Fyn [43].

Both the  $\alpha6\beta4$  integrin and Fyn have been reported to be palmitoylate-modified on their N-termini [52, 87, 178]. The palmitoylated  $\alpha6\beta4$  integrin localization in cell surface microdomains has been under debate. Some researchers believe that palmitoylation modification is required for localizing  $\alpha6\beta4$  in lipid rafts where other palmitoylated signaling proteins, such as some G proteins, H-Ras and SFKs are concentrated [87]. Other researchers argue that the palmitoylated  $\alpha6\beta4$  doesn't associate with lipid rafts at all, instead it incorporates into Tetraspanin enriched microdomains (TEMs) [52]. In either case, it seems that palmitoylation modification is essential for the  $\alpha6\beta4$  integrin to be relocalized on the cell surface with its targets to function properly. This has been substantiated by my data showing that palmitoylation deficient Fyn transfected cells lost not only their signaling function, but also their ability to promote cancer cell invasion in response to  $\alpha6\beta4$  engagement (Fig. 3-8; Fig. 3-10). Since Fyn can be both myristoylated and palmitoylated on its N-terminus, the palmitoylation deficient Fyn used in my study should still be recruited to the cell surface, but not into the same microdomain as  $\alpha6\beta4$ . This underscores the importance of being localized in close vicinity for them to function properly [178-180].

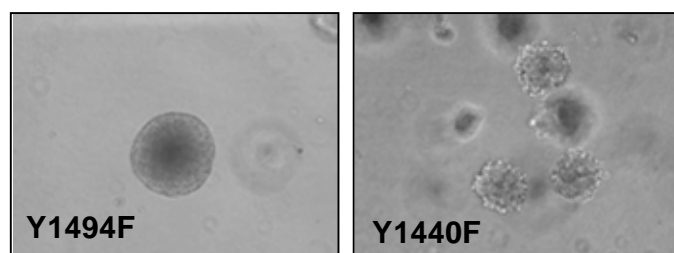
The cell surface laminin receptor  $\alpha6\beta4$ , the cytosolic tyrosine phosphatase SHP2 and the tyrosine kinase Fyn have all been implicated in tumor initiation and progression. Each of them makes an attractive cancer therapy target [38, 68, 110, 114, 120, 138]. In my current study, I have identified a novel mechanism for the SHP2-dependent activation of Fyn by  $\alpha6\beta4$  ligation. Blocking each step of the  $\alpha6\beta4$ -SHP2-Fyn signaling axis inhibited the  $\alpha6\beta4$ -dependent tumor invasion (Fig. 2-6, D; Fig. 3-9; Fig.3-10). This

implies that further studies on antibody mediated or chemical mediated blocking of this signaling pathway to inhibit cancer cell invasion are greatly needed. Of clinical relevance, phospho-Y580-SHP2 and phospho-Y418-SFKs are potential biomarkers of invasive breast cancer because their expression is elevated in high-grade breast tumors (Fig. 3-11, C, D).



**Figure 4-1. Schematic of  $\alpha 6\beta 4$ -SHP2-Fyn signaling**

In the “Inactive” state, the  $\alpha 6\beta 4$  integrin and Fyn are localized in proximity in the membrane through their palmitoylation. However, neither Fyn nor SHP2 are active because of intramolecular inhibitory interactions. Upon engagement of the  $\alpha 6\beta 4$  integrin with its ligand laminin (“Active” state), the  $\beta 4$  cytoplasmic domain is phosphorylated, which recruits SHP2 through an interaction of its C-SH2 domain with pY1440 and activates SHP2 catalytic activity through the interaction of its N-SH2 domain with pY1494. Fyn is recruited to the complex and activated upon binding of its SH2 domain to pY580 in the SHP2 C-terminal domain. Although SHP2 phosphatase activity is required for Fyn activation, the specific SHP2 substrates involved are unknown. The localized activation of this  $\alpha 6\beta 4$ /SHP2/FYN signaling pathway promotes carcinoma invasion. The selective activation of Fyn over Src is due to colocalization of palmitoylated  $\alpha 6\beta 4$  integrin and Fyn. Black star symbol, phosphorylation event.



**Figure 4-2. 3D invasion assay of  $\beta 4$  mutant cells**

Representative images captured at 20X magnification of MDA-MB-435 cells transfected with the indicated  $\beta 4$  mutant constructs for 10 days in 3D Matrigel culture.

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