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Dynamic Src Tyrosine Kinase Signaling Directs Invadopodia Formation and Function in Head and Neck Cancer: Novel Insights into the Original Oncogene

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**Dynamic Src Tyrosine Kinase Signaling Directs Invadopodia
Formation and Function in Head and Neck Cancer: Novel Insights into
the Original Oncogene**

Laura Catherine Kelley

Dissertation Submitted to the School of Medicine at West Virginia University
In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Cancer Cell Biology

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Abstract

Dynamic Src Tyrosine Kinase Signaling Directs Invadopodia Formation and Function in Head and Neck Cancer: Novel Insights into the Original Oncogene

Laura Catherine Kelley

Cancer cell invasion and motility is mediated through actin-rich protrusions that facilitate migration through extracellular matrix and degradation of tissue barriers. Src tyrosine kinase is an essential catalyst of motile actin networks and is a potent mediator of the metastatic program. The Src substrate cortactin is a critical scaffold that links kinase signaling to cytoskeletal dynamics. Both Src and cortactin are overexpressed in several human cancers and their expression is associated with poor prognosis. These proteins are not associated with the initiation of tumorigenesis, but are thought to play a vital role in the metastatic process. The overall aim of my work is to determine the molecular mechanisms by which Src and cortactin promote the invasive cancer phenotype. Further understanding of these processes provided by the following studies, could provide clinical benefits for cancer patients with advanced disease. Study one demonstrates treatment with the Src-targeted small molecule inhibitor saracatinib impairs tumor cell invasion *in vitro* and lymph node metastasis *in vivo*. Further, we identify that Src inhibition decreased invadopodia formation and MMP expression in HNSCC cell lines. Study two identifies regulated WT Src activity as essential for governing invadopodia maturation in HNSCC cells and Src transformed fibroblasts. In addition, we establish cortactin phosphorylation downstream of Src as central to this process. In study three we examine the role of the EGFR/MEK pathway upstream of Src and cortactin in regulating cell migration and lamellipodia dynamics. Lastly, study four outlines an attempt to create a transgenic model of HNSCC tumor cell invasion in which cortactin is overexpressed in the oral cavity of tumorigenic mice.

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In 2004 I moved from California to West Virginia to be a scientist. I found a career which harnessed my ultra-rational way of thinking and my love of problem solving with something that actually matters- understanding human disease. I am extremely grateful for my training in Cancer Cell Biology at WVU and I could not have achieved the work contained in this document without the help of many extraordinary individuals.

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Glossary

A	alanine
Å	angstrom
ABP	actin binding protein
ADP	adenosine diphosphate
AFAP110	actin filament-associated protein of 110 kDa
Arp2/3	actin related protein 2/3
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
C	Celcius
CAS	Crk-associated substrate
CEF	chicken embryo fibroblast
Cer	cerulean fluorescent protein
CMV	cytomegalovirus promoter
CTTN	human cortactin gene
Cttn	murine cortactin gene
Csk	C-terminal Src kinase
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DRF	Diaphanous-related formin
ECIS	Electric Cell-Substrate Impedance Sensing
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
ERK1/2	extracellular signal regulated kinase 1/2
F	filamentous
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
G	globular
GFP	green fluorescent protein
GTP	guanosine triphosphate
HGF	hepatocyte growth factor
HNSCC	head and neck squamous cell carcinoma
IHC	immunohistochemistry
kDa	kiloDalton
MAPK	mitogen-activated protein kinase
mCh	mCherry fluorescent protein
MEF	mouse embryonic fibroblast
MEK1/2	MAP kinase kinase 1/2
MMP	matrix metalloprotease

MT1-MMP	matrix bound matrix metalloprotease 1
NPF	nucleation-promoting factor
NTA	N-terminal acidic domain
Pak	p21-activated protein kinase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PI3K	phosphoinositide-3 kinase
PIP2	phosphatidylinositol bisphosphate
PIP3	phosphatidylinositol 3, 4, 5-triphosphate
PKC	protein kinase C
PLL	poly-L-lysine
PTB	phosphotyrosine binding domain
PTP1B	protein tyrosine phosphatase 1B
PRR	proline-rich region
Pyk2	proline-rich tyrosine kinase 2
R	Arginine
RhoA	Ras homolog gene family, member A
RIPA	radioimmunoprecipitation assay
RNAi	RNA interference
RSV	Rous sarcoma virus
RTK	receptor tyrosine kinase
SFK	Src family kinases
SH2	Src homology 2
SH3	Src homology 3
SHP-2	Src homology 2-containing tyrosine phosphatase
SiRNA	small interfering ribonucleic acid
SNARE	Soluble NSF Attachment Protein Receptors
S	serine
SYF	Src ^{-/-} Yes ^{-/-} Fyn ^{-/-}
SYF ^{+/+}	Src ^{+/+} Yes ^{-/-} Fyn ^{-/-}
Tg	transgenic
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
Trp53	transformation related protein 53 gene
tsLa29	temperature-sensitive viral Src clone 29
VASP	vasodilator-stimulated phosphoprotein
VEGF	vascular endothelial growth factor
W	tryptophan
WASp	Wiscott Aldrich Syndrome protein
WAVE	WASp family Verprolin-homologous protein
WH2	WASp homology domain
WIP	WASp interacting protein
WT	wild-type
Y	tyrosine

Literature Review

Introduction and significance: Cancer cell invasion and metastasis

Cancer is a group of diseases that arises when normal cells undergo a series of genetic modifications that lead to uncontrolled growth. Malignant transformation requires the acquisition of multiple mutations that lead to the production of oncogenes that actively promote tumorigenesis, or the loss of tumor suppressor genes that restrain tumorigenesis (Weinberg, 1994). There are presumably hundreds of combinations of genetic mutations that support uncontrolled growth. To this end, a multitude of cancers exist which are classified by tissue of origin, and subsequently by the genetic or epigenetic abnormalities they possess. Nevertheless, it is generally accepted that for any tumor type to become malignant the cell must gain several distinct properties. These include the ability to promote uncontrolled growth, evade apoptosis, sustain angiogenesis, continuously replicate, and be able to invade local tissues in order to eventually metastasize to distant sites (Hanahan and Weinberg, 2000). This dissertation focuses on two proteins that are overexpressed in invasive tumor types; Src tyrosine kinase and the adapter protein cortactin. How these proteins contribute to dynamic, motile protrusions that propel the invasive/metastatic program of epithelial derived tumors (carcinomas) is discussed.

The majority of cancer deaths result from metastasis of the primary tumor (Steeg, 2006). Metastasis- the process by which malignant cells spread from the tissue of origin to colonize local and/or distant organs- requires multiple discrete steps (Nguyen et al., 2009). First, the primary tumor progresses into an invasive carcinoma, priming it to

respond to cues from the surrounding microenvironment, whereby it takes on a migratory or invasive phenotype and locally invades the surrounding tissue. Tumor cells then reach and invade the dense vascular-endothelial basement membrane to enter the circulation, a process termed intravasation. In the blood stream or lymphatic system, cells are required to survive anchorage independent growth (anoikis) in the circulation until the cells arrest and extravasate at the metastatic location. During extravasation, cells again invade through basement membranes and the surrounding tissue to seed and colonize distant sites. Tumor cell invasion, a critical step at multiple points during metastasis as outlined above, is a process which is dependent on temporal and spatial rearrangement of the actin cytoskeleton (Yamaguchi et al., 2005b). Understanding the function of proteins involved in invasion is prerequisite for the development of anti-invasive therapies that have the potential to directly improve patient outcome. To this end, it is critical to gain understanding of the molecular mechanisms that drive tumor cell invasion and metastasis.

Tumor cell invasion is facilitated through highly regulated changes in plasma membrane dynamics, resulting from reorganization of the underlying actin cytoskeleton (Yamaguchi et al., 2006). The ATP-binding protein actin is one of the most abundant proteins in eukaryotic cells. Spatial and temporal dynamic regulation of globular (G) or monomeric actin into filamentous (F) actin provides the structural network and the protrusive forces that govern cell motility and invasion (Campellone and Welch, 2010). Actin filaments possess a fast growing barbed (+) end and a slower growing pointed (-) end that have structurally and biochemically distinct properties. Construction of a new actin filament is facilitated through the generation of a free barbed end, termed actin

nucleation. ATP bound actin monomers are then added to the + end and the dynamics of the growing filament are tightly coupled to ATP-hydrolysis. Regulation of this highly specialized cellular process is facilitated by an abundance of actin-binding (ABP) proteins that direct actin polymerization/depolymerization necessary for functional cell movement. There are several classes of ABPs that bind to G- or F-actin to control actin filament dynamics through numerous biochemical processes, including nucleation, capping, severing, crosslinking, bundling, anchoring, depolymerizing, sequestering, branch formation, monomer delivery, nucleotide exchange, cytoskeletal linkers, sidebinding, and signaling (Winder and Ayscough, 2005). Not surprisingly, several actin-associated regulatory proteins are overexpressed or dysregulated in tumor cells, altering actin dynamics in a manner conducive to driving the invasive cancer phenotype (Iwaya et al., 2007; Kelley et al., 2008; Otsubo et al., 2004; Semba et al., 2006; Wang et al., 2004; Wang et al., 2005).

Figure 1 Model depicting the tumor cell invasion mediated by invadopodia. Carcinoma cells gain an invasive phenotype and actively degrade the underlying basement membrane. Once the cells are detached from the primary tumor, cancer cells migrate along/through the ECM. Tumor cells must then invade the blood vessel wall in order to enter the circulatory system (intravasation) where they eventually arrest and extravasate before metastatic colonization at the secondary site. Illustration adapted from Yamaguchi et al., 2005b.

Actin-based specialized motility structures formed by cancer cells include lamellipodia, filopodia, podosomes and invadopodia (Buccione et al., 2004). These differing types of membrane protrusions are developed in response to a variety of extracellular chemoattractants including ECM components, growth factors and chemokines. Upon binding of chemoattractants to extracellular cell surface receptors, intracellular signaling pathways are triggered leading to specific, highly organized changes in cell shape that are regulated by Rho family GTPases (Ridley, 2001a; Ridley, 2001b; Ridley, 2004; Ridley, 2006). Actin nucleation downstream of these signals is facilitated through activation of the actin related protein (Arp)2/3 complex, which results

in the formation of *de novo* branched actin networks (Higgs and Pollard, 2001; Pollard and Beltzner, 2002). The activation of Arp2/3 complex is regulated by actin nucleation promoting factors (NPFs) including the WASp (Wilskott-Aldrich syndrome protein) family proteins and cortactin (Urano et al., 2003; Weaver et al., 2001; Weed et al., 2000). Recently, additional actin nucleators including diaphanous-related formins (DRFs), WH2 containing proteins (spire, cordon-bleu, and leimodin), and two additional WCA containing NPFs (WHAMM and JMY) were discovered that facilitate elongated, unbranched actin polymerization necessary in extended membrane protrusive structures such as filipodia and invadopodia (Campellone and Welch, 2010).

The type of protrusion formed by a motile cancer cell is highly dependent on the selective pressure of the environment (Wolf and Friedl, 2009; Yamaguchi and Condeelis, 2007). In 2D environments, such as cells plated on glass coverslips or coverslips coated with thin ECM such as fibronectin, tumor cells form sheet-like projections called lamellipodia and filopodia at the leading edge. Cancer cells plated on thick ECM, such as collagen and Matrigel substrates, form rod-shaped, elongated cell protrusions perpendicular to the cell body termed invadopodia. Invadopodia contain proteolytic activities that facilitate clearing of the ECM around the protrusions. Additionally, invadopodia produced by cancer cells also facilitate breaching of live basement membrane explants and invadopodia-like protrusions have been imaged *in vivo* (Condeelis and Segall, 2003; Hotary et al., 2002; Yamaguchi et al., 2005b). Therefore, these specialized structures are thought to be the critical machinery that allows cancer cells to migrate through ECM and to cross tissue barriers during multiple steps during the metastatic process *in vivo* (see Figure 1).

Invadopodia: Invasion machinery of cancer cells

Invadopodia are actin-rich ventral membrane protrusions found in oncogenic kinase-transformed fibroblasts and metastatic cancer cell lines (Ayala et al., 2006; Buccione et al., 2004; Weaver, 2006; Yamaguchi et al., 2006). Invadopodia are most similar in structure to podosomes, which are produced in untransformed, non-metastatic cell lineages such as endothelial, smooth muscle and monocytic lines (osteoclasts, macrophages, monocytes, and dendritic cells)(Linder, 2009). Invadopodia and podosomes share similar protein components, but differ in their number, size and dynamics. Invadopodia are present in smaller numbers (<10 versus >100 per cell), but they are much larger (~8, compared to 1 μ M) and more persistent than podosomes (lasting hours as opposed to minutes).

There is no unique biochemical marker for invadopodia. *In vitro*, invadopodia are identified by plating cells on top of fluorescently labeled ECM, usually collagen (gelatin), where invadopodia protein components colocalize with areas of degraded matrix (Figure 2). In three-dimensional matrices, cancer cells develop numerous lateral spikes that are enriched with invadopodia markers including the matrix metalloproteinase MT1-MMP (Li et al., 2010; Wolf and Friedl, 2009). *In vivo* imaging of invadopodia is extremely difficult due to dynamic nature of the process and the inaccessible tissue environment in which they occur. However, intravital imaging of GFP-labeled tumor cells demonstrated “invadopodia-like” cell protrusions extending into the vasculature during intravasation (Wyckoff et al., 2004; Yamaguchi et al., 2005b). Until the imaging technology advances to the point enabling identification of

invadopodia markers at high resolution during the metastatic process, the question of whether invadopodia form, or what form they take *in vivo* remains unanswered. Recently, membrane protrusions highly resembling invadopodia structure and function were identified and imaged in *C. elegans*. In this system, cells that cross basement membranes during normal worm development use invadopodia-like structures to degrade and invade surrounding tissues. Due to the transparent nature of these worms, several invadopodia markers were visualized by live cell imaging including F-actin, Src non-receptor tyrosine kinase (Src), phospholipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), and proteases (Hagedorn et al., 2009; Sherwood et al., 2005; Ziel et al., 2009). The ability of cancer cell lines to form invadopodia and actively degrade matrix is associated with increased invasive capacity measured by other experimental parameters such as transwell invasion assays and xenograft mouse models (Ammer et al., 2009; Bowden et al., 1999; Coopman et al., 1998; Thompson et al., 1992).

Figure 2 Invadopodia formed in Src transformed fibroblasts. Invadopodia are actin rich aggregates that contain cortactin and MT1-MMP. Underlying invadopodia are dark areas in the labeled gelatin (white) where the ECM has been degraded by invadopodia. Arrows point to areas with invadopodia, bar = 10μM.

Invadopodia were first identified by the Chen laboratory in the 1980's as specialized proteolytic surface protrusions formed in chicken embryonic fibroblasts (CEF) transformed by Rous sarcoma virus (RSV)(Chen, 1989; Chen et al., 1985). Throughout the 1990's, the Chen laboratory further characterized these structures as containing integrins, fibronectin (Mueller and Chen, 1991), tyrosine phosphorylated proteins (YPPs)(Mueller et al., 1992), and soluble (Monsky et al., 1993; Monsky et al., 1994) and membrane bound proteases (Nakahara et al., 1997). In addition, invadopodia were discovered to promote focal degradation of a variety of ECM components including fibronectin, laminin, type I collagen, and type IV collagen (Kelly et al., 1994).

During the last decade, considerable work on invadopodia biology has

substantially expanded our understanding of the molecular machinery within these organelles where protein networks coordinating cell signaling, actin polymerization, vesicle trafficking, and ECM hydrolysis converge to create organelles necessary for initiating cellular invasion.

It is currently unclear whether the initial trigger of invadopodia formation is integrin-based adhesion to ECM, or if the formation of F-actin-rich structure precedes and supports integrin clustering. It is clear that integrins are necessary to target and/or activate proteases at invadopodia (Artym et al., 2002; Deryugina et al., 2001), and the integrin combination that localizes to invadopodia is dependent on cell type and the specific ECM engaged (Badowski et al., 2008; Block et al., 2008; Mueller and Chen, 1991). Also, Invadopodia contain several adhesion proteins that have well-characterized roles in focal adhesions, cell-matrix contacts that provide mechanical force during cell movement, including talin (Mueller et al., 1999) and paxillin (Bowden et al., 1999). Surprisingly, vinculin (Gimona et al., 2008) is notably absent from these structures. Focal adhesion kinase (FAK) does not localize to invadopodia and is not required for invadopodia formation or activity (Vitale et al., 2008). However, depletion of FAK increases the number of active invadopodia per cell and redistributes phosphotyrosine-containing proteins from focal adhesions to invadopodia, but FAK depleted cells showed impaired invasion through Matrigel (Chan et al., 2009). These results suggest that a balance of “adhesive” and “invasive” ECM-cell contacts is necessary for optimal cancer cell invasion.

Since invadopodia are absent in non-cancerous cell types, it is intriguing to speculate that cancer cells gain specific mutations that alter actin cytoskeletal signaling

to support the formation of invadopodia. For instance, invadopodia can be triggered only at permissive temperatures when using a temperature-activated Src (See Study 1), supporting an inside-out mechanism of invadopodia formation. However, it is clear that external signals regulate invadopodia assembly and function. Addition of epidermal growth factor (EGF) enhances invadopodia numbers (Yamaguchi et al., 2005a), and increasing substrate rigidity enhances the number and ECM degradation activity of invadopodia (Enderling et al., 2008; Parekh and Weaver, 2009). These results suggest that once tumor cells are “primed” to form invadopodia, the extracellular environment modulates the invasiveness of these cells. Surprisingly, addition of protease inhibitors blocks degradation and the formation of invadopodia in cancer cells that form spontaneous invadopodia (Ayala et al., 2008), suggesting that an outside-in signaling loop is necessary to support invadopodia biogenesis.

Invadopodia contain branched and bundled actin filament networks. Like the formation of lamellipodia, initial invadopodia protrusions are dependent on N-WASp-activated Arp2/3-mediated actin polymerization. Both N-WASp and Arp2/3 localize to invadopodia (Baldassarre et al., 2006; Clark and Weaver, 2008; Lorenz et al., 2004; Yamaguchi et al., 2005a) and knockdown or expression of dominant negative mutants of these proteins substantially decreases invadopodia formation (Mizutani et al., 2002; Schoumacher et al., 2010; Yamaguchi et al., 2005a). Upstream activators of N-WASp, including WASp-interacting protein (WIP), Nck1, Tks5 (Oikawa et al., 2008), CDC42, and the CDC42 GEF (guanine exchange factor that stimulates the exchange of GDP for GTP) Fgd1 are also necessary for invadopodia assembly (Ayala et al., 2009; Oser et al., 2009; Styli et al., 2009; Yamaguchi et al., 2005a). Accordingly, CDC42 seems to be

the dominant member of the Ras superfamily of GTPases that is responsible for invadopodia assembly. CDC42 localizes to invadopodia sites whereas RhoA and Rac1 are not detectable (Baldassarre et al., 2006). Cells with invadopodia are substantially decreased in cancer cell lines transfected with CDC42 siRNA or dominant-negative CDC42 (Yamaguchi et al., 2005a). Very recently, several papers have been published suggesting that the Arp2/3-N-WASp network activated downstream of CDC42 is necessary for the initial formation of the dendritic actin structure at invadopodia, but unbranched, bundled actin structures are necessary for invadopodia elongation and stability. Diaphanous-related formins (DRFs 1-3), which polymerize linear actin filaments in stress fibers and filopodia, are necessary for invadopodia formation and efficient degradation (Lizarraga et al., 2009). In addition, several filopodia-associated proteins have been localized to elongated invadopodia tips, including fascin, α -actinin, VASP, T-fimbrin, and myosinX (Li et al., 2010; Schoumacher et al., 2010). In addition to these, several other actin binding proteins function in invadopodia assembly or stability, including gelsolin, zyxin (Spinardi et al., 2004), cofilin (Yamaguchi et al., 2005a), and cortactin (Bowden et al., 1999).

Early invadopodia studies identified the serine protease seprase (Monsky et al., 1994), and the urokinase-type plasminogen activator (uPA) (Artym et al., 2002; Kindzelskii et al., 2004) as essential proteases regulating degradation at invadopodia. However, today it is widely recognized that the family of zinc-dependant matrix metalloproteinases (MMPs) are the key enzymes that mediate cell invasion (Deryugina and Quigley, 2006; Egeblad and Werb, 2002; Hotary et al., 2006; Poincloux et al., 2009). All MMPs, which contain propeptide, catalytic, and hemopexin-like-C-terminal

domains, are generated as proenzymes (zymogens) and become activated through proteolytic cleavage of the propeptide. MMP activation and protein levels are increased in cancer and their expression is associated with poor prognosis (Deryugina and Quigley, 2006; Littlepage et al., 2010; Scherer et al., 2008). The secreted proteases MMP-2 and MMP-9 have been localized to invadopodia in many systems and increased amounts or activity of these secreted proteins have been correlated to invadopodia activity and cell invasion (Ammer et al., 2009; Artym et al., 2006; Clark and Weaver, 2008; Clark et al., 2007). However, the membrane-bound MT1-MMP has emerged as the indispensable mediator of focal degradation at invadopodia (Artym et al., 2006; Hotary et al., 2006; Li et al., 2008; Nakahara et al., 1997; Poincloux et al., 2009; Sabeh et al., 2004; Sakurai-Yageta et al., 2008; Steffen et al., 2008). MT1-MMP overexpression (Hotary et al., 2006; Nakahara et al., 1997; Sabeh et al., 2004) or knockdown (Artym et al., 2006; Hotary et al., 2006; Steffen et al., 2008) increases or decreases ECM degradation, accordingly. In agreement with this, treatment of cells with a broad-spectrum MMP inhibitor prevents focal invadopodia ECM degradation activity (Ayala et al., 2008).

In order for invadopodia to functionally degrade the ECM, the cytoskeletal structure must be tethered to the cell membrane, and invadopodia must facilitate the focal delivery of proteases. In this way, invadopodia are highly specialized structures in which actin remodeling is coupled to vesicular transport in order to deliver new membrane components and actively remove ECM. Delivery of MMPs to invadopodia and uptake of ECM most likely occurs via the *trans*-Golgi network (Ayala et al., 2006). This finding is consistent with the observation that the Golgi is often found oriented

towards, and in close proximity to invadopodia (Baldassarre et al., 2003). Two ADP-ribosylation factors (Arf1 and Arf6) that regulate membrane trafficking and actin remodeling have been identified as contributing to invadopodia formation (Hashimoto et al., 2004; Onodera et al., 2005; Tague et al., 2004). Arf1 is mainly found at the Golgi where it is known to act upstream and downstream of CDC42 (the main GTPase in invadopodia) to regulate actin polymerization (Chen et al., 2005; Dubois et al., 2005; Wu et al., 2000). In addition, AMAP1, a GTP-Arf6 activating protein (GAP) and known Src substrate, localizes to and regulates invadopodia formation (Bharti et al., 2007). The transport vesicle-tethering protein exocyst complex (Sakurai-Yageta et al., 2008), and docking/fusion protein v-SNARE TI-VAMP/VAMP7 (Steffen et al., 2008) are both required for the delivery of MT1-MMP to invadopodia sites. The GTPase dynamin 2 (Dyn2) which is required for endocytosis (Hinshaw, 2000), and protein trafficking from the Golgi (Jones et al., 1998), is required for invadopodia formation in Src transformed fibroblasts (Baldassarre et al., 2003; Buccione et al., 2004; McNiven et al., 2004).

Until recently, the role of directional, microtubule-based vesicle trafficking in invadopodia formation was unknown. IQGAP1, a protein that links actin and microtubule cytoskeletal networks, is critical for delivery of MT1-MMP to invadopodia (Sakurai-Yageta et al., 2008). In addition, electron micrographs of elongated invadopodia revealed the presence of 1-2 microtubules decorated with many vesicles/endosomes (Schoumacher et al., 2010). Immunofluorescent staining of these structures confirmed the presence of tubulin throughout the length of the invadopodia, with γ -tubulin (indicative of dynamic microtubule networks) concentrated the invadopodia base. Treatment of invadopodia with nocodazole (facilitating microtubule

depolymerization) did not change the number of invadopodia structures, but significantly decreased invadopodia length. In the same report, similar results were found for vimentin, suggesting a role for intermediate filaments in invadopodia elongation. In addition to actin, regulation of other cytoskeletal elements is an area of emerging interest in invadopodia biology.

Cortactin: Scaffolding invasive actin networks

Cortactin is an essential link between kinase signaling and dynamic actin networks in cell adhesion, migration, endocytosis, and tumor invasion (Ammer and Weed, 2008). Accordingly, cortactin localizes to actin-based protrusions, including lamellipodia, filopodia, dorsal waves, podosomes and invadopodia. Cortactin is a multi-domain scaffolding protein identified by the Parsons laboratory in the early 1990s in a screen for prominent Src substrates (Kanner et al., 1990; Wu and Parsons, 1993; Wu et al., 1991). The cortactin (CTTN) gene encodes a 550 amino acid product that is highly conserved among vertebrate species (Schuuring et al., 1993; Wu and Parsons, 1993; Zhan et al., 1993). Electron microscopy studies of purified recombinant cortactin have proposed that cortactin is a monomeric, asymmetric, rod-shaped molecule ~220 Å in length (Weaver et al., 2002). However, cortactin has recently been reported to also exist in a partially globular conformation whereby the C-terminal region of cortactin folds back towards the N-terminus resulting in a “closed” conformation (Cowieson et al., 2008). Cortactin is predicted to have a molecular mass ~62 kDa. However, cortactin migrates as a doublet upon SDS/PAGE to 80kDa (p80) and 85kDa (p85)(Schuuring et al., 1993; Wu et al., 1991). The distinct bands are proposed to represent post-translational

modifications of cortactin, but the nature of these modifications remains controversial. A single band is formed when the proline rich region (PRR) of cortactin is deleted or when SDS/PAGE is performed in the presence of 5 M urea (Campbell et al., 1999; Huang et al., 1997a; Huang et al., 1997b). In addition, a shift from the 80 to the 85kDa form is seen in response to EGF treatment and the resulting upper band is associated with serine phosphorylation at residues S405 and S418 (Campbell et al., 1999). In this system, the 80 to 85kDa shift is blocked by inhibition of the Erk activating kinase MEK, suggesting that ERK is responsible for serine phosphorylation at these sites. However, prolonged treatment with MEK inhibitors alone failed to change the ratio of the 80/85kDa forms, pointing to a model in which MEK-independent pathways may also regulate the shift. The p85 form was identified as the predominant form of cortactin in colorectal cancers compared to equal ratios of the p80 and p85 in non-cancerous tissues (Zhang et al., 2006), suggesting a functional preference for the p85 cortactin conformation in tumor cell invasion.

Based on primary structure analysis, cortactin predominantly consists of five distinct domains (see Figure 3). Cortactin activates the Arp2/3 complex via a NPF conserved DDW motif at the N-terminal acidic domain (NTA). Distinct from other NPF's that bind actin monomers, cortactin interacts with F-actin via a tandem repeat region containing six and a half 37 amino acid cortactin repeats. This F-actin binding region is located adjacent to the NTA (Weed et al., 2000). Cortactin is a weaker activator of Arp2/3 than WASp family proteins, yet it has a 20-fold higher affinity for F-actin than the Arp 2/3 complex (Urano et al., 2001). In addition, cortactin preferentially binds to newly polymerized actin filaments with monomers containing ATP or ADP-Pi, instead of older

filaments consisting of ADP monomers (Bryce et al., 2005). These data suggest that cortactin is targeted to sites of dynamic actin remodeling where it stabilizes newly formed branches (Weaver et al., 2001). Accordingly, cortactin is a ubiquitously expressed protein that participates in diverse actin-based subcellular functions in several distinct cell types. These processes include cell migration, invasion, and metastasis (Li et al., 2001; Yamaguchi and Condeelis, 2007), axon guidance (Knoll and Drescher, 2004), neuronal morphogenesis (Gray et al., 2005; Martinez et al., 2003), integrin and cadherin-mediated signaling (Ren et al., 2009), bone reabsorption (Matsubara et al., 2006), endocytosis (Orth and McNiven, 2006), and pathogenic cellular entry of bacteria and viruses (Selbach and Backert, 2005). The regulation of cortactin needed to orchestrate these distinct and complex processes is mediated by the carboxyl-terminal end of the molecule. Specifically, cortactin is phosphorylated by multiple tyrosine and serine/threonine kinases within its proline rich domain (PRR), and interacts with several binding partners via a C-terminal Src homology 3 (SH3) domain (Figure 3).

Figure 3 Domain structure of cortactin and illustration of interacting partners. The N-terminal acidic domain (NTA) contains a conserved DDW motif that binds and activates Arp2/3, followed by the repeat region (R) that interacts with F-actin. Src family tyrosine kinases and MAPK serine kinases phosphorylate cortactin in the proline-rich region (PRR). The SH3 domain of cortactin is necessary for binding to several proline-rich proteins that regulate a wide array of cellular processes.

Cortactin was originally identified as a prominent substrate of the v-Src oncogene, and cortactin colocalizes with actin to invasive structures produced in these cells (Wu and Parsons, 1993). The phosphorylation of cortactin by Src occurs on tyrosine residues 421, 470, and 486 (Huang et al., 1998; Huang et al., 1997a). Phosphorylation of cortactin at these sites is important in many systems. Specific to my work, the level of tyrosine phosphorylation of cortactin is positively associated with

increased cell motility and invasion *in vitro* and tumor cell metastasis *in vivo* (Bourguignon et al., 2001; Huang et al., 1998; Huang et al., 2003; Li et al., 2001; Liu et al., 1999). Conversely, non-functional mutants of these sites inhibit these processes. Tyrosine phosphorylated cortactin is enriched in lamellipodia (Head et al., 2003), invadopodia (Study 2) and dorsal waves (Boyle et al., 2007). Cortactin is also selectively targeted by calpain in the regulation of cell movement (Huang et al., 1997b; Perrin et al., 2006). In addition to Src, several other non-receptor tyrosine kinases and serine/threonine kinases have been shown to target cortactin. These include multiple Src family tyrosine kinases (Fyn, Fer, Syk, Abl, and Arg), extra-cellular signal-related kinase (Erk), and p21-activated kinase (PAK) (Boyle et al., 2007; Campbell et al., 1999; Craig et al., 2001; Gallet et al., 1999; Huang et al., 2003; Vidal et al., 2002; Webb et al., 2006). Erk phosphorylates cortactin on S405 and S418 downstream of epidermal growth factor (EGF) stimulation, and results in a molecular weight shift from 80 to 85kDa on SDS-PAGE (Campbell et al., 1999; Martinez-Quiles et al., 2004; Stuible et al., 2008). How tyrosine and serine phosphorylation impacts cortactin localization has been an open question in the field for many years, yet remains poorly understood.

Phosphorylation of cortactin regulates the interaction of cortactin with other proteins through the creation of docking sites. This occurs either as a direct result of phosphorylation (pY/SH2 interactions) or by binding of the SH3 domain to the PRR of binding partners. The cortactin SH3 domain binds several regulators of the actin cytoskeleton, including MLCK, N-WASp, WIP (WASp-interacting protein), Dynamin-2, and CD2AP (CD2-associated protein) (Dudek et al., 2002; Kinley et al., 2003; Lynch et al., 2003; Martinez-Quiles et al., 2004; Zhu et al., 2007). It was initially reported that

tyrosine phosphorylation of cortactin does not affect its ability to activate the Arp2/3 complex, since cortactin lacking the C-terminal region activated Arp2/3 similarly to the full-length protein (Weaver et al., 2001). However, it is now clear that phosphorylation of cortactin increases Arp2/3 activation indirectly through the recruitment of a trimeric phosphocortactin/Nck1/N-Wasp or /WIP complex (Tehrani et al., 2007).

Recently, the ability of cortactin to bind and regulate N-WASp activity led to a proposed regulatory mechanism termed the “Serine-Tyrosine (SY) switch”. In this model, inactive cortactin is proposed to exist in an auto-inhibited form by intramolecular binding through the SH3 and PRR regions (Martinez-Quiles et al., 2004). This inhibition is released via ERK phosphorylation on serine residues 405 and 418, allowing the cortactin SH3 domain to bind and activate N-WASP. The result is synergistic activation of Arp2/3 by cortactin (via the NTA domain) and N-WASP by the SH3 domain to promote actin polymerization and stabilization required for cellular motility. Src phosphorylation of cortactin downregulates the binding of the cortactin SH3 domain to N-WASp and therefore inhibits N-WASp activity. This “on-off” view of cortactin regulation by Erk and Src phosphorylation is limited by the fact that it is solely based on data in relation to cortactin’s interaction with one SH3-binding partner (N-WASp) and is derived *in vitro* using purified proteins. In addition, data from our laboratory demonstrate that the ability of cortactin to be phosphorylated on tyrosine 421 is not dependent on serine phosphorylation status (Study 3). This suggests that phosphorylation of S405 and S418 are not necessary to relieve the putative SH3-PRR intermolecular interactions that would subsequently expose the Src-targeted tyrosine 421 residue. While it is clear that Src and Erk phosphorylation serve to regulate cortactin function, the precise

mechanisms, as well as the degree of interplay between these two kinases in governing cortactin function remains largely unknown.

Cortactin overexpression is observed in several human cancers and tumor-derived cell lines, including carcinomas of the head and neck, lung, esophagus, lung, bladder and breast (Chuma et al., 2004; Luo et al., 2006; Schuuring et al., 1993; Schuuring et al., 1992; Yuan et al., 2003). The cortactin gene (CTTN) maps to chromosome 11q13.3. This region is amplified in 33% of HNSCC and 13% of breast carcinomas, resulting in cortactin overexpression in these tissues. Amplification of 11q13 in HNSCC is associated with tumor aggressiveness, increased metastasis, and poor prognosis (Meredith et al., 1995; Takes et al., 1998; Williams et al., 1993). Cortactin is overexpressed in 50% of cell lines derived from HNSCC's, and increased mRNA levels in patient tumor samples have been found to be an independent predictor of death (Patel et al., 1996; Rodrigo et al., 2000).

HNSCC is a highly aggressive and invasive disease largely driven by overexpression of EGFR (Kalyankrishna and Grandis, 2006). Recently, our laboratory found a link between CTTN copy number and enhanced tumor cell invasion and motility downstream of EGFR through comparison of HNSCC cells with and without 11q13 amplification (Rothschild et al., 2006). The increase in cell motility demonstrated by cells that overexpress cortactin is associated with increased Arp2/3 binding/activation and tyrosine phosphorylation of cortactin. Pretreatment of cells with the EGFR inhibitor, gefitinib, blocked cortactin tyrosine phosphorylation and cell motility. While these experiments indicate that cortactin overexpression directly drives HNSCC motility and

invasion, validation of the role of cortactin in tumor progression can only be accomplished by the use of an *in vivo* cancer model.

Cortactin is a core invadopodia component and its abundance at invadopodia sites has made it the most recognized invadopodia marker. Early studies identified cortactin in a complex with PKC μ and paxillin in these structures whereby the level of tyrosine phosphorylation in invadopodia correlates with their ability to degrade the extracellular matrix (Bowden et al., 1999). Cortactin is necessary for invadopodia formation in several invadopodia-forming cell systems including transformed fibroblasts (Webb et al., 2007) and metastatic cell lines derived from multiple cancer types (Artym et al., 2006; Ayala et al., 2008; Clark et al., 2007; Oser et al., 2009). A stepwise model of invadopodia formation has been proposed in which cortactin is recruited to sites of actin assembly and matrix adhesion resulting in pre-invadopodia complexes (Artym et al., 2006). The membrane-bound matrix metalloproteinase MT1-MMP is subsequently recruited to these sites, allowing for matrix degradation and invadopodia maturation.

Three separate reports have investigated the functional domains of cortactin necessary for invadopodia formation. The first, which used constitutively active cSrc to drive invadopodia formation in fibroblasts, concluded that the actin-binding repeat (ABR) alone is required for functional invadopodia (Webb et al., 2007). In addition, full-length cortactin with tyrosine to phenylalanine mutations at 421, 466, 482 was not able to rescue invadopodia formation, indicating that cortactin tyrosine phosphorylation is also a requirement for invadopodia formation. The second report, determined the DDW motif, SH3 domain, and the phosphorylation sites targeted by Src (tyrosine 421, 466, and 470), ERK (serine 405 and 418), and PAK (serine 113) are all needed for efficient ECM

degradation in melanoma cells (Ayala et al., 2008). The most recent report (Oser et al., 2009) evaluated invadopodia in MTLn3 breast cancer cells concluding that the DDW motif and SH3 domain are necessary for invadopodia formation. However for efficient degradation at invadopodia sites, tyrosines 421, 466, and 470 are needed. Taken together, these data suggest a model in which cortactin acts as a scaffold during invadopodia formation where it may link Arp2/3 activation, actin stabilization, and recruitment of SH3 binding partners such as N-WASp. However, during invadopodia maturation cortactin is regulated by phosphorylation to direct ECM degradation.

Oser et al. (Oser et al., 2009) recently proposed a three step phosphorylation model whereby dephosphorylated cortactin is targeted to invadopodia and recruits N-WASp, Arp2/3, and cofilin. Cortactin is then tyrosine phosphorylated to release cofilin to drive actin polymerization through barbed end production, and creates phosphotyrosine docking sites for the adapter protein Nck1 (Tehrani et al., 2007). Finally, cortactin is dephosphorylated to sequester cofilin, release Nck1 and stabilize invadopodia. This study identifies cortactin as a critical molecular “hub” that regulates the stages of actin assembly and stabilization. However, it still remains how cortactin is spatially and temporally regulated through upstream kinase-based signaling to drive invadopodia maturation.

While the above studies identify cortactin as a critical regulator at the level of the invadopodia actin cytoskeleton, other reports point to cortactin playing a major role in the secretion of MMPs (Clark and Weaver, 2008; Clark et al., 2007). Varying cortactin protein levels (through overexpression or RNAi) in HSNCC cells results in dramatic changes in matrix degradation that cannot be attributed to increased invadopodia

numbers. In addition, RNAi knockdown of cortactin results in decreased secretion of MMP-2, MMP-9 and surface expression of MT1-MMP. While cortactin overexpression resulted in increased secretion/expression of these MMPs, altering cortactin levels also affected the secretion of Apo-1, a protein not linked to invadopodia or proteases, suggesting that cortactin has a general role in regulating secretory cell pathways rather than invadopodia-specific MMP targeting and secretion.

Src tyrosine kinase: Master regulator of the invasive phenotype

Src tyrosine kinase is the first discovered proto-oncogene and is one of the most well-studied proteins to date. Since it was first described in the 1980s as the cellular homologue (cSrc) of the viral Src gene (vSrc) encoded by the Rous Sarcoma virus, Src has been identified in regulating a host of cellular functions/processes important in cancer cell biology including proliferation and survival, cytoskeletal dynamics, cell shape, cell-cell contacts, epithelial to mesenchymal transition (EMT), adhesion, motility and invasion. Even though substantial progress has been made in identifying Src as a master regulator of the tumor cell phenotype, its role in cancer is not completely understood.

Src is the archetypical member of the membrane-bound non-receptor tyrosine kinase family which includes Fyn, Yes, Blk, Yrk, Fgr, Lck, and Lyn (Parsons and Parsons, 2004). Three of these kinases, Src, Yes and Fyn are ubiquitously expressed. The members of this family share a conserved domain structure which contains a N-terminal myristoylated acid moiety necessary for insertion into the cytoplasmic face of the cell membrane, a Src homology 3 (SH3) domain able to bind proline-rich

sequences, a Src homology 2 (SH2) domain which interacts with phosphorylated tyrosines, a flexible linker domain, a tyrosine kinase domain, and a short C-terminal tail (Brown and Cooper, 1996)(Figure 4). Src kinase activity is regulated by conformational changes that govern whether the kinase domain is accessible to interact with substrates (including autophosphorylation of Src itself) (Yamaguchi and Hendrickson, 1996). In its inactive form, Src is phosphorylated on Tyr527 (530 in humans) and is held in a “closed” conformation through intermolecular binding of Tyr527 to the SH2 domain, and additional SH3 domain interactions with the linker region. Src is activated when Tyr527 is dephosphorylated, as the molecule adopts an “open” conformation allowing for autophosphorylation of Tyr416, phosphorylation of Src substrates and potential interaction with binding partners through SH2 domain-mediated interactions. Phosphorylation and dephosphorylation of Src at Tyr527 is facilitated by c-terminal Src kinase (Csk) (Cooper et al., 1986) and the protein tyrosine phosphatase PTP1B (Bjorge et al., 2000), respectively. Independent of phosphorylation on Tyr527, Src can be activated through SH2- and SH3-mediated binding to focal adhesion kinase (FAK), CRK-associated substrate (CAS), and a multitude of receptor tyrosine kinases (RTKs). These interactions “force” Src to adopt the open conformation, leading to kinase activation. Src is also regulated through subcellular localization. Inactive Src resides in the perinuclear region associated with the microtubule organizing center, colocalizing with endosomal markers (Kaplan et al., 1992). Once activated, Src translocates to the cell periphery in an endosomal- and actin-dependent fashion (Fincham et al., 1996; Sandilands et al., 2004). The location in the cell where Src is targeted is contingent on the activation of specific Rho GTPases (Timpson et al., 2001), whereby activation of

Rac1 targets Src to lamellipodia, CDC42 targets it to filopodia, and RhoA targets it to focal adhesions.

Figure 4 Structure and activation of Src tyrosine kinase. (Left) The molecular structures of cSrc and vSrc. Src tyrosine kinases contain the following domains: a N-terminal myristylation domain that allows for interaction with the plasma membrane; a unique or SH4 domain; a Src homology 3 (SH3) and 2 (SH2) domain facilitating binding with interaction partners; a kinase domain with an autophosphorylation site at tyrosine 416 (Y416); a C-terminal regulatory region containing the auto-inhibitory tyrosine 527. (Right) In the inactive conformation the SH2 domain binds the phosphorylated C-terminal Y527, and the SH3 domain interacts with the kinase domain promoting a “closed” conformation in which limits the interaction of the kinase domain with potential substrates. When Src is activated, Y527 is dephosphorylated and the inhibition of the kinase domain is released. Src is in an “open” conformation leading to autophosphorylation on Y416. vSrc has multiple point mutation throughout the molecule and lacks the C-terminal auto-inhibitory Y527 making the molecule constitutively-active and highly transforming. Illustration adapted from Yeatman, 2004.

Much of the seminal work on Src activity was facilitated through the use of temperature sensitive mutants of vSrc (Yeatman, 2004). In 1970 it was discovered that vSrc alone was the necessary and sufficient transforming agent responsible for Rous sarcoma virus (RSV) in chickens (Martin, 1970). The vSrc transformed cell phenotype includes a marked reorganization of the actin cytoskeleton, with disruption of cell-matrix (focal adhesion) and cell-cell adhesions, leading to rounded cell morphology. Unlike its regulated cellular counterpart (cSrc), vSrc has a C-terminal truncation that removes the critical Tyr527 residue, rendering the kinase constitutively active and oncogenic (Takeya

et al., 1982; Takeya and Hanafusa, 1982). Additionally, vSrc contains several mutations throughout its domain structure, but how these differences may cooperate in enabling transforming ability is unknown (Jove and Hanafusa, 1987; Parsons and Weber, 1989).

Unlike vSrc, cSrc is poorly transforming and lacks oncogenicity even when expressed at high levels. While activating cSrc mutations have been identified in a subset of human cancers, these cases are extremely rare (Daigo et al., 1999; Irby et al., 1999; Nilbert and Fernebro, 2000; Sugimura et al., 2000). Src is overexpressed or hyperactivated in a myriad of carcinomas and sarcomas (Guarino, 2010; Irby and Yeatman, 2000; Summy and Gallick, 2003; Yeatman, 2004), and Src has been shown to have a distinct, predictive oncogenic signature of pathway deregulation in cancer cell lines (Bild et al., 2006), supporting a substantial role for Src activity in tumor progression. Increased Src activity in tumors most likely occurs as a result of hyperactivated upstream signaling of RTKs and integrins (Abram and Courtneidge, 2000; Summy and Gallick, 2003). In this way, Src is a necessary and potent mediator of the metastatic cascade regulating anoikis (Windham et al., 2002), EMT, cell migration and invasion and metastasis (Guarino, 2010). In addition, Src activity is not required for cancer cell proliferation *in vitro* or for tumor growth *in vivo* (Brunton et al., 2005). Therefore, it is highly likely that cSrc is not an initiator of cell transformation in human cancers, but instead is a powerful driver of tumor progression.

Invadopodia were initially identified in vSrc transformed cells (Chen, 1989), and exogenous expression of constitutively active Src remains the best known “trigger” to produce invadopodia in invadopodia-null lines. cSrc activity is also important in cells that form spontaneous invadopodia, since Src inhibitors ablate invadopodia formation and

activity (Ammer et al., 2009; Ayala et al., 2008; Pichot et al., 2009). In fact, Src activity levels seem to be intimately related to the formation and the degradative capacity of invadopodia forming cells, since cells expressing increasingly active Src constructs exhibit a dosage effect (Artym et al., 2006). Cells overexpressing WT Src have increased invadopodia number and ECM degradation over control cells, and addition of constitutively active Src enhances this result. Conversely, expression of a kinase dead Src mutant inhibits invadopodia formation and ECM degradation to well below control cell levels. However, extremely high Src activity (via the expression of vSrc) in breast cancer cell lines has been reported to lead to a increased number of invadopodia/cell at the expense of a decrease in invadopodia lifetime, which results in a net decrease in degradation area per invadopodia (Oser et al., 2009).

It is not completely known how Src regulates invadopodia in cancer cell lines. It is clear that Src has an important role in the initiation of actin-based invadopodia assembly, since Src activity is tightly linked to the invadopodia formation. It is easily assumed that Src phosphorylation acts as the initial catalyst that recruits invadopodia-associated proteins through a series of phosphorylation events. Invadopodia are enriched with phospho-tyrosine containing proteins (Bowden et al., 2006), and numerous Src substrates localize to invadopodia including cortactin (Bowden et al., 1999), N-WASp (Yamaguchi et al., 2005a), dynamin2 (Baldassarre et al., 2003), AMAP1 (Onodera et al., 2005), paxillin (Bowden et al., 1999), p130Cas (Brabek et al., 2004), Tsk5 (Seals et al., 2005), p190RhoGAP (Nakahara et al., 1998), AFAP110 (Gatesman et al., 2004), caveolin (Yamaguchi et al., 2009), and MT1-MMP (Nyalendo et al., 2008; Nyalendo et al., 2007). Several groups have reported that dynamic phosphorylation

events are necessary to regulate signaling pathways during invadopodia stability and maturation (Oser et al., 2009; Stylli et al., 2009). It is known that these events are dependent on upstream Src activity as the initial activator; however it is unclear what role Src plays after it acts as the initial trigger. It is possible that Src itself regulates additional downstream phosphorylation events during invadopodia maturation. However, it is also likely that Src activates or localizes additional kinases to invadopodia important in initiating and maintaining matrix degradation.

FAK has been shown to act upstream of Src as a negative regulator of invadopodia (Chan et al., 2009). FAK depletion from breast cancer cells increases the number of invadopodia formed and switches the distribution of phosphotyrosine-containing proteins from focal adhesion complexes to invadopodia. It is clear that FAK, a prominent focal adhesion protein, is absent from invadopodia structures. However, Src is known to complex with FAK to regulate focal adhesion dynamics, suggesting that Src may be critical in regulation cross-talk between these two structures. Even though decreasing FAK levels increase invadopodia formation, invasion through matrigel is inhibited. These data suggest that FAK-dependant spatial and temporal Src regulation is needed to regulate efficient cell invasion. Calpain 2 is another known upstream regulator of Src in invadopodia formation. In a model proposed by the Huttenlocher lab, calpain-2 cleaves PTP1B, which results in the de-phosphorylation of Src at Tyr527, leading to increased Src activity initiating cortactin-dependant invadopodia assembly (Cortesio et al., 2008). However, calpain-2 also functions downstream of Src to regulate invadopodia disassembly through proteolysis of cortactin. It has since been discovered that cortactin is also a substrate of PTP1B (Stuible et al., 2008), making it likely that

PTP1B knockdown in this study was additionally affecting invadopodia assembly through direct regulation of cortactin phosphorylation. These two recent papers from the Huttenlocker laboratory have confirmed the role of Src as the essential kinase mediating invadopodia assembly through regulated targeting of actin-associated substrates to invadopodia sites. Future studies are necessary to define the role of Src in the later stages of invadopodia lifetimes. Src is known to promote the expression of several MMPs that localize to invadopodia, including MMP-2, MMP-9 (Artym et al., 2006; Hsia et al., 2003; Van Slambrouck et al., 2009) and MT1-MMP (Artym et al., 2006; Wu et al., 2005). In addition, Src is known to regulate activity of MT1-MMP to promote invasion (Sabbota et al., 2010; Wu et al., 2005). These data strongly suggest that Src may play an important role in delivery and/or activation of MMPs necessary for efficient focal ECM degradation at invadopodia sites.

Because Src has been identified as an important mediator of tumor malignancy, it is a prime target for the development of anti-invasive therapies in multiple cancer types, including HNSCC (Egloff and Grandis, 2008). Src is overexpressed in HNSCC (van Oijen et al., 1998) and is activated downstream of EGFR (Xi et al., 2003) to promote tumor cell proliferation and invasion (Zhang et al., 2004). In addition, several Src substrates important in tumor cell motility and invasion are overexpressed in HNSCC (including cortactin-mentioned previously), that may serve to amplify the effects of Src hyperactivation resulting in increased metastatic potential (Kelley et al., 2008). Dasatinib and saracatinib are ATP-binding site small molecule Src inhibitors that are currently in phase II clinical trials (NIH, 2010a; NIH, 2010b). Src inhibitors are being tested on patients with metastatic solid tumors refractory to standard therapies. Data

thus far demonstrate that Src inhibitors may be promising for treatment alone, however substantial evidence suggest that these compounds would be more effective in combination with EGFR inhibitors or conventional chemotherapy regimens (Egloff and Grandis, 2008; Pichot et al., 2009).

It is clear that Src and the Src substrate cortactin have substantial roles in tumor progression. However, the precise roles in which these molecules play in advancing tumor cell invasion and metastasis is incomplete. The following studies demonstrate advances in understanding the molecular mechanisms that promote invasion in HNSCC, with Src and cortactin being central to this process. In Study 1 we reveal an important and previously unappreciated function of the Src inhibitor saracatinib to impair invadopodia formation and associated matrix degradation activity in HNSCC cells, resulting in decreased invasiveness. Ablation of invadopodia formation in response to saracatinib is associated with a decrease in tyrosine phosphorylation of several invadopodia-associated Src substrates, including cortactin. Study 2 further defines the role of WT Src in invadopodia formation and function in HNSCC. We confirm that activated Src is responsible for the initiation of invadopodia formation, and demonstrate the novel finding that regulated Src activity has an important role in invadopodia maturation. We also identify regulated cortactin tyrosine phosphorylation downstream of Src as central to this process. The regulation and interplay of cortactin by Src-mediated tyrosine phosphorylation and Erk-mediated serine phosphorylation downstream of EGFR is evaluated in Study 3. We reveal that both pathways are important to promote cell motility in cancer cell lines. Finally, Study 4 illustrates a transgenic model of HNSCC where mice were designed to overexpress cortactin specifically in the oral cavity of

tumorigenic mice to provide an *in vivo* model evaluating the role of cortactin in promoting tumor cell invasion.

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Study 1: Saracatinib Impairs Head and Neck Squamous Cell Carcinoma

Invasion by Disrupting Invadopodia Function

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Running title: Saracatinib inhibits invadopodia activity

Abstract

Elevated Src kinase activity is linked to the progression of solid tumors, including head and neck squamous cell carcinoma (HNSCC). Src regulates HNSCC proliferation and tumor invasion, with the Src-targeted small molecule inhibitor saracatinib displaying potent anti-invasive effects in preclinical studies. However, the pro-invasive cellular mechanism(s) perturbed by saracatinib are unclear. The anti-proliferative and anti-invasive effects of saracatinib on HNSCC cell lines were therefore investigated in preclinical cell and mouse model systems. Saracatinib treatment inhibited growth, cell cycle progression and transwell Matrigel invasion in HNSCC cell lines. Dose-dependent decreases in Src activation and phosphorylation of the invasion-associated substrates focal adhesion kinase, p130 CAS and cortactin were also observed. While saracatinib did not significantly impact HNSCC tumor growth in a mouse orthotopic model of tongue squamous cell carcinoma, impaired perineural invasion and cervical lymph node metastasis was observed. Accordingly, saracatinib treatment displayed a dose-dependent inhibitory effect on invadopodia formation, extracellular matrix degradation and matrix metalloprotease 9 activation. These results suggest that inhibition of Src kinase by saracatinib impairs the pro-invasive activity of HNSCC by inhibiting Src substrate phosphorylation important for invadopodia formation and associated matrix metalloprotease activity.

Keywords: Saracatinib, Src, Head and Neck cancer, invadopodia, invasion, MMP

Abbreviations

CAS- Crk-associated substrate

c-Src- cellular Src kinase

ECM- extracellular matrix

FACS- fluorescence-activated cell sorting

FAK- focal adhesion kinase

FITC- fluorescein isothiocyanate

HNSCC- head and neck squamous cell carcinoma

IHC- immunohistochemistry

SFK- Src family kinase

Introduction

Tumor cell invasion and metastasis is a compounding problem in cancer management, with therapeutic intervention of tumor invasion becoming recognized as an increasingly relevant clinical factor (Dolgin, 2009). Increased activation of the proto-oncogene c-Src (Src) has been established in enhancing tumor progression in human cancer and corresponds with poor clinical outcome (Irby & Yeatman, 2000; Yeatman, 2004). Src is responsible for governing signaling pathways that regulate proliferation, angiogenesis, resistance to apoptosis, adhesion, motility and invasion (Summy & Gallick, 2006). High Src expression and/or activity is observed in metastases, supporting a role for Src in tumor progression by enhancing tumor invasion and metastatic potential (Summy & Gallick, 2003; Yeatman, 2004). Small molecules targeting Src kinase activity suppress proliferation, invasion and metastasis in preclinical settings (Summy & Gallick, 2006), and are currently being evaluated in clinical trials (Kopetz et al., 2007).

Head and neck squamous cell carcinoma (HNSCC) is highly invasive, frequently metastasizing to cervical lymph nodes and corresponds with poor prognosis (Kramer et al., 2005). Src overexpression is common in HNSCC (van Oijen et al., 1998) and is activated following engagement of the epidermal growth factor receptor (EGFR), where it modulates HNSCC growth and invasion through several signaling pathways (Zhang et al., 2004). The small molecule Src kinase inhibitor dasatinib suppresses motility and invasion of HNSCC cells *in vitro* and in mouse xenografts models, corresponding with decreased Src activation and invasion-associated substrate phosphorylation (Johnson et al., 2005; Sen et al., 2009). Amplification and/or overexpression of Src substrates in

HNSCC correlates with poor clinical outcome, potentially serving to magnify Src pathway effects on HNSCC invasion and metastasis (Kelley et al., 2008).

HNSCC invasion and metastatic spread is mediated in part by the action of matrix metalloproteases (MMPs), with MMP1, MMP2, MMP9 and MT1-MMP activity associated with poor outcome (Rosenthal & Matrisian, 2006). MT1-MMP, MMP2 and MMP9 localize to invadopodia, actin-based ventral protrusions in invasive tumor cells that mediate focalized proteolysis of the extracellular matrix (ECM) (Linder, 2007; Weaver, 2006). Invadopodia formation is dependent on Src activity, which enhances MMP2 and MMP9 secretion (Hsia et al., 2003; Mueller et al., 1992), and matrix degradation in HNSCC cells (Clark et al., 2007). The collective localization and action of MMPs at invadopodia allows matrix remodeling to accommodate primary tumor growth and to allow dissemination of encapsulated tumor cells to local and distant sites (Gimona et al., 2008).

Saracatinib (AZD0530) is a recently developed anilinoquinazoline inhibitor designed to disrupt Src kinase activity (Hennequin et al., 2006; Summy & Gallick, 2006). Saracatinib exhibits inhibitory effects on tumor growth in some model systems (Herynk et al., 2006), but several preclinical reports suggest that the primary anticancer effects of saracatinib are impaired tumor cell migration and invasion in HNSCC and other cancer types (Green et al., 2009; Koppikar et al., 2008; Nozawa et al., 2008). The anti-invasive effects of saracatinib are consistent with the effects of Src kinase inhibition in HNSCC by dasatinib, another Src-targeted inhibitor (Johnson et al., 2005). Saracatinib

is currently being evaluated in phase I/II clinical trials for efficacy against advanced stage HNSCC and other tumor types (Kopetz et al., 2007).

Although saracatinib and other Src inhibitors are effective anti-invasive compounds, a complete understanding of the how therapeutic Src inhibition perturbs tumor invasion at the cellular level is lacking. We show that saracatinib inhibited Src activation and phosphorylation of the invadopodia regulatory proteins focal adhesion kinase (FAK), p130 Crk-associated substrate (CAS) and cortactin in HNSCC cells. Saracatinib suppressed HNSCC growth and cell cycle progression in a subset of HNSCC cell lines. Administration of saracatinib to nude mice containing orthotopic HNSCC tongue tumors inhibited Src activity, cortactin phosphorylation, perineural invasion and lymph node metastasis. We also demonstrated that saracatinib prevented invadopodia formation and ECM degradation in invasive HNSCC cells, as well as secretion and activation of MMP9. Collectively these results suggest that saracatinib exhibits anti-tumor effects in HNSCC by inhibiting invasion through the prevention of invadopodia formation. The ability of saracatinib to prevent invadopodia-mediated ECM proteolysis reveals a cellular process perturbed by Src inhibitors that is likely utilized in the progression of HNSCC and other invasive carcinomas containing high Src activity.

Materials and Methods

Cell lines, antibodies and Western blotting

HNSCC cell lines 1483, HN31, UMSCC 1, UMSCC19 and MSK 921 were maintained as described (Rothschild et al., 2006). Western blotting of cell lysates was conducted essentially as before (Rothschild et al., 2006). Western blotting of secreted MMP2 and 9 was conducted on conditioned media, with volumes adjusted to compensate for variations in cell numbers using dimethyl sulfoxide (DMSO)-treated cell numbers as controls.

Antibodies for immunoblotting included anti-Src (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pY418 Src (1:1000; Invitrogen, Carlsbad, CA, USA), anti-p130CAS (1:1000; BD Biosciences, San Jose, CA, USA), anti-pY410 p130CAS (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-FAK (1:1000; BD Biosciences), anti-pY861 FAK (1:1000; Invitrogen) and anti-cortactin (1µg/ml; 4F11 (Rothschild et al., 2006)). For detection of human pY421 cortactin, a custom antibody was developed by 21st Century Biochemicals (Marlboro, MA, USA). Briefly, a synthetic cortactin peptide encompassing the sequence around tyrosine 421 (NH₂-VpYEDAASF~~K~~L-COOH) was synthesized, phosphorylated and injected into rabbits. Immune serum was passed through a column containing agarose beads coupled to the equivalent non-phosphorylated peptide, and then passed over a second agarose column containing a partially overlapping phosphorylated peptide (NH₂-LPSSPVpYEDAA-COOH). Bound antibodies were eluted, concentrated and screened for specificity by Western blotting against recombinant cortactin mutant proteins harboring phenylalanine-tyrosine point mutations at tyrosine 421 (Fig. 1, Supplemental

Material). Anti-ERK1/2 (Cell Signaling) and anti-pT202/pY204 ERK1/2 (Cell Signaling) were used at 1:1000. Anti-MMP2 (1:500; Millipore, Billerica, MA, USA) was used to detect cellular MMP2 levels. Secreted MMP2 was detected with antibody CA-4001 (1:100; Millipore). Cellular and secreted MMP9 was detected with monoclonal antibody 9D4.2 (1:100; Millipore). All Western blots were quantified by densitometry and ImageJ analysis, and band intensities determined relative to non-treated controls.

Cell proliferation and cell cycle progression assays

For cell proliferation assays, 4,000 cells were seeded overnight and treated with 0-10 mM saracatinib (AstraZeneca, Cheshire, UK) for 5d. 100 µg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich, St. Louis, MO, USA) was added to each saracatinib treatment condition for 4h, cells were washed and the reduced dye extracted with a 75% isopropanol/2% HCl/23% H₂O mixture. Dye absorbance was read at 490 nm with an automated plate reader.

Cell cycle distribution was determined by fluorescence activated cell sorting (FACS) as previously described (Frederick et al., 2007).

Invasion assays

BioCoat Matrigel invasion chambers (BD Biosciences) were rehydrated with serum-free DMEM media for 2 h. 1×10^5 cells suspended in serum-free media were plated in the chamber insert and incubated for 2 h to allow attachment. The media in the upper and lower chambers was replaced, with serum-free DMEM added to the upper chamber and DMEM containing 5% FBS added to the lower chamber to generate a chemotactic

gradient. Increasing doses of saracatinib (0-1 μ M) of saracatinib as indicated (Fig. 1D) to the upper and lower chambers. Cells were allowed to invade for 12-24 h (depending on cell line), fixed with 10% buffered formalin phosphate (Fisher Scientific, Hanover Park, IL, USA) and rinsed with PBS. Non-invasive cells were removed from the interior of the chamber insert with a swab and the remaining cells were stained with 0.4% Crystal Violet solution (Fisher) for 15 min. Invasion was assessed by counting cells in four random 20X microscopic fields.

Orthotopic xenograft assay of HNSCC invasion

An *in vivo* mouse model of oral tongue squamous cell carcinoma was established as described (Myers et al., 2002) with minor modification using female athymic Foxn1^{nu/nu} mice 4-5 weeks of age (Harlan Laboratories, Indianapolis, IN, USA). All animal procedures were conducted according to an approved protocol by the West Virginia University Animal Care and Use Committee. Anesthetized mice were injected with 2.5×10^4 UMSCC1 cells suspended in DMEM into the anterior ~1/3 of the tongue. After 10 d, treatment was initiated by daily oral gavage of 25mg/kg saracatinib suspended in a sterile solution of 0.5% methyl cellulose/0.1% polysorbate 80 (Tween 80, Sigma-Aldrich). Control animals were gavaged with the methylcellulose/Tween 80 vehicle. No overt difficulties were encountered when gavaging mice over time as the tumor size increased. Six mice were used for each treatment group. After 30 d of treatment, mice were euthanized by carbon dioxide inhalation and tumor volumes determined as described (Huang et al., 2002). Tongues, sublingual tissue containing the superficial cervical lymph nodes and the tracheoesophageal region, deep cervical and mediastinal

lymph nodes, liver and lung were removed from each animal, rinsed, fixed and paraffin embedded for routine histological evaluation.

Immunohistochemistry

Human HNSCC cases were obtained from the West Virginia Tissue Bank and used under the approval of West Virginia University Institutional Review Board. Five-micrometer sections from human HNSCC and mouse tissue blocks were processed and immunolabeled or hematoxylin and eosin (H&E) stained using a Discovery XT automated staining system (Ventana Medical Systems, Tucson, AZ, USA). For immunohistochemistry, primary antibody conditions were: anti-total Src (Cell Signaling) 1:600 in Dako diluent (Dako, Carpinteria, CA, USA) for 1 h, anti-pY416 Src family kinase (SFK) (Cell Signaling) 1:25 in phosphate saline solution (PSS) (Ventana) for 12 h, anti-total cortactin (Novus Biologicals, Littleton, CO, USA) 1:700 in PSS for 1 h, anti-pY421 cortactin 1:50 in Tris-buffered saline containing 4% BSA for 1 h, and prediluted anti-cytokeratin 14 (Abcam, Cambridge, MA, USA) for 20 min. Primary antibodies were detected using the Omnimap antibody horseradish peroxidase kit (Ventana) and slides were counterstained with hematoxylin. Images were acquired as described (Rothschild et al., 2006). For quantifying pY416 Src and pY421 cortactin staining intensities, brightfield images from at least 5 randomly selected images were captured on an Olympus ZX70 Provis microscope (Olympus, Center Valley, PA, USA) with a 20x/0.70 UPlanApo objective and an Optronics MicroFire 1600x1200 color CCD camera (Optronics Inc, Goleta, CA, USA) using the StereoInvestigator imaging package (MBF Bioscience, Williston, VT) with the same camera settings and brightfield correction

enabled to ensure even illumination across the image. Brown 3,3'-diaminobenzidine (DAB) staining was separated from blue hematoxylin staining using the color deconvolution plug-in function of ImageJ (NIH) as described (Park et al., 2008). The vector values for the DAB staining were determined from ROIs with brown staining (R=0.425, G=0.600 and B=0.677 for pY421 cortactin; R=0.475, G= 0.653 and B=0.686 for pY416 Src). Brown images were inverted and intensities were measured inside the tumor tissue. The mean DAB intensities were averaged within the group to calculate ratios of phosphorylation-specific staining in treated vs. control tissues.

Immunofluorescence labeling, confocal microscopy and image analysis

UMSCC1 cells were plated on fluorescein isothiocyanate (FITC)-gelatin (Sigma) coated coverslips as described (Artym et al., 2006) for 2 h. Cells were left untreated or treated with saracatinib for 6 h, rinsed and fixed with 4% formaldehyde. Cells were permeabilized with 0.4% Triton-X/PBS for 4 minutes, then blocked in 5% BSA/PBS for 1 h. To identify invadopodia, cells were incubated with rhodamine-conjugated phalloidin (1:1000; Invitrogen), anti-cortactin monoclonal antibody 4F11 (1 µg/ml) and polyclonal pTyr-100 (1:200; BD Biosciences) in 5% BSA/PBS for 1 h. After washing, cells were incubated in 5% BSA/PBS containing AlexaFluor 405 goat anti-rabbit and AlexaFluor 647 goat anti-mouse secondary antibodies (Invitrogen) at 1:1000. Cells were rinsed and mounted in Fluoromount-G (SouthernBiotech, Birmingham, AL, USA).

For quantifying saracatinib effects on invadopodia incidence and matrix degradation, eight-bit 1024x1024 pixel confocal images were acquired with a Zeiss LSM510 confocal microscope using AIM software (Carl Zeiss MicroImaging, Thronwood, NY, USA).

Images were scanned with a 63x/1.4 NA Oil Plan-Apochromat objective at 1.3x zoom, yielding a resolution of 10.14 pixels/ μm . All images of the FITC-gelatin were taken with the same parameters (pinhole size, laser intensity and gain) so image intensity would be comparable between samples. For invadopodia formation, a minimum of six independent fields comprising ≥ 50 cells were analyzed for cells containing invadopodia compared to total cell number. For matrix degradation, cells were analyzed using ImageJ software. Actin images were adjusted to threshold values to include all cellular regions, and the resulting images were used to calculate total cell areas in μm^2 . For quantifying matrix degradation, FITC-gelatin images were inverted so that regions with increased degradation would yield higher intensity values, ensuring selection of all areas of matrix degradation. The integrated density was reported as the amount of degradation per total cell area. A minimum of 15 cells was analyzed for each saracatinib concentration.

Gelatin zymography

UMSCC1 and 1483 cells were plated overnight at 5×10^6 in complete media and were treated with saracatinib at increasing dosage for 24 h, rinsed and incubated for 24 h in serum-free media containing the equivalent saracatinib dose. Cells were counted, and conditioned media collected and concentrated by ultrafiltration using Amicon Ultra-4 centrifugal filter devices with a 10kDa molecular weight cutoff (Millipore). Zymography was conducted as described (Clark et al., 2007) with minor modification. Conditioned media (35 μl) was diluted in 2X non-reducing SDS-PAGE sample buffer and resolved on 8% SDS-PAGE gels containing 1 mg/ml bovine gelatin (Sigma). Aliquots of serum-free DMEM and DMEM containing 10% FBS were used as negative and positive controls,

respectively. MMP activity was renatured by washing gels in 2.5% Triton X-100 for 30 min, followed by washing gels in Developing Buffer (50 mM Tris, 0.2M NaCl, 5mM CaCl₂, 0.02% Brij 35) for 30 min at room temperature. Gels were then incubated in renewed Developing buffer for 24h at 37° C to allow MMP activity to proceed. Gels were stained with Coomassie Brilliant Blue R-250 (0.5% w/v) for 30 min, followed by destaining in water. Resulting gels were scanned using a FotoAnalyst Investigator (Fotodyne Inc, Hartland, WI, USA) and areas of gelatinase activity quantified using ImageJ. Results were adjusted relative to control DMSO treatment for gelatinase activity and cell counts for each treatment to compensate for variations in final cell numbers.

Statistical analysis

Differences in mean values between saracatinib treatment groups for invasion, invadopodia and gelatinase assays were evaluated using one-way ANOVA, followed by Student-Newman-Keuls post hoc testing. Differences were considered significant at $P < 0.05$, with all experimentation conducted at least in triplicate.

Results

Saracatinib effects on HNSCC proliferation and invasion

As a first step in our work, we characterized several HNSCC cell lines previously determined to have varying degrees of invasive and metastatic potential (Rothschild et al., 2006; Sano & Myers, 2007; Yang et al., 2004) for their response to saracatinib. To determine the effect of saracatinib treatment on proliferation in these lines, growth inhibition was assessed by 5d 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays for cells treated with increasing doses of saracatinib (Fig. 1A). The HN31 and UMSCC1 lines were sensitive to growth inhibition by saracatinib, as indicated by sub-micromolar IC_{50} values (Fig. 1A). In contrast, the 1483 line demonstrated a marked resistance to saracatinib, with an $IC_{50} = 7.60\mu M$. Corresponding effects on cell cycle progression are also observed, with increasing saracatinib concentrations resulting in enhanced G_1 checkpoint arrest in HN31 and UMSCC1 lines determined by FACS analysis (Fig. 1B). Saracatinib treatment did not alter the percentage of cells undergoing G_2 -M transition for either line. Arrest of G_1 was not evident in 1483 cells treated with saracatinib concentrations up to $1\mu M$, in agreement with resistance of this line in proliferation analysis (Fig. 1A). Erk1/2 activation, a potent driver of mitogenesis, was impaired in HN31 and UMSCC1 cells at concentrations above $0.5\mu M$ as determined by Western blotting (Fig. 1C). Similar results were obtained for the invasive HNSCC lines MSK921 and UMSCC19 (data not shown). Erk1/2 activation was elevated in 1483 cells at doses up to $1\mu M$ (Fig. 1C), in agreement with the high IC_{50} value for this line (Fig. 1A). These data indicate that the HNSCC lines used in this study vary in their proliferative response to saracatinib

treatment, and can be segregated into sensitive (HN31 and UMSCC1) and resistant (1483) populations.

Next we evaluated the effect of saracatinib on HNSCC invasion *in vitro* using modified Boyden chamber transwell assays. In the absence of saracatinib, UMSCC1 and HN31 cells displayed an invasive response to serum, averaging 4150 cells/aggregate field and 1719 cells/aggregate field, respectively (Fig. 1D). 1483 cells were weakly invasive, with an average of 243 cells/aggregate field (Fig. 1D). Increased concentrations of saracatinib resulted in dose-dependent inhibition of HNSCC invasion for all tested lines, with reduced invasion compared to control levels of 88% in UMSCC1, 70% in HN31, and 78% in 1483 cells at the highest evaluated dose (1.0 μ M) (Fig. 1D). These results indicate that saracatinib directly impacts the ability of HNSCC cell lines to invade in an *in vitro* setting.

Saracatinib inhibits Src activation and substrate phosphorylation in HNSCC cells

To determine the impact of saracatinib on Src activity and phosphorylation of invadopodia-related Src substrates, dose-dependence experiments were performed on HN31, UMSCC1 and 1483 cells (Fig. 2). Cell lines were treated with increasing doses of saracatinib for 24 hours, lysed and assessed for phosphorylation by immunoblotting using anti-phosphorylation site-specific antibodies to detect Src activation (pY418) and specific Src phosphorylation sites in downstream substrates (pY410 p130 CAS, pY421 cortactin and pY861 FAK). The phosphorylation of Src at Y418 was inhibited by saracatinib in all tested lines at concentrations between 0.5 and 1.0 μ M (Fig. 2). Phosphorylation of cortactin at tyrosine 421 and FAK at tyrosine 861 was also reduced

within the same range of saracatinib concentrations (FAK phosphorylation in 1483 cells could not be evaluated due to the absence of detectable FAK expression in this line). While phosphorylation of tyrosine 410 in p130 CAS was inhibited within this same dose range in UMSCC1 cells, we observed that p130 CAS phosphorylation was consistently inhibited at lower dose ranges (0.01-0.05 μ M) in HN31 and 1483 cells (Fig. 2). Immunoblotting with antibodies against total p130 CAS, cortactin and FAK indicate that the expression levels of these proteins are somewhat reduced at high dose levels of saracatinib treatment (0.5-1 μ M), but not at levels accountable for the resultant decrease of tyrosine phosphorylation at the assayed sites (0.1-0.5 μ M).

Saracatinib inhibits Src activation, invasion and cervical lymph node metastasis in an orthotopic mouse model of oral squamous cell carcinoma

A mouse orthotopic model of tongue squamous cell carcinoma (Myers et al., 2002) that phenotypically mimics human HNSCC (Fig. 3) was utilized to evaluate the effects of saracatinib on HNSCC progression and invasion in an *in vivo* setting. Athymic mice with UMSCC1 tongue tumors were randomized and treated with either vehicle or daily with 25mg/kg saracatinib. At the end of treatment (40d), mice from both groups had similar weight (24g) and mean tumor volumes ($54.1\text{mm}^3 \pm 2.3\text{mm}$ for controls; $43.7\text{mm}^3 \pm 4.6\text{mm}$ with saracatinib treatment). The modest impact of saracatinib on UMSCC1 tumor growth was not statistically significant. To determine the impact of saracatinib on Src activity, primary tumors were evaluated for Src and cortactin phosphorylation by immunohistochemistry. Active Src (determined by pY416 SFK staining) and pY421 cortactin labeling in UMSCC1 tumors displayed similar patterns

compared to human HNSCC (Fig. 3A). Saracatinib treatment reduced the ratios of pY416 SFK and pY421 cortactin compared to control-treated mice (Figure 3A).

Human HNSCC often displays perineural invasion with regional lymph node involvement. Given the invasive nature of UMSCC1 cells (Fig. 1D), we evaluated the impact of saracatinib treatment on loco-regional tissue invasion and cervical lymph node metastasis in treated mice. Soft tissues from the submental space through the tracheoesophageal region were evaluated for perineural invasion and cervical lymph node metastasis by immunostaining for the epithelial marker cytokeratin 14. Similar to human tumors, extensive perineural invasion and metastasis to the superficial cervical lymph nodes was evident in 5/6 control treated mice (Fig. 3B). Invasion and lymph node metastasis was found in 1/6 saracatinib-treated mice, and the remaining mice displayed a complete absence of cytokeratin-positive cells associated with nerves, sublingual glands, connective tissue or cervical lymph nodes (Fig. 3B). These data demonstrate that the *in vivo* ability of saracatinib to down-regulate Src activity and cortactin phosphorylation correlates with decreased invasion and local lymph node metastasis.

Saracatinib inhibits invadopodia formation and matrix degradation in UMSCC1 cells

UMSCC1 cells plated on fluorescently-labeled gelatin formed centrally localized ventral puncta enriched with cortactin, filamentous (F)-actin and phosphotyrosine (Fig. 4A, DMSO), three markers that define invadopodia (Bowden et al., 2006). Spontaneous invadopodia formation was observed in 51% of UMSCC1 cells by confocal microscopy at a given time, corresponding with focalized areas of matrix clearing (FITC-gelatin)

(Fig. 4B, 0 μ M dosage point). The gelatin matrix underneath UMSCC1 cells typically displayed degradation encompassing ~53% of the overlying cell area, reflecting the action of invadopodia-associated MMP activity (Fig. 4C).

UMSCC1 cells plated on FITC-coated gelatin were treated with increasing concentrations of saracatinib, and invadopodia formation and matrix degradation was evaluated by confocal microscopy (Fig. 4). The number of cells containing invadopodia was significantly decreased with increasing saracatinib dosage, with < 2% of cells having formed invadopodia at concentrations at or above 0.5 μ M (Fig. 4B). Effects on matrix degradation were more pronounced, where increased saracatinib dosage resulted in similar incremental decreases in degradation (Fig. 4C). UMSCC1 cells treated with 1.0 μ M saracatinib did not contain invadopodia and were incapable of degrading matrix (Fig. 4A). These cells also lacked focal cortactin localization, had diminished phosphotyrosine levels at focal adhesions and contained disorganized F-actin puncta on the ventral membrane surface where invadopodia typically form (Fig. 4A, arrowheads).

Saracatinib inhibits MMP9 secretion from HNSCC cells

In addition to MT1-MMP, secretion and activation of MMP2 and MMP9 at invadopodia has been reported to be partially responsible for the observed effects on matrix degradation (Linder, 2007). Src activity regulates MMP2 and MMP9 secretion in fibroblasts (Hsia et al., 2003). We therefore determined the effect of saracatinib on MMP secretion. Confocal immunofluorescence microscopy indicated that UMSCC1 cells have MMP9-containing vesicles localized to invadopodia at sites that correspond

with gelatin degradation (Fig. 5A), indicating that MMP9 is concentrated in UMSCC1 invadopodia. To evaluate the impact of saracatinib on MMP2 and MMP9 secretion and activity in HNSCC cells, total cell lysates and conditioned media from saracatinib-treated 1483 and UMSCC1 cells were analyzed for the presence of cellular and secreted MMP2 and MMP9 by Western blotting (Fig. 5B). Saracatinib treatment resulted in modest decreases (up to 24%) in cellular MMP2 levels at concentrations to 1 μ M, while cellular MMP9 levels demonstrated up to a two-fold increase under the same concentration range. Although secreted MMP2 was not detected in the media of either cell line, both lines secreted detectable amounts of MMP9 (Fig. 5B). Treatment of either line with saracatinib inhibited MMP9 secretion, and each line displayed differential drug sensitivity. Detectable MMP9 secretion from 1483 cells was largely absent at the lowest evaluated concentration (0.01 μ M) whereas secretion from the more invasive UMSCC1 line was inhibited at concentrations of 0.5 μ M and above (Fig. 5B). The secreted MMP9 from both lines displayed proteolytic activity when assayed by gelatin zymography (Fig. 5C). Saracatinib concentrations up to 0.1 μ M did not significantly affect MMP9 activity as determined by ANOVA analysis for both lines, although mean values for 1483 cells treated with these lower doses were consistently below control levels (Fig. 5C). Higher saracatinib concentrations (0.5 μ M and 1.0 μ M) reduced MMP9 activity to respective mean values of 39% and 25% for control levels in 1483 cells, and 22% and 12% in UMSCC1 cells (Fig. 5B,C). These data indicate that saracatinib treatment of HNSCC cells leads to selective inhibition of MMP9 secretion in 1483 and UMSCC1 cells, preventing efficient enzymatic degradation of extracellular matrix components.

Discussion

The present study demonstrates that inhibition of HNSCC invasion in preclinical *in vitro* and *in vivo* settings by saracatinib directly corresponds to disruption of HNSCC invadopodia formation and function, identifying invadopodia as a potential downstream target of therapeutic Src kinase inhibition in HNSCC and other invasive human cancers. Invasive HNSCC presents a difficult problem in patient care, given the proximity of most tumors to multiple vital organ sites in the head and neck region. Disregulation of signaling pathways that promote and sustain invasion impinge on adhesion- and cytoskeletal-associated proteins. These proteins function in concert with MMPs to enable tumor cells to degrade and protrude through an encapsulating ECM, allowing movement into neighboring tissues. The ability of saracatinib to ablate invadopodia and the associated invasive behavior of HNSCC cells in mice provides further evidence for a direct link between invadopodia activity and tumor invasion, shedding light on the specific invasion-promoting cellular processes perturbed by Src kinase inhibition.

The HNSCC lines used in this study displayed differential responses to saracatinib in terms of anti-proliferate effects, with some lines (HN31 and UMSCC1) having submicromolar sensitivity, cell cycle inhibition and decreased ERK1/2 activity and others (1483) demonstrating resistance to the drug at concentrations up to 1 μ M (Fig. 1A-C). While a recent study reported IC₅₀ saracatinib values near 1 μ M for five different HNSCC lines (Koppikar et al., 2008), our findings are in line with the wider range of IC₅₀ values reported for different HNSCC lines treated with the non-related Src kinase inhibitor dasatinib (Johnson et al., 2005) as well as in other tumor cell types (Boyer et

al., 2002; Johnson et al., 2005; Jones et al., 2002). These reports taken together with our data suggest that the HNSCC lines utilized in this study fall within the typical *in vitro* proliferative response profile to therapeutic Src inhibition. While the underlying compensatory mechanism for saracatinib resistance in 1483 cells is unknown, future expression profiling of resistant and sensitive lines may provide insight into the molecular nature of saracatinib resistance, as has been recently conducted for the EGFR inhibitor gefitinib in a variety of HNSCC lines (Frederick et al., 2007).

Saracatinib treatment resulted in *in vitro* anti-invasive activity, impaired Src activation and tyrosine phosphorylation of FAK and p130 CAS in all analyzed HNSCC lines (Fig. 1D; Fig. 2). This is in accord with other studies on other HNSCC lines utilizing saracatinib or dasatinib as single agents (Johnson et al., 2005; Koppikar et al., 2008; Nozawa et al., 2008). Tyrosine phosphorylation of FAK and p130 CAS have been commonly utilized as downstream indicators for preclinical therapeutic anti-Src efficacy, since Src-mediated phosphorylation of these proteins are critical events in enabling tumor invasiveness (Brabek et al., 2005; Zhao & Guan, 2009). In addition, we show that cortactin tyrosine phosphorylation was also reduced following saracatinib treatment (Fig. 2). Cortactin is a Src substrate commonly overexpressed in invasive HNSCC and regulates invadopodia formation (Rodrigo et al., 2000; Rothschild et al., 2006; Weaver, 2008). Cortactin phosphorylation is important for tumor cell motility and matrix degradation at invadopodia (Ayala et al., 2008; Oser et al., 2009; Rothschild et al., 2006). These results suggest that cortactin tyrosine phosphorylation status can serve

as an additional downstream monitor of Src activity and invasive potential in HNSCC cells where Src kinase function is impaired.

While saracatinib was able to modestly decrease the size of *in vivo* UMSCC1 tongue tumors, the reduction in tumor size was not significant compared to untreated controls. UMSCC1 cell growth is inhibited by saracatinib *in vitro* (Fig. 1A), implying that microenvironmental factors such as inflammatory cytokines, growth factors, neoangiogenic and hypoxic aspects responsible for promoting and maintaining HNSCC growth (Pries & Wollenberg, 2006; Timar et al., 2005) may partially circumvent the growth-inhibitory effects of saracatinib in UMSCC1 xenografts. On the other hand, saracatinib displays *in vivo* anti-invasive properties by potently inhibiting perineural invasion and cervical lymph node metastasis. Suppressed Src activation and cortactin tyrosine phosphorylation in primary tumors (Fig. 4A) supports this conclusion, as signaling through these proteins promotes invasion and metastatic spread (Weaver, 2008; Yeatman, 2004). Recent findings in a comparable xenograft system utilizing mice treated with dasatinib demonstrated reduced Src and FAK activity (Sen et al., 2009). EGFR overexpression is common in HNSCC, resulting in enhanced Src activity, cortactin phosphorylation and tumor invasiveness (Koppikar et al., 2008; Rothschild et al., 2006). Clinical EGFR inhibitors also display anti-invasive activity and impair invadopodia formation in preclinical settings (Huang et al., 2002; Yamaguchi et al., 2005; Yang et al., 2004), suggesting in light of our findings that inhibiting EGFR activity in HNSCC impairs Src activation and substrate phosphorylation required for invasion.

This is supported by emerging rationale for dual targeting of EGFR and Src in treating advanced HNSCC (Egloff & Grandis, 2008).

Elevated Src activity is necessary and essential for invadopodia formation (Chen et al., 1985; Chen et al., 1984). The ability of saracatinib to ablate invadopodia and associated matrix degradation demonstrates that a clinically utilized Src inhibitor disrupts a vital subcellular structure required for tumor invasion. Src-induced invadopodia formation in carcinoma cells first targets cortactin and F-actin formation at matrix adhesion sites, forming a core preinvadopodia complex. Recruitment of MT1-MMP to preinvadopodia initiates matrix degradation and invadopodia maturation, with further maturation involving dissolution of the cortactin-F-actin complex, focal retention of MT1-MMP and continued proteolytic activity (Artym et al., 2006). While rudimentary invadopodia-like F-actin structures formed in UMSCC1 cells treated with inhibitory concentrations of saracatinib, they do not contain cortactin (Fig. 4A), which is essential for invadopodia formation (Artym et al., 2006), indicating that Src kinase activity is required for cortactin localization to invadopodia. Similar results have been shown through the use of kinase-inactive Src constructs (Bowden et al., 2006). Tyrosine phosphorylation of invadopodia proteins is strongly linked with the ability to degrade extracellular matrix (Bowden et al., 2006), with Src phosphorylation of cortactin (Ayala et al., 2008), paxillin (Badowski et al., 2008), and ASAP1 (Bharti et al., 2007) requisite for invadopodia formation and/or proteolytic activity. Src kinase inhibition therefore displays at least a two-fold effect on substrates in invadopodia by impairing proper preinvadopodia targeting and perturbing phosphotyrosine-based signaling dynamics

involved in regulating invadopodia maturation and function. Tyrosine phosphorylation in peripheral focal adhesions was observed in UMSCC1 cells, indicating saracatinib may also perturb focal adhesion formation and/or function given the critical role for Src and related kinases in these structures (Frame, 2004).

MMP activity is essential for HNSCC invadopodia formation and function (Clark et al., 2007). The impairment of MMP9 secretion and activation from HNSCC cells treated with saracatinib indicates that Src kinase activity is required for targeting and secretion of MMP9-containing vesicles at invadopodia. This is in agreement with observed effects of Src kinase inhibition on MMP9 secretion in other tumor types (Cortes-Reynosa et al., 2008; Lee et al., 2005). The lack of apparent MMP2 secretion in the HNSCC lines used in our studies precluded evaluation of this metalloproteinase, and is likely characteristic to these lines since other HNSCC cells secrete MMP2 (Clark et al., 2007). Localization of transmembrane and secreted MMPs to invadopodia involves directed trafficking of vesicles emanating from the *trans*-Golgi network, where a dynamin-2-N-WASp-Arp2/3-cortactin complex has been implicated in coupling cortical actin regulation with invadopodia membrane dynamics (Buccione et al., 2004). Cortactin has been implicated as a key regulator of MT1-MMP surface expression and MMP2 and MMP9 secretion in HNSCC (Clark & Weaver, 2008; Clark et al., 2007). While it is currently unknown how cortactin regulates the targeting of MMP-containing vesicles, Src-mediated phosphorylation may play a vital role since tyrosine phosphorylation of cortactin increases its binding to vesicle-associated proteins (Ammer & Weed, 2008) and is required for efficient invadopodia-mediated ECM degradation (Ayala et al., 2008; Webb et al., 2007).

Our results indicate that disruption of Src activity by saracatinib impairs HNSCC cell invasion and lymph node metastasis by preventing invadopodia formation and function, identifying a cellular mechanism that may be universally impacted by Src inhibition in invasive carcinoma cells. In addition to Src and related kinases, saracatinib also inhibits Abl kinase, an activity that has been exploited to evaluate imatinib-resistant chronic myelogenous leukemia (CML) cases expressing the constitutively active BCR-Abl Philadelphia chromosome gene product (Gwanmesia et al., 2009). Along with the tumor-promoting activities in CML, elevated Abl kinase activity has been shown to be important in breast (Srinivasan & Plattner, 2006) and non small cell lung cancer (Lin et al., 2007), indicating Abl kinase activity has a functional role in solid tumor progression (Lin & Arlinghaus, 2008). Abl expression has been evaluated in oral squamous cell carcinoma and correlates with tumor stage (Yanagawa et al., 2000), suggesting that elevated Abl expression may contribute to HNSCC progression. While a mechanism pertaining to Abl function in solid tumor invasion or invadopodia function has not been reported, Abl does bind and phosphorylate cortactin, (Boyle et al., 2007), raising the potential for Abl kinase to play a role in cortactin-based invadopodia function in HNSCC and other invasive Abl-expressing solid tumors. Such a role for Abl in regulating tumor invasion would also likely be impaired by saracatinib and other dual Src/Abl inhibitory compounds.

In addition to carcinoma invadopodia, Src-mediated processes in non-cancerous cell types involved in promoting invasion may also be impacted by Src family kinase inhibition. The motility and protease remodeling ability of tumor stromal fibroblasts involved in enabling collective HNSCC invasion could be affected by impairing Src

family kinases *in vivo* (Gaggioli et al., 2007), as well as the proinvasive properties of tumor-associated macrophages (Condeelis & Pollard, 2006). The ability of saracatinib and other Src inhibitors to impair functions of different cellular types that propagate tumor invasion provides the opportunity for the future discovery of additional Src-based cellular mechanisms utilized during tumor progression.

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Figure Legends

Figure 1. Effects of saracatinib on HNSCC proliferation, cell cycle progression, Erk1/2 activation and *in vitro* invasion. **A.** IC₅₀ values for cell growth determined by 5 day MTT assays for the indicated HNSCC lines treated with 0-10 mM saracatinib. Mean values are shown for each line from three independent assays. **B.** Impact of saracatinib on HNSCC cell cycle progression. HNSCC cell lines were treated with the indicated amounts of saracatinib for 24 hours, fixed labeled with propidium iodide to assess DNA content, and analyzed for cell cycle status by fluorescence-activated cell sorting. Results show the average percentage of cells in each cell cycle phase as indicated on the *left*. Bars, SD of two independent experiments. **C.** Effects of saracatinib on Erk1/2 activity. HNSCC cells were treated with saracatinib for 24 hours at the indicated doses, lysed and analyzed by Western blotting with phosphorylation-specific (pErk1/2) and total Erk1/2 antibodies. Blots shown are representative of three different experiments, with indicated band intensities shown relative to no treatment (0 mM) for each cell line. **D.** Saracatinib inhibits *in vitro* HNSCC invasion. HNSCC cells (1×10^5) were plated in Matrigel-coated transwells alone or with increasing concentrations of saracatinib. After 2 h, invasion was stimulated with 5% FBS and cells were allowed to invade for 12 h (UMSCC1) or 24 h (HN31 and 1483). Invaded cells were quantified by brightfield microscopy. Bars, SEM of three independent experiments.

Figure 2. Saracatinib inhibits Src activity and downstream Src substrate phosphorylation in HNSCC cell lines. HN31, UMSCC1 and 1483 cells were treated with DMSO vehicle or saracatinib (0.01-1 μ M) for 24 h. Cells were lysed and total protein amounts were analyzed by Western blotting with total or phosphorylation site-specific antibodies for Src and the indicated substrates. Blots shown are representative of at least four independent experiments, with band intensities for each substrate quantified relative to the untreated (0 mM) condition for each cell line.

Figure 3. Saracatinib inhibits Src activity, perineural invasion and cervical lymph node metastasis in orthotopic UMSCC1 tongue tumors. **A.** UMSCC1 tongue tumors from representative control-treated or saracatinib-treated mice were sectioned and stained with hematoxylin and eosin (H&E) or by IHC with the indicated antibodies (*left*). A case of human HNSCC was evaluated in parallel as a positive control. The pY416 SFK and pY421 cortactin ratios from saracatinib treated to control levels are indicated. *Bars*, 100 μ m. **B.** Locoregional invasion and lymph node metastasis is inhibited by saracatinib. Submental and associated tracheoesophageal tissue from control treated and saracatinib treated mice was immunostained for cytokeratin 14 to detect cells of epithelial origin. Magnified regions containing a single sublingual nerve and superficial cervical lymph node are shown for clarity. *Inset* shows a magnified cortical region of superficial cervical lymph nodes from control and saracatinib treated mice. Arrowheads denote metastasized UMSCC1 cells. N; sublingual nerve, ED; excretory duct. *Bars* 100 μ m; inset, 50 μ m.

Figure 4. Saracatinib inhibits invadopodia formation and ECM degradation. **A.** Representative images of UMSCC1 cells treated with different saracatinib concentrations. UMSCC1 cells plated on FITC-gelatin coated coverslips (pseudocolored *white*) for 2 h were treated with saracatinib as indicated (*left*) for 6 h. Cells were labeled to visualize F-actin (*red*), cortactin (*green*) and phosphotyrosine (*blue*). Arrows denote invadopodia and corresponding colocalized areas of focal matrix degradation with invadopodia components. Treatment with 1.0 μ M saracatinib resulted in F-actin aggregates lacking cortactin but accumulated at cytoplasmic sites where invadopodia typically occur (*arrowheads*). Bar, 10 μ m. **B.** UMSCC1 cells treated with increasing concentrations of saracatinib were stained as in **A** and quantified to determine the percentage of cells that produced functional invadopodia, presented as the mean $\pm$ SEM. All treatment groups were significantly different from each another based on a one-way ANOVA ($p < 0.05$) except 0 and 0.01 μ M, and 0.5 and 1.0 μ M pairs. **C.** Saracatinib decreases the ability of UNSCC1 cells to degrade ECM. The percentage of gelatin degradation per cell area for the cell population analyzed in B is shown with the mean $\pm$ SEM.

Figure 5: MMP9 secretion and ECM degradation activity in HNSCC cells is blocked by saracatinib. **A.** Localization of MMP9-containing vesicles in UMSCC1 invadopodia. *Top:* UMSCC1 cells plated on FITC-coated gelatin coverslips for 2 h were fixed and labeled with antibodies against cortactin and MMP9. The merged image indicates areas of cortactin and MMP9 co-localization (*yellow*; *white arrows*) that correspond with sites of focal gelatin degradation (*black arrows*). Bar; 10 mM, Asterisk; regions of global

matrix degradation due to secreted protease activity. *Bottom*: Magnified view of indicated *Top* region. **B.** Inhibition of MMP9 secretion by saracatinib. Total cell lysates (*cell*) and aliquots of normalized conditioned media containing secreted MMPs (*sec*) from 1483 and UMSCC1 cells treated with increasing doses of saracatinib (*bottom*) were assayed for the presence of MMP2 and MMP9 by immunoblotting. Band intensities relative to control (0 mM) are shown for each treatment condition; secreted MMP2 was not detected and therefore not quantified. **C.** Gelatin zymography of MMP9 activity. Representative zymograms from conditioned media of 1483 or UMSCC1 cells cultured with the indicated saracatinib concentrations (*bottom*). DMEM was used as a negative control (M), DMEM containing 10% FBS (FBS) was used as a positive control for zymography. *Graphs*, densitometric analysis of MMP9 zymography. Percentage of MMP9 gelatin clearing is represented and the mean $\pm$ SEM for each cell line from three independent experiments.

Ammer et al. Figure 1

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Supplementary Figure 1: Specificity of human anti-pY421 cortactin antibody. SYF fibroblasts lacking Src, Yes and Fyn were cotransfected with the temperature-sensitive Src allele La29 along with expression vectors encoding recombinant wild-type human cortactin (WT) or cortactin mutants containing tyrosine-phenylalanine substitutions at the indicated Src-targeted amino acids and held at the non-permissive temperature (41°C) or switched to the activating permissive temperature (35°C). TYM; triple tyrosine-phenylalanine cortactin mutant lacking all three Src-targeted sites. Cells were lysed and analyzed by Western blot analysis with anti-pY421 cortactin (*left*). The blot was stripped and reprobbed with anti-cortactin monoclonal antibody 4F11 (*right*). The position of molecular weight markers is noted on the *left* in kilodaltons.

Study 2: Oncogenic Src requires a wild-type counterpart to regulate invadopodia maturation

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Summary

The proto-oncogene Src tyrosine kinase (Src) is overexpressed in human cancers and is a current target of anti-invasive therapies. Src activation is an essential catalyst of invadopodia production. Invadopodia are cellular structures that mediate extracellular matrix (ECM) proteolysis, allowing invasive cell types to breach confining tissue barriers. Invadopodia assembly and maturation is a multistep process, first requiring the targeting of actin-associated proteins to form pre-invadopodia. Pre-invadopodia subsequently mature by recruitment and activation of matrix metalloproteases (MMPs) that facilitate ECM degradation. We demonstrate that active, oncogenic Src alleles require the presence of a wild-type counterpart to induce ECM degradation at invadopodia sites. In addition, we identify the phosphorylation of the invadopodia regulatory protein cortactin as an important mediator of invadopodia maturation downstream of WT Src. Distinct phosphotyrosine-based protein binding profiles in cells forming pre- and mature invadopodia were identified by SH2-domain array analysis. These results indicate that while elevated Src kinase activity is required to target actin-associated proteins to pre-invadopodia, regulated Src activity is required for invadopodia maturation and matrix degradation activity. Our findings describe a previously unappreciated role for proto-oncogenic Src in enabling the invasive activity of constitutively active Src alleles.

Introduction

Src is the first described proto-oncogene and a current target for anti-invasive compounds in clinical trials (Brunton and Frame, 2008; Yeatman, 2004). Src participates in a vast array of cellular functions that include the regulation of cell proliferation, adhesion, migration, and invasion (Guarino, 2010; Thomas and Brugge, 1997). In normal cells, Src activity is tightly controlled through intramolecular regulation, subcellular localization and protein expression levels. Elevated or aberrant Src activity is a potent mediator of cell transformation and tumor progression, and is associated with the majority of human cancers including head and neck squamous cell carcinoma (HNSCC) (Summy and Gallick, 2003).

One of the most evident phenotypes of Src transformed cells is the formation of actin rich ventral membrane protrusive structures that actively degrade ECM (Chen, 1989). These structures, termed invadopodia, are made by metastatic cancer cells and Src-transformed fibroblasts (Linder, 2009). Invadopodia spontaneously form in tumor cells directly cultured from patient samples and are hypothesized to facilitate breaching of basement membranes during metastasis (Clark et al., 2007; Yamaguchi and Condeelis, 2007). Src activity is absolutely necessary for invadopodia formation and function, with the level of tyrosine phosphorylation at invadopodia positively correlating with the degree of ECM degradation (Bowden et al., 2006; Spinardi et al., 2004). The molecular components that make up invadopodia include proteins that facilitate actin-assembly, membrane trafficking, and focal degradation. Src substrates participate in all of these functions and include the proteins cortactin (Bowden et al., 1999), N-WASp

(Yamaguchi et al., 2005), dynamin2 (Baldassarre et al., 2003), AMAP1 (Onodera et al., 2005), paxillin (Bowden et al., 1999), p130Cas (Brabek et al., 2004), Tsk5 (Seals et al., 2005), p190RhoGAP (Nakahara et al., 1998), AFAP110 (Gatesman et al., 2004), and caveolin (Yamaguchi et al., 2009). Several studies have evaluated Src activity in invadopodia formation through the ectopic expression of constitutively active Src alleles (Artym et al., 2006; Oser et al., 2009; Stylli et al., 2009). However, these activating Src mutants are rarely found in human tumors, which instead typically contain increased levels of wild-type (WT) Src expression and/or aberrant WT Src activity due to hyperactivation of upstream pathways (Yeaman, 2004). The role of WT Src in invadopodia formation and function is unknown.

Invadopodia assembly has been proposed to involve several stages that regulate the progression from pre-invadopodia (non-degradative) complexes to functional, mature invadopodia containing active MMPs that degrade ECM (Artym et al., 2006; Oser et al., 2009). In current models of invadopodia formation, filamentous (F)-actin and the actin-associated protein cortactin are recruited to sites of matrix adhesion, resulting in pre-invadopodia complexes (Artym et al., 2006). The membrane-bound matrix metalloproteinase MT1-MMP (MMP 14) is subsequently recruited to these sites, allowing for matrix degradation and invadopodia maturation. Cortactin is an actin-binding protein phosphorylated by Src kinase (Head et al., 2003), and is a core invadopodia component. Knockdown of cortactin expression results in decreased invadopodia formation (Artym et al., 2006; Webb et al., 2007) and MMP secretion (Clark and Weaver, 2008; Clark et al., 2007), whereas phosphorylation of cortactin is important in regulating matrix degradation at invadopodia (Ayala et al., 2008).

Recent work on discerning the molecular mechanism regulating actin polymerization prior to MMP recruitment has identified dynamic regulation of cortactin phosphorylation/dephosphorylation downstream of Src as central to this process (Oser et al., 2009). In this model, cortactin sequesters the actin-severing protein cofilin within pre-invadopodia. Tyrosine phosphorylation of cortactin releases cofilin, which in turn accelerates actin polymerization through the severing of existing invadopodial actin filaments. In addition, tyrosine phosphorylation of cortactin creates docking sites for the adaptor protein Nck1, which binds and activates the Arp2/3 activator N-WASp. The combined effect of cofilin activation and N-WASp-mediated Arp2/3 activity serves to increase actin polymerization as pre-invadopodia mature and obtain the ability to degrade ECM. Dephosphorylation of cortactin is proposed to stabilize maturing invadopodia by downregulating actin polymerization through liberation of the Nck1/N-WASp complex, coupled with rebinding of inactive cofilin. While these data implicate cycles of cortactin phosphorylation/dephosphorylation as critical in invadopodia maturation, how the phosphorylation of cortactin and other invadopodia maturation-associated proteins is spatially and temporally orchestrated through upstream kinase-based signaling to drive invadopodia maturation is unknown. In this study we have determined that the presence of endogenous, regulated WT cSrc is required for pre-invadopodia complexes induced by oncogenic Src activity to mature into degradative invadopodia. In addition, we distinguish cortactin phosphorylation downstream of WT cSrc as an important mediator of the maturation process.

Results

Elevated Src activity regulates invadopodia formation in HNSCC cell lines.

The introduction of constitutively-active viral Src (vSrc) or cellular Src (Src527F) has been examined in invadopodia formation in cancer cell lines (Artym et al., 2006; Buschman et al., 2009; Oikawa et al., 2008; Stylli et al., 2009), but the role of endogenous cSrc in invadopodia function is unclear. We analyzed a panel of HNSCC lines for endogenous cSrc activity and total cSrc protein levels (Fig. 1A), as well as the ability of these lines to form spontaneous invadopodia on FITC-gelatin matrix (Fig. 1B, supplementary material Fig. S1A). Two (UMSCC1 and OSC19) out of the six tested lines generate invadopodia that can be identified by the colocalization of actin and cortactin-rich yellow aggregates in merged images (Fig. 1B, white arrows) coinciding with areas of focal gelatin degradation (black arrows). UMSCC1 and OSC19 cells have substantially elevated cSrc expression and cSrc activity compared to the UMSCC2, 1483, and MSK921 invadopodia-null lines (Fig. 1A). The FADU cells have elevated cSrc activity but fail to generate spontaneous invadopodia, suggesting that elevated Src expression alone is not sufficient to drive invadopodia biogenesis in this line. However, invadopodia are formed and matrix degradation occurs in all the HNSCC cell lines that do not form spontaneous invadopodia (UMSCC2, 1483, FADU, and MSK921) following exogenous Src527F expression (Fig. 1B, supplementary material Fig 1B). These data suggest a cell line-specific threshold of Src activity (highest in FADU cells) that must be reached to support invadopodia formation and matrix degradation. These results are consistent with elevated Src activity driving invadopodia biogenesis, and are in agreement with previous work with small molecule Src inhibitors in HNSCC cell lines decreasing invadopodia formation and matrix degradation in a dose dependent manner

(Ammer et al., 2009). Similar results were also found in breast cancer cells (Pichot et al., 2009).

Endogenous Src expression is required for efficient invadopodia-based matrix degradation in HNSCC cells expressing constitutively-active Src. To test the effect of depleting endogenous cSrc on HNSCC invadopodia formation and function, cSrc expression was knocked down in UMSCC1 cells by RNA interference (SrcSi) (Fig. 2A) and assayed for invadopodia formation and gelatin degradation (Fig. 2B). Endogenous cSrc was depleted by 58% two days, and 70% three days post-transfection. SrcSi cells had no statistically significant difference in the number of cells with invadopodia or the number of invadopodia per cell (Fig. 2C, top and middle panels). However Src knockdown cells exhibited a 2.3-fold decrease in gelatin degradation compared to control cells (Ctl) (Fig. 2C, lower panel). Rescue of WT Src expression in SrcSi cells (SrcSi+WT) restored matrix degradation to above control cell levels (1.7-Fold) (Fig. 2C). Increases above control are presumably attributed to the additive effect of remaining endogenous Src from incomplete knockdown coupled with the modest overexpression of the SrcWT construct (Fig. 2B).

We also examined the effect of silencing endogenous cSrc in the presence of constitutively active Src (Fig. 2B, C). Consistent with previous reports (Artym et al., 2006; Oser et al., 2009), expression of Src527F in control cells (Ctl+527) or SrcSi cells (SrcSi+527F) resulted in increases in cells with invadopodia (~30%) and the number invadopodia per cell (~36%). Surprisingly, cSrc knockdown markedly blunted the increased degradation due to Src527F expression, a 2.5-fold (SrcSi+Src527F cells) increase compared to a 4.8-fold (Ctl+527F) increase over control cells (Fig. 2C). These

results demonstrate that constitutively active Src cannot completely rescue endogenous cSrc function in regulating matrix degradation. Collectively these data indicate that while increased Src activity enhances invadopodia formation as previously reported, the presence of endogenous cSrc is required for optimal matrix degradation. Similar results were found in OSC19 cells (supplementary material Fig. S2).

Constitutively active Src is sufficient to promote invadopodia formation but not ECM degradation in Src-null cells. Complete cSrc knockdown in our HNSCC lines is technically problematic, and additional Src family kinases (Yes and Fyn), with unknown functions in invadopodia biology, are present and maintained in OSC19 and UMSCC1 cell lines treated with Src RNAi (Fig. 3A). We therefore utilized Src/Yes/Fyn-deficient (SYF) fibroblasts to further evaluate the role of WT Src in invadopodia function. A GFP-tagged temperature-sensitive mutant of vSrc (*tsLa29-GFP*) was generated and expressed in SYF cells to dynamically regulate Src activity and invadopodia formation. Src kinase activation occurs within 15 min when cells are switched from the non-permissive temperature (41°C) to the permissive temperature (35°C) (Fig. 3B). vSrc inactivation occurs within 30 min when cells are shifted back to 41°C. Activation of vSrc leads to the phosphorylation of cortactin on tyrosine 421, indicating that *tsLa29-GFP* regulates phosphorylation of a known downstream Src target critical for invadopodia assembly. In agreement with previous reports (Walker et al., 2007), invadopodia formation is induced when cells expressing *tsLa29-GFP* are switched to the permissive temperature (Fig. 3C). These invadopodia are enriched with active vSrc and phosphorylated cortactin (Fig. 3C, supplementary material Fig. S3A). As reported in other invadopodia forming cell systems (Artym et al., 2006; Ayala et al., 2008; Clark et

al., 2007; Webb et al., 2006), inhibition of cortactin expression by siRNA diminishes the ability of *tsLA29*-GFP to induce invadopodia formation at the permissive temperature (supplementary material Fig. S3B, C).

To evaluate the functionality of invadopodia in this system, we plated SYF cells on FITC-gelatin coated coverslips to assay ECM degradation. Invadopodia induced by *tsLa29*-GFP in the Src-null fibroblasts fail to degrade the ECM at periods up to and >48 h, indicating they remain in a pre-invadopodia state (Fig. 3D). Experiments with an untagged vSrc produced a similar result, ruling out improper activation or localization related to the addition of GFP. We also attempted to rescue invadopodia maturation in the SYF cells with *tsla29*-vSrc. Manipulating *tsla29*-vSrc activity over the 24 h incubation period by switching cells from permissive to the non-permissive temperatures did not result in invadopodia maturation (data not shown). These results demonstrate that constitutively active vSrc activity is responsible for the induction of the initial phosphorylation cascade that drives recruitment of invadopodia components to form pre-invadopodia complexes, but these vSrc induced complexes are insufficient to direct matrix degradation in SYF cells.

Wild-type Src kinase is necessary for invadopodia maturation. Based on our findings we hypothesized that either: 1) Another ubiquitously expressed Src family kinase (Yes and/or Fyn) absent from SYF cells is required for invadopodia maturation in addition to active Src, or 2) WT Src or “regulated” Src must also be present with active Src for degradation of ECM to occur. To test these hypotheses, we utilized a SYF cell line with two copies of WT Src genetically reintroduced to restore normal WT Src expression (SYF^{+/+}; Fig. 3A, 4A). The percentages of cells forming invadopodia and the

percentage of invadopodia-forming cells degrading matrix were assessed following introduction of vSrc or Src527F. Transfection with activated Src constructs in the form of *tsLa29* or Src527F induces mature matrix-degrading invadopodia in SYF^{+/+} cells, in contrast to pre-invadopodia formation in SYF cells (Fig. 4B). Approximately 55% of SYF^{+/+} cells forming invadopodia contain invadopodia that actively degrade matrix, compared to 2% of SYF cells (Fig. 4C). There is no difference in the percentage of cells forming invadopodia (pre and mature) in SYF or SYF^{+/+} cells expressing Src527F (Fig. 4C), similar to results in UMSCC1 cells (Fig. 2C). Also, the level of general phosphotyrosine-containing proteins localized to invadopodia is unchanged in SYF527F or SYF527F^{+/+} (Fig. 4B). Taken together, these results support that catalytically active Src alone promotes the assembly of pre-invadopodia complexes and targets tyrosine phosphorylation of proteins within these structures, but WT Src is necessary for pre-invadopodia maturation required to induce ECM degradation.

To further confirm these findings, WT Src expression was transiently restored in SYF cells and assayed for ECM degradation. Monitoring co-expression of Src527F and SrcWT was achieved by creating carboxyl-terminal linker fusions with mCherry (Src527F-mCh) and cerulean (Src-Cer) fluorescent proteins (see supplementary material Fig. S4). Transfection efficiency of the co-transfected Src constructs is consistently greater than 90% and imaging reveals that nearly all SYF cells express both Src alleles (supplementary material Fig. S5A). In SYF cells expressing SrcWT alone, Src has a perinuclear localization consistent with previous reports (Sandilands et al., 2004) (supplementary material Fig. 4B, C). Co-expression of Src527F with SrcWT results in recruitment of SrcWT to invadopodia where it colocalizes with Src 527F (Fig.

5A). In addition, cells co-expressing these constructs regain the ability to degrade ECM (Fig. 5B, C). To further verify the functional requirement for WT Src in invadopodia maturation, we conducted Src WT-specific staining of SYF cells containing Src527F. Src-WT colocalizes with cortactin to areas of ECM degradation, further demonstrating that Src-WT localizes to mature invadopodia (Fig. 5D, upper panels). In addition, direct visualization of WT Src-Cer and Src527F-mCh in SYF cells demonstrates a concentration of Src-Cer at areas of ECM degradation, with Src527F-mCh localized to the same vicinity (Fig. 5D, lower panels).

Since the localization of endogenous Src to invadopodia is required for invadopodia maturation, we determined if catalytically-inactive Src could substitute for WT Src and rescue ECM degradation in SYF cells. This result would suggest that two separate and distinct pools of Src (constitutively-active and kinase-inactive) were necessary and sufficient for maturation. To test this hypothesis, constitutively-active Src527F-mCh was coexpressed with a cerulean-tagged kinase-inactive Src (Src295M-Cer) in SYF cells. Like SrcWT, Src295M is largely perinuclear when expressed alone in SYF cells (supplementary Fig. 4B, C), but is recruited to invadopodia when co-expressed with Src527F (Fig. 5A). However, Src295M fails to rescue invadopodia maturation (Fig. 5B, C), indicating that catalytically inactive (Src295M) Src does not substitute for WT Src function. Since constitutively active and kinase dead Src alleles cannot support invadopodia maturation, this suggests that WT Src kinase activity is dynamically regulated to promote invadopodia maturation. Along these lines, cell staining for active Src (pY418) localizes to pre-invadopodia in SYF cells and mature invadopodia in SYF^{+/+} cells (Fig. 5E), ruling out the possibility that catalytically-active

Src drives invadopodia assembly, is inactivated, and remains inactive during maturation. Taken together, these results demonstrate that regulated WT Src kinase activation/inactivation within pre-invadopodia complexes is necessary to govern the downstream signaling events required for invadopodia maturation and ECM degradation.

Regulated Src activity directs cortactin phosphorylation dynamics to control invadopodia maturation. In proposed models of invadopodia maturation, dynamic cortactin tyrosine phosphorylation is required for pre-invadopodia maturation (Oser et al., 2009). In order to identify a potential mechanism of invadopodia maturation affected by Src activation/inactivation, we assessed the role of Src kinase activity on cortactin phosphorylation. In the SYF/SYF^{+/+} system, cortactin phosphorylated on tyrosine 421 localizes to pre- and mature invadopodia (Fig. 6A) suggesting that cortactin phosphorylation is important in pre-invadopodia assembly and invadopodia maturation. This is consistent with a well-described role for cortactin in invadopodia maturation in other systems (Artym et al., 2006; Clark et al., 2007; Oser et al., 2009). Stable cell lines were generated expressing endogenous levels of wild-type cortactin (SYF^{+/+} CortWT) or a cortactin mutant in which the three Src-targeted tyrosine residues (421, 470, 486) are mutated to phenylalanine (SYF^{+/+} CortTYM) (Fig. 6B). Endogenous murine cortactin was silenced with siRNA (CortSi, knockdown >90%), resulting in the exclusive expression of WT or TYM human cortactin (Fig. 6C). SYF^{+/+} CortWT and SYF^{+/+} CortTYM cell lines treated with cortactin siRNA were transfected with Src527F to promote invadopodia formation. WT human cortactin expression rescued the inhibitory effects of cortactin knockdown on invadopodia formation, resulting in the formation of mature invadopodia

(~60% of total cells with invadopodia, Fig. 6D, E). However, only ~10% of SYF^{+/+} CortTYM cells produce degrading invadopodia. Consistent with previous reports (Oser et al., 2009), there were no differences observed in the percentage of cells forming actin/cortactin aggregates in SYF^{+/+} CortWT and SYF^{+/+} CortTYM cells, demonstrating that cortactin is targeted to pre-invadopodia independent of tyrosine phosphorylation. However, expression of CortTYM in SYF^{+/+} cells completely blocks the upstream function of WT Src, rendering the SYF^{+/+} Src527F cells with a degradation profile similar to that seen in cells lacking WT Src (SYF Src527F, Fig. 4B, C). These results indicate that there is differential phosphotyrosine signaling in SYF cells with WT Src that supports invadopodia maturation downstream of constitutively-active Src.

Cells forming pre- and mature invadopodia have distinct phosphotyrosine signatures. To examine if distinct tyrosine phosphorylation signatures occur in cells that form pre- and mature invadopodia, we conducted a non-biased, comprehensive and quantitative SH2-domain screen (Machida et al., 2007) to identify differences in potential phosphotyrosine binding proteins under conditions of pre- and mature invadopodia formation (Fig 7, supplementary material Fig. S6). Non-transfected SYF and SYF^{+/+} cells had minimal differences in SH2 domain binding profiles. Expression of Src527F in either cell type enhanced overall SH2 domain signal binding intensity, indicating a broad increase in phosphotyrosine signaling and creation of new SH2 domain docking sites. This result is anticipated with expression of constitutively-active Src. However, cells that form pre-invadopodia (SYF Src527F) have distinct differences in their SH2 binding intensity from cells that form functional mature invadopodia (SYF^{+/+} Src527F) (bottom row, difference). The variations in binding intensity that arise in SYF

and SYF^{+/+} cells expressing Src527F indicate a fundamental mechanistic difference in the concentration of phosphotyrosine binding sites for several SH2 domain containing proteins likely playing key roles in invadopodia maturation. Interestingly, tyrosine phosphorylated cortactin is known to interact with several high intensity “hits”, including Arg, Abl (Boyle et al., 2007), Fer (El Sayegh et al., 2005), Crk (Bougneres et al., 2004), and Nck (Tehrani et al., 2007), consistent with its role in invadopodia maturation. Experiments to elucidate additional proteins involved in these signaling complexes are currently underway.

Discussion

In this study we investigated the role of endogenous or WT cSrc, and the interplay between constitutively-active Src and cellular Src in invadopodia formation. Previous studies on Src in invadopodia formation have exclusively manipulated Src activity in cells containing WT cSrc. Tumor cells that form spontaneous invadopodia presumably have upstream oncogenic signals such as over-activation/expression of epidermal growth factor receptor (EGFR) that drives cSrc activation (Xue et al., 2006). Accordingly, increased Src kinase activity (through overexpression of constitutively active Src, or overexpression of WT Src) in tumor cells that form spontaneous invadopodia is associated with increased invadopodia formation and matrix degradation (Artym et al., 2006; Oser et al., 2009). Unlike previous reports (Oser et al., 2009), we did not observe that tumor cells overexpressing constitutively active Src have less degradation per invadopodia than spontaneous invadopodia formed in control cells (Fig. 2, supplementary material Fig 2). However, these differences may be attributed to a much larger capacity for invadopodia formation in UMSCC1/OSC19 cells compared to

MtLn3 cells (~25 vs. ~two invadopodia formed in control cells, respectively). In HNSCC cells and Src-null fibroblasts, we propose that constitutively-active Src acts as an oncogenic “trigger” that promotes pre-invadopodia formation, whereas WT Src acts downstream to direct invadopodia stability and maturation. Though models of spontaneous invadopodia formation are invaluable to the field, our novel finding that Src cycling is critical for invadopodia maturation could only be completely uncovered through the use of the SYF/SYF^{+/+} system.

In this study, we show that WT Src is indispensable for invadopodia maturation driven by elevated Src activity. We hypothesize that distinct, temporally and spatially regulated Src function is necessary to regulate the phosphorylation of cortactin. It is likely that Src regulates other scaffolding proteins important in invadopodia stability and maturation, such as dynamin (Caldieri et al., 2008), Tks5 (Seals et al., 2005), IQGAP1 (Sakurai-Yageta et al., 2008), and paxillin (Bowden et al., 1999). It is possible that Src is activated prior to its localization with downstream actin-associated substrates in pre-invadopodia. Once pre-invadopodia assembly is complete, Src is inactivated (presumably by COOH-terminal Src kinase (Csk) acting on pY527 (Okada et al., 1991)) and released to regulate additional proteins involved in actin dynamics and MMP delivery to invadopodia. In this way, Src may act as a regulator of several sequentially coordinated protein interaction events that directs the diverse array of cellular processes at invadopodia, including actin assembly, membrane trafficking, and ECM degradation.

It is known that invadopodia produced by cancer cells and transformed fibroblasts use similar signaling pathways and contain many of the same proteins as podosomes produced by osteoclasts, dendritic cells and macrophages (Linder, 2009).

Src-null osteoclasts have been used to study the role of Src in podosome assembly and dynamics (Destaing et al., 2008). WT Src and Src527F were both able to restore normal podosome organization, whereas Src 295M did not. These data in podosomes support our findings in invadopodia, but since this report did not address the functionality of these cells to degrade ECM, it is not known if WT Src is needed for maturation in this system. These results are particularly interesting since Src has been suggested to act as a “molecular switch” to regulate dynamin-Cbl signaling complexes (Bruzzaniti et al., 2005) and as having an important adapter (kinase-independent) function (Bruzzaniti et al., 2009) in osteoclast podosomes. Manipulation of Src activity in WT osteoclasts also suggest a dual function for Src in regulating actin dynamics through cortactin phosphorylation in podosome assembly and subsequent maturation into higher organized structures known as sealing zones (Luxenburg et al., 2006).

We identify cortactin as a key regulator of invadopodia maturation downstream of WT Src activity. We show that constitutively-active Src is sufficient to target cortactin to pre-invadopodia (Fig. 3C, 4B), but cortactin is targeted independent of its tyrosine phosphorylation (Fig. 6D, E). This is in agreement with previous reports demonstrating that dephosphorylated cortactin acts as a scaffold to recruit Arp2/3, N-WASp, and cofilin (Oser et al., 2009). Interestingly, cortactin phosphorylated at tyrosine 421 is enriched in pre-invadopodia (SYF Src527F) and in matrix degrading mature invadopodia (SYF^{+/+} Src527F) (Fig. 6A, supplementary material Fig. S3A). In addition, tyrosine phosphorylation of cortactin is required for invadopodia maturation and ECM degradation (Fig. 6 D, E; (Ayala et al., 2008; Desmarais et al., 2009; Webb et al., 2007)). These data are consistent with phosphorylation of cortactin occurring before

ECM degradation. However, in our SYF/SYF^{+/+} model this initial cortactin phosphorylation is not sufficient to drive maturation in the absence of WT Src, suggesting that cortactin must be dephosphorylated before maturation can occur. Our proposed mechanism of dynamic Src activity on cortactin phosphorylation in invadopodia maturation is consistent with the model of cyclical cortactin phosphorylation regulating actin polymerization and invadopodia stabilization recently proposed by the Condeelis laboratory (Oser et al., 2009).

Here we show that Src activation and inactivation regulates cortactin phosphorylation during invadopodia maturation. Future studies will be required to determine if Src kinase is directly responsible for initially phosphorylating cortactin to promote invadopodia assembly, or in subsequent step(s) to regulate maturation (Huang et al., 1998). Fer (El Sayegh et al., 2005) and Abl family kinases (Boyle et al., 2007) are also present in SYF cells and may act downstream of Src to regulate one or more of these steps. It also follows that tyrosine phosphatases are critically important in the regulation of invadopodia maturation, since cortactin and other possible targets downstream of Src kinase activity in our system require dynamic cycles of phosphorylation and dephosphorylation to drive ECM degradation. Protein tyrosine phosphatase 1B is a good candidate for this process since it is known to regulate Src in invadopodia dynamics (Cortesio et al., 2008) and has subsequently been shown to regulate cortactin dephosphorylation (Stuible et al., 2008). Nonetheless, we show that WT Src kinase is the critical upstream regulator of other downstream kinases/phosphatases important to these processes.

Invadopodia maturation is associated with the delivery of MMPs to invadopodia sites (Artym et al., 2006; Clark et al., 2007). MT1-MMP is cited as the key MMP regulating ECM degradation at invadopodia (Poincloux et al., 2009), and Src kinase activity is known to regulate phosphorylation of MT1-MMP and proteins associated with its trafficking to the cell membrane (Nyalendo et al., 2008; Nyalendo et al., 2007). Future studies should address whether WT Src is necessary to control trafficking to, or activation of, MT1-MMP at invadopodia. These studies are further warranted since it has been proposed that a major role of cortactin in invadopodia function involves the targeting and delivery of MMPs to invadopodia enhancing ECM degradation (Clark and Weaver, 2008; Clark et al., 2007).

This report identifies a dominant oncogene that requires the proto-oncogenic complement to reach its full spectrum of transforming functionality. Unlike tumor suppressor proteins that often require loss of non-mutated alleles, little is known about the potential effect of the presence or absence of proto-oncogenes on their oncogenic counterparts during tumorigenesis. A function in Ras-induced tumorigenesis has been described for Ras proto-oncogenes (Singh et al., 2005), where WT Ras has been reported to regulate the functioning of oncogenic Ras with regards to cell proliferation. Our results are consistent with the majority of solid human tumors that contain elevated cSrc expression or specific activity due to hyperactivation of upstream regulatory pathways rather than activating point mutations (Yeatman, 2004). To our knowledge this is the first description of such a finding. Similar mechanisms may be required for other transforming kinases involved in driving tumor progression.

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Materials and Methods

Cell culture

HNSCC cell lines UMSCC1, FADU and OSC19 were obtained from Jeffery Myers (MD Anderson Cancer Center). These lines along with UMSCC2 (Tom Carey, University of Michigan), 1483 (David Raben, University of Colorado), and MSK921 (Peter Sacks, New York University) were cultured as previously described (Rothschild et al., 2006). SYF ($\text{Src}^{-/-}\text{Yes}^{-/-}\text{Fyn}^{-/-}$) and SYF^{+/+} ($\text{Src}^{+/+}\text{Yes}^{-/-}\text{Fyn}^{-/-}$) cells were obtained from the American Type Culture Collection. Control non-silencing and human Src targeted siRNA (5'-AAACTCCCCTTGCTCATGTACTT-3') were from Dharmacon. OSC19 cells stably expressing vector control or Src targeted shRNA were created by infection with control or human Src specific lentivirus (Santa Cruz) and cultured according to manufacturer's instructions. SYF or SYF^{+/+} cells stably expressing GFP-tagged human cortactin (wild-type [WT] or Y421F, Y466F, Y482F [triple point mutant (TPM)]) were created using the Flp In™ system (Invitrogen). These lines were transfected with siRNA targeting murine cortactin (5'-GCTTCGAGAGAATGTCTTC-3') (siCTTN, Dharmacon). For transient transfections, 3×10^6 cells were incubated with 2 μ g plasmid construct or siRNA. Fibroblast lines were transfected with the Nucleofector I device (Amaxa

Biosystems), and HNSCC lines were transfected with TurboFect™ transfection reagent (Fermentas).

Immunofluorescence labeling and confocal microscopy

Cells were plated on FITC-gelatin (Sigma) coated coverslips as described (Artym et al., 2006) for 12-24 hours. Cells were fixed with fresh 4% formaldehyde and permeabilized with 0.4% Triton-X/PBS. Primary antibodies were diluted in 5% BSA/PBS. Antibodies used were: cortactin 4F11, cortactin EP1922Y (Novus Biologicals), human cort-pY421 (Ammer et al., 2009), Src-pY418 (Biosource), Src GD11 (Upstate), GFP/Cerulean 3E6 (Invitrogen), pTyr-100 (BD Biosciences), Alexa Fluor 405 goat anti-rabbit and Alexa Fluor 647 goat anti-mouse (Molecular Probes). F-actin was labeled with rhodamine-conjugated phalloidin (Molecular Probes). Cells were mounted in Fluoromount-G (Southern Biotech) and imaged with a Zeiss LSM510 confocal microscope using AIM software (Carl Zeiss MicroImaging). A Nikon Swept-Field using Nikon Elements software (Nikon Instruments Inc.) was used for imaging of Src-Cer.

Invadopodia and matrix degradation assays

Cells with invadopodia were identified by the presence of at least one actin/cortactin aggregate within the cell ($N \geq 300$). The number of invadopodia per cell was calculated by counting the number of actin/cortactin aggregates within invadopodia forming cells ($N \geq 150$). Degradation per cell area was analyzed using ImageJ as described previously (Clark et al., 2007). Cells with invadopodia degrading matrix were identified by at least one actin/cortactin aggregate colocalizing with a “dark hole” corresponding to degraded

FITC-matrix ($N \geq 50$). Data were pooled from multiple independent experiments; N represents the number of cells analyzed within each experimental group.

Antibodies and Western blotting

Western blotting of cell lysates was conducted as described (Rothschild et al., 2006). The following antibodies were used: 4F11, Src clone GD11 (Upstate); β -actin (Calbiochem); Living Colors GFP clone JL-8 (BD); Cort-pY421, Src-pY418 (Biosource); avian Src clone EC10 (Millipore), and Yes, Fyn (Cell Signaling).

Plasmids

The Src-GFP linker constructs (WT, 527F, and 295M) were a gift from Margaret Frame (The Beatson Institute for Cancer Research, Glasgow, United Kingdom). Substitution of green fluorescent protein (GFP) with cerulean or mCherry fluorescent protein was accomplished through digestion of Src-pEGFP-N1, pmCherry-C1, and mCerulean-C1 fluorescent vectors with AgeI and BsrGI. The resulting mCherry and mCerulean fragments (~700 bps) were ligated into the GFP-digested Src-containing pEGFP-N1 vector (BD). Temperature-sensitive vSrc (*tsLA29*) was subcloned from pCMV-*tsLA29* vector into EGFP-N1 using unique EcoRI and BamHI restriction sites.

Human cortactin constructs.

A single-stranded primed cDNA library (Invitrogen) was used for cloning the human cortactin (CTTN) cDNA. The cDNA was PCR amplified to produce a 965bp KpnI-HincII fragment and a 688bp HincII-EcoRI fragment. Fragments were ligated into KpnI/EcoRI digested pcDNA3FLAG2AB to generate the 1653bp full-length CTTN cDNA. The

CTTN triple tyrosine mutant (TYM) was generated using the QuickChange kit with primers designed to alter codons 421, 470 and 486 from tyrosine to phenylalanine and confirmed by DNA sequencing. WT and TYM CTTN cDNAs were subsequently amplified as EcoRI/KpnI fragments and subcloned into pEGFP-N1 (WT) or pAcGFP-N1 (TYM). GFP-CTTN WT and TYM fragments were PCR amplified and subcloned into pEF5/FRT/V5-D-TOPO (Invitrogen) and stable SYF and SYF^{+/+} cell lines generated using the Flp-In system according to manufacturer's instructions.

Immunoprecipitations

Cells were lysed in NP40 Buffer (20 mM Hepes-KOH, pH 7.8, 50 mM KCl, 1 mM EDTA, and 1% NP40). Anti-cortactin (4F11, 5ug) was incubated with 0.5 mg of clarified lysates for 2 h at 4°C, then incubated with 40 µl of Protein A/G Beads (Thermo Scientific) for 1 h at 4°C. Immune complexes were collected by centrifugation, washed twice with NP40 Buffer, separated by SDS-PAGE, and Western blotted with antibodies as described.

SH2/PTB binding assay

SH2/PTB domain binding assay was performed as described (Dierck et al., 2009; Machida et al., 2007). Briefly, SYF cell lysates were spotted in duplicate on a nitrocellulose membrane in register with the wells of a 96-well chamber plate. Each well was separately incubated with purified GST-SH2 or -PTB domains (~100 nM) for 2 h. Probe binding was detected by enhanced chemiluminescence (ECL) (PerkinElmer) and digitally captured (Kodak Image Station). Two independent experiments were performed in duplicate, providing four quantifiable data points for each probe. The array images

were background-subtracted and the integrated density of each spot was measured using ImageJ (v1.40).

Statistical analysis

Differences in mean values between groups were evaluated using a student's T-test (2 groups) or a one-way ANOVA (multiple groups) followed by Scheffe post hoc testing.

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Figure legends

Fig 1. Src activity regulates invadopodia formation in HNSCC lines. (A) Protein levels of active cSrc (Src-pY⁴¹⁸), total cSrc (Src), and β -actin (loading control) in HNSCC lines. For quantification of active Src and total Src, expression levels were normalized to MSK921 cells, a line with low Src activity that does not form invadopodia. Multiple bands in the Src-pY⁴¹⁸ panel presumably represent additional Src Family kinases; see Fig. 3A. (B) HNSCC cell lines with or without Src-527F were incubated on FITC-gelatin (pseudocolored white) coverslips for 12 hrs and labeled with TRITC-phalloidin (red) and anti-cortactin (green). Invadopodia are identified by the yellow aggregates in the merged images of actin and cortactin (white arrows) that localize with the dark holes in the FITC-gelatin (black arrows). Bars, 10 μ m.

Fig 2. Inhibition of endogenous Src expression decreases matrix degradation independent of changes in invadopodia number or increased Src activity. (A) UMSCC1 cells transfected with Src SiRNA (SrcSi) or a non-targeting SiRNA (Ctl) were evaluated for Src knockdown two and three days post-transfection by anti-Src Western blotting. β -actin blotting was used as a loading control. (B) Cell lysates from UMSCC1 cells transfected with non-targeting SiRNA (Ctl) or Src SiRNA (Si) alone, or in combination with cerulean-tagged SrcWT (WT) or Src-527F (527) were evaluated by immunoblotting. Lysates were probed with anti-Src and β -actin antibodies. Filled arrowheads point to exogenously expressed Src (WT or 527F), open arrowheads denote endogenous Src (C) Representative confocal images of UMSCC1 cells transfected with non-targeting SiRNA (Ctl) or Src SiRNA (Si) alone, or in combination with cerulean-tagged SrcWT (WT) or Src-527F (527). Cells were plated on FITC-gelatin

coated (pseudocolored white) cover slips for 10 hrs and immunolabeled with TRITC-phalloidin (red) and cortactin (green). Bars, 10 μ m. (D) Quantification of the percent of cells displaying invadopodia (top), the number of invadopodia per cell (middle), and the amount of matrix degradation per cell (bottom) were examined for each line evaluated in (B). Data are represented as mean $\pm$ s.e.m., # statistically different from ctl, * groups under the bar are statically different ($p \leq 0.01$).

Fig 3. Invadopodia produced by constitutively active Src in Src/Yes/Fyn-null fibroblasts fail to degrade ECM. (A) Src family kinase expression in HNSCC and fibroblast cell lines. Clarified cell lysates from SYF, SYF^{+/+}, UMSCC1 (treated with control and SrcSiRNA), and OSC19 (expressing control vector and SrcShRNA) cells were resolved by SDS-PAGE and immunoblotted with anti-Src, anti-Yes, anti-Fyn, and anti- β -actin antibodies. (B) Time course of *tsLa29* vSrc activation and resulting cortactin phosphorylation. Cells transfected with *tsLa29* were incubated at 35°C (permissive temperature) for the indicated times and were lysed or returned to 41°C (non-permissive temperature) for 15 or 30 minutes and analyzed for Src-pY⁴¹⁸, GFP, cortactin, and cortactin-pY⁴²¹. (C) Invadopodia formation in vSrc expression cells. SYF cells were transfected with *tsLa29*-GFP (pseudocolored light blue) and incubated at 41°C or 35°C and labeled with TRITC-phalloidin (red) and cortactin (green). Cells were visualized by confocal microscopy and Z-stack sectioning. Invadopodia are visible in Z-stack images as actin/cortactin rich puncta that are several microns in length (white arrows). Bars, 10 μ m; 5 μ m (z-stacks). (D) Defective ECM degradation in vSrc expressing SYF cells. SYF cells transfected with *tsLa29*-vSrc and non-transfected UMSCC1 cells (positive control) were plated directly onto FITC-gelatin coverslips. After 24 hrs cells were labeled

with TRITC-phalloidin and anti-cortactin antibodies. Bars, 10 μ m; white arrows point to invadopodia and black arrows point to areas of degraded matrix (C and D).

Fig 4. WT Src rescues invadopodia maturation in cells expressing constitutively active Src. (A) SYF, SYF^{+/+}, and UMSCC1 cells were non-transfected (NT), transfected with Src-527F or *tsv*Src were lysed, resolved by SDS-PAGE and immunoblotted with anti-Src-pY418, anti-Src clone EC10 (only recognizes avian Src), anti-cortactin-pY421, anti-cortactin (4F11), and anti- β -actin antibodies. (B) SYF, SYF^{+/+}, and UMSCC1 cells that were non-transfected (NT), transfected with SRC-527F, or *tsLa29-v*Src were plated onto FITC-conjugated gelatin coverslips and evaluated by confocal microscopy. Cells were labeled with TRITC-phalloidin (red), cortactin (green), and anti-phosphotyrosine (light blue) antibodies. Invadopodia are identified by the yellow aggregates in the merged image of actin and cortactin in cells containing phosphotyrosine. In addition, degrading (mature) invadopodia localize with the dark holes (arrows) in the FITC-gelatin (white). (C) Quantification of the percentage of cells forming invadopodia structures (actin/cortactin aggregates, left), and the percentage of invadopodia forming cells that contain matrix degradation (mature invadopodia, right). Data are represented as mean $\pm$ s.d., * groups are statically different ($p \leq 0.05$).

Fig 5. Expression of regulated Src is necessary for ECM degradation at invadopodia. (A) Confocal imaging of SYF cells co-expressing Src-cerulean and Src527F-mCherry, or Src-295M-cerulean and Src527F-mCherry. Cells were immunolabeled with anti-cortactin and AlexaFluor 647- phalloidin. Dashed boxes represent enlarged image

regions shown below. Arrows denote invadopodia. (B) Representative fields of SYF and SYF^{+/+} cells expressing Src527F-mCherry, SYF cells co-expressing Src527F-mCherry and Src-cerulean, or SYF cells co-expressing Src527F-mCherry and Src-295M-cerulean. Cells were plated on FITC-gelatin coated cover slips for 24 hours and immunolabeled with anti-cortactin. Areas of gelatin degradation and clearing appear black against the pseudocolored white background. (C) Assessment of the % cells with forming invadopodia (actin/cortactin aggregates, left), and % of invadopodia forming cells with degraded matrix (mature invadopodia, right) from the experimental conditions shown in (B). Data are represented as mean $\pm$ s.d., * groups that are statistically different from groups without asterisks ($p \leq 0.05$). (D) Confocal imaging (upper panel) or swept field imaging (lower panel) of SYF cells co-expressing cSrc527F and Src-cerulean. Cells were plated on FITC-gelatin coated cover slips and immunolabeled with anti-cerulean (dark blue) and anti-cortactin (yellow) (colocalization appears white in merged image; upper panel) or directly imaged for Src527F-mCherry (red) and Src-cerulean (dark blue) (lower panels). Arrow indicates co-localization of Src 527F-mCherry and WT Src-cerulean at sites of gelatin degradation. (E) Confocal imaging of Src activity in SYF and SYF^{+/+} cells expressing Src527F. Cells were incubated on FITC-gelatin coated coverslips for 24 hrs, fixed and immunolabeled with TRITC-phalloidin (red), anti-pY418 Src (light blue), and anti-Src (green) antibodies. White arrows denote invadopodia; black arrows matrix degradation. Bars, 20 μ m (A); 10 μ m (A, D, E).

Fig 6. WT Src regulates cortactin phosphorylation during invadopodia maturation. (A) SYF and SYF^{+/+} cells expressing Src527F were incubated on FITC-gelatin coated coverslips for 24 hrs, fixed and immunolabeled with TRITC-phalloidin (red), human anti-

pY421 cortactin (light blue), and anti-cortactin (green) antibodies. Note that cortactin is phosphorylated in pre-invadopodia and in mature invadopodia as defined by the absence or presence of matrix degradation. (B) Validation of GFP-tagged cortactin expression in stable cell lines. Cellular extracts from SYF or SYF^{+/+} cells stably expressing GFP-CortWT, or GFP-CortTYM were resolved by SDS-PAGE and immunoblotted with an anti-cortactin (4F11) antibody. * endogenous cortactin, ** GFP-tagged cortactin (the slight mobility disparity is due to the size difference in AcGFP and EGFP tags); ratios of exogenous (WT and TYM) to endogenous cortactin is depicted under the blot. (C) Clarified lysates from SYF^{+/+} GFP-CortWT and SYF^{+/+} GFP-CortTYM cells were transfected with Cort-SiRNA alone or in combination with Src527F. Cortactin was immunoprecipitated with the anti-cortactin (4F11) antibody, immune complexes resolved by SDS-PAGE and immunoblotted with human anti-pY421 cortactin and anti-cortactin (4F11) antibodies. Total cell lysates were immunoblotted with anti- β -actin for a loading control. (D) SYF^{+/+} cells stably expressing human GFP-CortWT or GFP-CortTYM were transfected with murine cortactin-targeted siRNA to eliminate endogenous cortactin expression. Two days later cells were transfected with Src527F and plated on FITC-gelatin coated coverslips for 24 hrs to promote gelatin degradation. Cells were assessed for the percent of cells forming invadopodia (actin/cortactin aggregates) and the percent of invadopodia forming cells with matrix degradation. Data are represented as mean $\pm$ s.d., * groups under the bar are statically different ($p \leq 0.05$). Bars, 10 μ m (A); 20 μ m (B).

Fig 7. Enhanced binding of SH2 domains binding in SYF^{+/+} cells expressing Src527F. In-vitro SH2/PTB domain binding profiles for SYF, SYF^{+/+}, and SYF and SYF^{+/+}

expressing Src527F. Binding of SH2/PTB domains to cell lysates are shown as a heat map where intensity of red indicates strength of binding. Domain names are on the top row, with a rank order of binding to SYF^{+/+} Src527F to denote potential importance in invadopodia maturation. Sample labels are on the left side. Difference = Signal[SYF^{+/+} Src527F] - Signal[SYF Src527F].

Supplemental Figure legends

Fig. S1. Confocal imaging of additional HNSCC cell lines assayed for Src activity and invadopodia forming capability in Fig 1A. (A) The FADU and MSK921 HNSCC cell lines were incubated on FITC-coated gelatin (white) coverslips for 12 hrs, then fixed and labeled with TRITC-phalloidin (red), and cortactin (green) (top panels). Both lines fail to make endogenous invadopodia or degrade matrix. (B) FADU and MSK921 cells transfected with Src527F-mCh (red) were incubated on FITC-coated gelatin (white) coverslips for 12 hrs, then fixed and labeled with cortactin (green) (top panels). Src-induced invadopodia are present and identified by the cortactin aggregates (white arrows) that localize with Src527F and the dark holes in the FITC-gelatin (black arrows). Bars, 10 μ m.

Fig. S2. Src regulates invadopodia maturation in OSC19 cells. (A) Src protein levels in OSC19 cells treated with with Src ShRNA (SrcSh) or a ShRNA vector control (Ctl). Expression SrcWT (WT) or Src-527F (527) in Ctl and SrcSh cells evaluated by immunoblotting (B), and confocal microscopy (C). Cells were plated on FITC-gelatin coated (pseudocolored white) cover slips for 10 hrs and immunolabeled with TRITC-phalloidin (red) Bars, 10 μ m. (D) The effect of Src expression on percent of cells

displaying invadopodia, the number of invadopodia per cell, and the amount of matrix degradation per cell were examined. Data are represented as mean $\pm$ SEM, groups are statically different (* $p \leq 0.01$, ** $p \leq 0.05$).

Fig. S3. (A) Invadopodia are formed in cells expressing *tsLa29*-GFP at the permissive temperature. SYF cells transfected with empty GFP vector (EV) or with *tsLa29* tagged with GFP on the carboxyl-terminus (*tsLa29*-GFP) were incubated at 41°C or 35°C. Cells were fixed, permeabilized, and dual-labeled with TRITC-phalloidin and a phospho-specific antibody for cortactin tyrosine 421. Cells were visualized by confocal microscopy through 2-D and 3-D (z-stack) sectioning. (B) Silencing of cortactin with siRNA prevents invadopodia formation. SYF cells were transfected with cortactin-targeted or control siRNA (Ctl) and incubated at 37°C for two days. Cells were then transfected with *tsLa29*-GFP and the experiment proceeded as described in (A). Cells were fixed, permeabilized, and immunolabeled with TRITC-phalloidin and an anti-cortactin (4F11) antibody. Invadopodia fail to form in cells treated with cortactin siRNA. (C) Clarified cell lysates (30µg) from cells transfected with mock- or Cort-SiRNA alone or in combination with *tsLa29*-GFP were incubated at 41°C or 35°C, resolved by SDS-PAGE and immunoblotted with anti-Src-pY418, anti-Src (EC10), anti-cortactin (4F11), anti-cortactin-pY421, anti-GFP (JL8) and anti- β -actin antibodies. (Bars = 10µm).

Fig. S4. Characterization of fluorescent protein-tagged Src constructs. (A) Determination of relative Src kinase activity. SYF cells expressing cSrc-GFP, cSrc-cerulean, cSrc527F-GFP, cSrc527F-mCherry, cSrc295M-GFP, or cSrc295M-cerulean

were lysed, resolved by SDS-PAGE and immunoblotted with anti-Src-pY418, anti-Src, and anti- β -actin antibodies. Quantification of the relative Src phosphorylation in transfected SYF cells was conducted using densitometry. Src-pY18 protein expression was normalized to total Src protein levels. (B) Swept field imaging of fluorescently labeled Src constructs. Fixed cells were imaged for expression and localization of GFP, mCherry, and cerulean-tagged Src fluorescent proteins by direct fluorescence. GFP, green fluorescent protein; CerFP, cerulean fluorescent protein; mChFP, mCherry fluorescent protein. (C) Confocal imaging of GFP-tagged SrcWT, Src295M, and Src527F. Transfected cells expressing the indicated Src constructs were fixed, permeabilized, and immunolabeled with TRITC-phalloidin and the anti-cortactin (4F11) antibody. Arrows point to invadopodia in cells expressing Src527F. (Bars = 10 μ m).

Fig. S5. Co-transfection and localization of fluorescent protein tagged Src constructs. (A) Representative images of non-transfected SYF cells (*left*) and SYF cells co-expressing WT Src tagged with Cerulean fluorescent protein (Src-Cer) and GFP-tagged Src527F (527F-GFP) (*right*). Cells were fixed, permeabilized, and immunolabeled with TRITC-phalloidin and anti-cortactin (4F11) antibody. Arrows denote invadopodia in the 527F-GFP transfected cells. Bar, 20 μ m. (B) SYF and SYF+/+ cells expressing cSrc527F-mCherry alone or in combination with cSrc-cerulean or cSrc295M-cerulean, were lysed and resolved by SDS-PAGE and immunoblotted with anti-Src-pY418, anti-Src, anti- GFP/cerulean (JL8, does not recognize mCherry), anti-actin, anti-cortactin-pY421, and anti-cortactin (4F11) antibodies.

Fig. S6. Comprehensive SH2/PTB binding assay. An *in vitro* binding assay was performed using 91 GST-SH2 domains and 3 GST-PTB domains representing nearly

the full complement of human phosphotyrosine binding domains. GST and mutated Abl SH2 domain (Abl R>K) were used as negative controls. SH2 binding to SYF cell lysate was determined by densitometric quantification of digitally captured chemiluminescence images. The mean raw binding intensities with SEM from two independent experiments are shown. Domains are ordered from left to right by their binding intensity to SYF^{+/+} cells expressing cSrc527F to reflect rank order relevance for invadopodia maturation.

Kelley et al., Figure 1

Kelley et al., Figure 2

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Kelley et al., Supplementary Figure 6

Study 3: Cortactin Phosphorylated by ERK1/2 Localizes to Sites of Dynamic Actin Regulation and is Required for Carcinoma Lamellipodia Persistence

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Running title: ERK1/2 regulates cortactin

Abstract

Background

Tumor cell motility and invasion is governed by dynamic regulation of the cortical actin cytoskeleton. The actin-binding protein cortactin is commonly upregulated in multiple cancer types and is associated with increased invasion and metastasis. Cortactin regulates actin nucleation through the actin related protein (Arp)2/3 complex, stabilizing the cortical actin cytoskeleton. Cortactin is regulated by multiple phosphorylation events, including phosphorylation of S405 and S418 by extracellular regulated kinase (ERK)1/2. ERK1/2 phosphorylation of cortactin has emerged as an important positive regulatory event, enabling cortactin to bind and activate the Arp2/3 regulator neuronal Wiskott-Aldrich syndrome protein (N-WASp), promoting actin polymerization and enhancing cell migration and tumor cell invasiveness.

Methodology/Principal Findings

In this report we have developed phosphorylation-specific antibodies against cortactin phosphorylated at S405 and S418 to analyze the subcellular localization of this cortactin form in tumor cells and patient samples by microscopy. We evaluated the interplay between cortactin S405 and S418 phosphorylation with cortactin tyrosine phosphorylation in regulating cortactin conformational forms by Western blotting. Cortactin is simultaneously phosphorylated at S405/418 and Y421 in tumor cells, and through the use of point mutant constructs we determined that serine and tyrosine phosphorylation events lack any co-dependency. Expression of S405/418 phosphorylation-null constructs impaired carcinoma motility and adhesion, and also inhibited lamellipodia persistence monitored by live cell imaging.

Conclusions/Significance

Cortactin phosphorylated at S405/418 is localized to sites of dynamic actin assembly in tumor cells. Concurrent phosphorylation of cortactin by ERK1/2 and tyrosine kinases enables cells with the ability to regulate actin dynamics through N-WASp and other effector proteins by synchronizing upstream regulatory pathways, confirming cortactin as an important node in actin-based signal transduction. Reduced lamellipodia persistence in cells with S405/418A expression identifies an essential motility-based process reliant on ERK1/2 signaling, providing additional understanding as to how this vital signaling pathway impacts tumor cell migration.

Introduction

Tumor cell motility and invasion is a central problem in cancer that is paramount in contributing to metastasis [1]. Tumor cells move through successive series of coordinated and integrated stages, with formation of protrusive membranous structures including filopodia, invadopodia and lamellipodia required for initiation and maintenance of invasion and migration [2,3,4,5]. Central to the movement of most carcinoma cell types undergoing single or collective migration is the production of lamellipodia at the leading edge of the cell. Lamellipodia are planar protrusive extensions of the plasma membrane produced by motile cells in two- and three-dimensional settings [6]. Lamellipodia extension drives cell migration through integrin-based adhesion with the underlying substratum, providing the necessary traction for contractile-based translocation of the cell body to generate productive movement [7]. It is generally accepted that dynamic regulation of the cortical actin cytoskeleton through cycles of actin polymerization and depolymerization are responsible for generating the propulsive force needed for lamellipodia extension [8].

The actin cytoskeleton within lamellipodia is governed by the activity of numerous actin-binding proteins. One element central to the formation of lamellipodia actin networks is activation of the actin-related (Arp) 2/3 complex, which nucleates filamentous (F-) actin polymerization within lamellipodia [9]. Arp2/3 complex binds to the sides of pre-existing F-actin, where upon activation the Arp2 and Arp3 subunits mimic the fast growing (“barbed” or “+”) end of an actin filament, allowing for the rapid addition of actin monomers to the complex and subsequent filament extension [4]. The resulting Arp2/3-F-actin networks comprise an organized branched array of F-actin filaments that

contribute to lamellipodia extension, with Arp2/3 localized at filament branch points [8,9]. Arp2/3 branch points are metastable, allowing for rapid breakdown of Arp2/3-F-actin networks by filament debranching [10]. Debranched F-actin filaments are further disassembled through the severing activity of members of the actin depolymerizing factor/cofilin family, which ultimately dissolve F-actin filaments to promote depolymerization, recycling actin monomers for additional rounds of polymerization and lamellipodia extension [11].

Arp2/3 activation is controlled by the activity of several actin nucleation promoting factors (NPFs). The best characterized NPFs to date are members of the Wiskott-Aldrich syndrome protein (WASp) family, which include the WASp and WAVE protein subgroups [12,13]. In many cell types, regulation of Arp2/3 activity by the WASp proteins N-WASp and WAVE2 are largely responsible for generating the actin network used for creating and regulating lamellipodia, filopodia and invadopodia [4,12,14], making these NPFs critical mediators of cell motility and invasion.

Another well-characterized NPF independent of the WASp protein family is the cortical actin-binding protein cortactin [15,16]. Cortactin directly binds Arp2/3 complex [17,18] and activates Arp2/3 complex nucleation activity in vitro, albeit at a lesser degree than WASp-family proteins [18,19]. In addition to actin nucleation, a unique function of cortactin is its ability to prevent spontaneous debranching of Arp2/3-F-actin networks by simultaneous binding to Arp2/3 and F-actin, prolonging the lifetime of branched filaments [19]. While the biochemical features of cortactin seem to point to a straightforward role in lamellipodia actin regulation, studies of cortactin function in lamellipodia have proven controversial, suggesting to a more complex role in cell

migration. For instance, RNA interference studies have yielded conflicting results in regards to lamellipodia dynamics, with cortactin knockdown resulting in decreased lamellipodia stability and reduced persistence [20,21,22], whereas similar studies in different cell types suggest cortactin downregulation increases the length of extending lamellipodia [23]. Furthermore, recent analysis of fibroblast lamellipodia dynamics in cortactin^{-/-} cells indicates that cortactin does not play a role in directly regulating lamellipodia protrusion or Arp2/3-based actin dynamics, but rather is important in mediating upstream activation of the small GTPases Rac1 and Cdc42, which in turn regulate WAVE2 and N-WASp activity [24]. While these reported discrepancies regarding cortactin function in lamellipodia have yet to be fully reconciled, it is clear that cortactin is an important regulator for normal and tumor cell migration in many cell systems [25,26]. An unambiguous role for cortactin has been shown in invadopodia, where removal of cortactin by RNA interference ablates invadopodia formation in multiple invasive tumor cell types [27,28,29].

Besides regulating Arp2/3-based cortical actin networks by direct interactions, cortactin also functions as a key mediator in several kinase-based signal transduction cascades that serve to indirectly govern Arp2/3 activity and subsequent cell movement. Cortactin is a well-defined target for Src kinase [30], phosphorylating human cortactin on tyrosine residues Y421, Y470 and Y486 within the proline-rich (PR) carboxyl-terminal domain [31]. Several other tyrosine kinases target these residues [32,33,34,35], indicating that they collectively form a “hot spot” region as a point of convergence for multiple signaling pathways. Cortactin phosphorylated at tyrosines 421, 470 and/or 486 creates Src homology (SH)2 docking sites for several phosphorylating kinases, as well as the

adaptor proteins Crk [36] and Nck1 [37]. In the case of Nck1, Nck1/cortactin complexes interact with N-WASp or WASp interacting protein through the Nck1 SH3 domain to stimulate Arp2/3-dependent actin nucleation [37], which in cooperation with Arg kinase has been recently demonstrated to be important for regulating lamellipodia protrusion and leading edge adhesion formation [38]. The cortactin/Nck1 complex is also required to stimulate actin polymerization essential for invadopodia function in invasive breast cancer cells [39]. These studies are in agreement with the localization of tyrosine phosphorylated cortactin within lamellipodia and invadopodia [40], lending mechanistic insight into the long recognized pro-migratory and pro-invasive properties associated with cortactin tyrosine phosphorylation [29,31,41,42].

In addition to tyrosine phosphorylation, cortactin is a target for multiple serine/threonine kinases [43]. Stimulation of tumor cells with epidermal growth factor (EGF) results in increased serine/threonine phosphorylation of serine residues 405 and 418 within the PR domain, coincident with a characteristic shift in cortactin electrophoretic mobility from 80 kDa to 85 kDa in SDS-PAGE [44,45]. The mobility shift and phosphorylation of S405/S418 are impaired by pharmacologic inhibition of mitogen activated protein/extracellular signal regulated kinase kinase (MEK)1/2, and direct biochemical evidence indicates that the MEK effector kinases ERK1/2 directly phosphorylate cortactin at these sites [45]. Phosphorylation of S405/S418 enhances binding of the cortactin carboxyl-terminal SH3 domain to N-WASp and activates N-WASp NPF activity, indicating a functional role in stimulating Arp2/3-mediated actin dynamics independent of tyrosine phosphorylation [46]. This is supported by studies expressing phosphorylation-null and phosphomimetic point mutant constructs in cells, suggesting

that S405/S418 phosphorylation plays a critical role in regulating cellular actin polymerization necessary to promote cell motility [47] and invadopodia function [29]. In addition, p21 activated kinase 1 (PAK1) phosphorylates cortactin at S405/S418, serving to stimulate N-WASp activity required for clathrin-independent endocytosis [48]. While studies to date implicate a positive regulatory function for cortactin S405/418 phosphorylation in promoting N-WASp-mediated Arp2/3 actin structures, the subcellular localization of phosphorylated S405/418 cortactin, as well as the precise role S405/418 phosphorylation plays in regulating lamellipodia dynamics have not been evaluated.

In this study, we have generated site-specific antibodies against phosphorylated cortactin S405 and S418 to determine the spatial and temporal localization of cortactin in dynamic actin structures and human tumors, and to evaluate signaling interplay between cortactin tyrosine and serine phosphorylation events. We also determined the effects of S405/418 cortactin phosphorylation on EGF-induced cell migration, adhesion and lamellipodia dynamics in carcinoma cells.

Methods

DNA Constructs and siRNA

For Myc-tagged human cortactin expression constructs, the wild-type human cortactin cDNA subcloned into pcDNA FLAG2AB [49] was used as a template for producing point mutants by site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA). Codon alterations in human cortactin were: S405A, S418A, S405A/S418A, Y421F, Y470F, Y486F, Y421F/Y470F/Y486F and W492K. Cortactin cDNAs were amplified by PCR as BamHI-EcoRI fragments and subcloned into BamHI-EcoRI digested pRK5Myc [50]. Murine GFP-tagged expression constructs were produced using pcDNA3FLAG2AB wild-type murine cortactin [17] as the template for mutagenesis, then subcloned as EcoRI-KpnI PCR fragments into pAcGFP-C1 (Clontech, Mountain View, CA). The temperature-sensitive vSrc LA29 construct was previously described [51]. mCherry-b-actin was obtained from D. Schafer (University of Virginia), with the parent construct produced by R. Tsien (University of California, San Diego). Small interfering (si)RNA targeting rodent cortactin (5'-GCTTCGAGAGAATGTCTTC-3') was purchased from Thermo Scientific (Waltham, MA)

Cell lines and Transfection

The HNSCC cell lines 1483 [52], UMSCC1 and UMSCC2 [53] were maintained as described [41]. SYF cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to the supplied protocol. The rat mammary adenocarcinoma line MTLn3 was maintained in aMEM supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin. Transient transfections

were conducted with 3×10^6 cells and 2mg of plasmid construct or siRNA using the Nucleofector I device (Amaxa Biosystems, Berlin, Germany).

Antibodies

Antibodies against phosphorylated serine 405 (pS405) and serine 418 (pS418) of human cortactin were produced by 21st Century Biochemicals (Marlboro, MA). Synthetic phosphorylated cortactin peptides containing the sequences NH₂-KTQTPPV[pS]PAPQPTC-COOH (cortactin pS405) and NH₂-TEERLPS[pS]PV-COOH (cortactin pS418) were produced, conjugated to keyhole limpet cyanine and injected into rabbits. Immune serum was screened by enzyme-linked immunosorbent assay against the appropriate phosphorylated cortactin peptide coupled to bovine serum albumin. High-titer bleeds were identified for each peptide, and immune serum was passed two successive times through chromatography columns containing agarose beads coupled to the equivalent non-phosphorylated peptide. The flow through material for each peptide was subsequently passed twice through chromatography columns containing beads conjugated to the matched phosphorylated cortactin peptide. After extensive washing, bound antibodies for each phosphorylation site were eluted, concentrated and screened for specificity by Western blotting against recombinant cortactin mutant proteins harboring alanine-serine point mutations at serine 405 or 418, respectively (Fig. 1A). The anti-pS405 and anti-pS418 cortactin antibodies are currently available through Protea Biosciences (Morgantown, WV). Anti-cortactin (4F11) was used as described [41]. Anti-pY421 cortactin and anti-pY418 Src were from Invitrogen (Carlsbad, CA). Anti-ERK1/2 and pERK1/2 were from Cell Signaling (Danvers, MA). Anti-Myc epitope tag (4A6) was from Millipore (Billerica, MA). Anti-GFP (JL-8) was from

Clontech (Mountain View, CA) and anti-b-actin was from EMD4Biosciences (San Diego, CA).

Western blotting and Immunoprecipitation

Western blotting was conducted as described [41]. Primary antibody dilutions used were: anti-pS405 cortactin (1:4000), anti-pS418 cortactin (1:500), anti-cortactin (1:1000), anti-pY-421 cortactin (1:2000), anti-ERK1/2 (1:2000), anti-pERK (1:2000), anti-pY418 Src, anti-GFP (1:1000) and anti-b-actin (1:5000). Immunoprecipitations were performed as described [40] using 5mg of precipitating antibody captured with 40ml of a 50% Protein A/G bead slurry (Thermo Fisher Scientific, Pittsburgh, PA). In some cases cells were treated with selumetinib (AZD6244; ARRY-142886) or saracatinib (AZD0530) for 24h prior to immunoprecipitation and Western blotting analysis.

Microscopy

UMSCC2 cells were plated on fibronectin-coated coverslips (10mg/ml; Sigma, St Louis, MO) and allowed to attach before serum starvation for 16h. Cells were stimulated with 100ng/ml EGF (Millipore) for 1h before fixation. UMSCC1 cells plated on FITC-gelatin (Sigma) for 8 h were processed for confocal microscopy using Zeiss LSM 510 Meta system (Thornwood, NY) as described [49]. Anti-pS418 cortactin was used at 1:1000, 4F11 at 1:500 and rhodamine-conjugated phalloidin at 1:1000 (Invitrogen, Carlsbad, CA).

For immunohistochemistry, HNSCC tissue blocks were obtained from the West Virginia University Tissue Bank and used under approval of the West Virginia University Institutional Review Board. Five-micrometer sections from formalin-fixed, paraffin-

embedded blocks were processed for immunostaining using the Discovery XT automated staining system (Ventana, Tucon AZ). Briefly, after deparaffinization and antigen retrieval, sections were incubated with monoclonal rabbit anti-cortactin (Novus, Littleton, CO) at 1:2000, anti-pS418 cortactin at 1:25 and anti-pERK1/2 at 1:100 dilutions. All primary antibodies were incubated in Dako diluent (Dako, Carpinteria, CA) for 1 h. Primary antibodies were detected with the Omnimap antibody horseradish peroxidase kit (Ventana). Slides were counterstained with hematoxylin and post-counterstained with bluing reagent (Ventana). Images were visualized with an Olympus AX70 microscope and captured using the MicroBrightfield system (Williston, VT).

Live cell imaging was conducted using MTLn3 cells starved for 3 h with serum-free media prior to stimulation with 100ng/ml EGF. Cells were plated on delta-T glass bottom dishes (Fisher) coated with 10mg/ml fibronectin (Sigma). Immediately following EGF addition, cells were imaged by differential interference contrast using a Nikon TE2000 inverted microscope equipped with a Roper CoolSNAP HQ charge-coupled device camera (Photometrics, Tucson, AZ). Images were captured every 5 s for 15 min (181 total frames). A Nikon LiveScan SFC swept field microscope was used for imaging cells expressing mCherry-actin. In all cases, GFP-cortactin expressing cells were identified by fluorescence microscopy prior to imaging. Kymograms were produced by extracting 1 pixel-width strips from each movie frame at points of initial and maximal lamellipodia extension, and assembled using ImageJ (v1.40).

Electric Cell Substrate Impedance Sensing

To assay cell motility and adhesion, 5×10^5 cells were plated into 8-well electric cell substrate impedance sensing dishes (ECIS; Applied Biophysics, Troy, NY). For motility

measurements, cells were allowed to adhere overnight on 8W1E dishes to form a monolayer. Adhesion was assayed immediately after plating cells onto 8W10E dishes. Measurements were conducted for 24 h at 45kHz, with reading taken at 1 min intervals. Cells treated with selumetinib were serum starved 24 h in the presence of drug prior to ECIS.

Statistical Analysis

Differences in mean groups for migration, adhesion and kymography between control and treated groups were evaluated using one way ANOVA, followed by Student-Newman-Keuls post hoc testing. All differences were considered significant at $p \leq 0.05$. A minimum of three experimental groups were used for all analyses.

Results

Localization of pS418 cortactin with dynamic cortical actin structures

We developed antibodies specific to phosphoserine 405 (pS405) and phosphoserine 418 (pS418) of human cortactin to facilitate analysis of these sites. To validate antibody specificity, epitope (Myc)-tagged cortactin constructs containing wild-type (WT) cortactin, cortactin with individual serine to alanine mutations at serine 405 (S405A), 418 (S418A) or with both mutated in tandem (S405,418A) were produced and transfected into 1483 cells. Total cell lysates were blotted with anti-pS405 or anti-pS418 antibodies (Figure 1A). The anti-pS405 antibody recognized the WT and S418A cortactin variants, failing to blot constructs containing the S405A mutation. Conversely, anti-pS418 blotted WT and S405A, failing to recognize cortactin constructs with S418A mutations. All cortactin variants were recognized by an anti-cortactin monoclonal antibody (Figure 1A), indicating equivalent expression of the assayed constructs. These results indicate that the anti-pS405 and anti-pS418 antibodies specifically recognize their cognate phosphorylated cortactin epitope, and that no interdependence exists between phosphorylation of cortactin S405 and S418.

To determine the subcellular localization of serine phosphorylated cortactin, we conducted indirect immunofluorescence studies on cells producing lamellipodia and invadopodia, two actin-based structures that depend in part on N-WASp activity. While the anti-pS405 antibody yielded non-specific staining in our hands (data not shown), anti-pS418 specifically labeled lamellipodia and cytoplasmic puncta (presumably vesicles) in UMSCC2 cells. In cells with a motile phenotype, anti-pS418 localized with cortactin and F-actin in these regions (Figure 1B, top row). Labeling of UMSCC1 cells

plated on FITC-coated gelatin matrix with anti-pS418 indicated specific localization to a subset of invadopodia that coincided with cortactin, F-actin and areas of gelatin clearing indicative of matrix metalloproteinase mediated invadopodia activity (Figure 1B, middle and bottom rows).

In solid human tumors, cortactin and cortactin phosphorylated on tyrosine 421 (pY421) localizes to invasive tumor fronts and to cell-cell junctions [41,49]. To determine the location of pS418 cortactin in tumor tissue, head and neck squamous cell carcinoma (HNSCC) cases were sectioned and stained with anti-pS418 (Figure 1C). Cortactin pS418 was abundant in HNSCC cell cytoplasm and was enriched in areas of cell-cell contact, displaying a pattern similar to sections labeled with a total cortactin antibody (Cort). These tumor regions also contained activated ERK1/2, as evidenced by pronounced cytoplasmic and nuclear staining of phosphorylated ERK1/2 in serial sections (Figure 1C).

Growth factor mediated phosphorylation of cortactin S405/418 is MEK dependent

Previous biochemical work has implicated chemical inhibition of MEK and subsequent blocking of ERK1/2 activation as a major pathway responsible for cortactin S405/418 phosphorylation [45]. To further evaluate the role of the MEK-ERK1/2 pathway on cortactin phosphorylation, we utilized the anti-pS405 and pS418 cortactin antibodies to directly test the effects of MEK inhibition on cortactin pS405/418. Western blot analysis of cell extracts from EGF- and serum-stimulated UMSCC1 cells with anti-pS405 and pS418 antibodies displayed similar phosphorylation kinetics of S405 and S418, with phosphorylation of both sites first evident 10 min after stimulation (Figure 2A) and remaining phosphorylated up to 2 h (data not shown). Treatment of UMSCC1 or 1483

cells with the small molecule MEK inhibitor selumetinib [54] reduced EGF-stimulated cortactin S405/418 phosphorylation in a dose-dependent manner, where near elimination of phosphorylation at both serine residues occurred at doses $\geq 1\text{mM}$ (Figure 2B). ERK1/2 activity was also reduced under similar dose conditions, although complete ablation of ERK1/2 phosphorylation was observed at doses $\geq 5\text{mM}$ (Figure 2B). These data suggest that the MEK-ERK pathway is largely responsible for growth-factor induced cortactin S405/418 phosphorylation in HNSCC cells, in agreement with previous findings in other cell types [45].

The 80kDa to 85kDa cortactin conformational shift is associated with serine and tyrosine phosphorylation

Based on sequence analysis, the largest and most prominent cortactin isoform (cortactin “A” or “SV1”) encodes a 61.5kDa protein [55,56]. This cortactin form frequently migrates as an 80/85kDa doublet in SDS-PAGE [30,57] that has been attributed to conformational alterations within the polypeptide chain [15,45]. Shifting from the 80kDa to 85kDa form is seen in response to EGF, with the resulting 85kDa band associated with S405/418 phosphorylation [44,45]. To directly assess the presence of pS405/418 in the two cortactin conformational isomers, serum-starved UMSCC2 (Figure 3A) and 1483 (Figure 3B) cells were stimulated with EGF and the cortactin forms in cell lysates were analyzed at successive time points with anti-pS405 and anti-pS418 antibodies. S405/418 phosphorylation was maintained in the 85kDa cortactin form in both cell lines following serum starvation, despite of the lack of ERK1/2 activity (0 min, Figure 3A and Figure 3B). EGF stimulation resulted in complete

conversion of the 80kDa to the 85kDa cortactin form by 1 h after EGF treatment in both cell lines (Figure 3A and Figure 3B). Cortactin pS405 and pS418 was observed primarily in the 85kDa form and increased at both sites during the entire time course, whereas ERK1/2 activity peaked at 15 min and rapidly declined afterwards (Figure 3A and Figure 3B). Interestingly, the phosphorylation of S405 was also associated with an increase appearance of cortactin degradation in UMSCC2 cells (Figure 3A). It is uncertain whether these products represent increased overall cortactin degradation, or if the net cortactin degradation is constant but is selectively identified by the pS405 antibody in response to EGF treatment and phosphorylation. EGF-induced Src activation and cortactin pY421 phosphorylation was sustained throughout the entire time course in UMSCC2 cells (Figure 3A), indicating that cortactin can be simultaneously phosphorylated by ERK1/2 and Src or potentially other EGF-stimulated cortactin tyrosine kinases. Pretreatment of UMSCC2 cells with the Src family kinase inhibitor saracatinib at 10mM or selumetinib at 1mM completely impaired the cortactin shift from 80kDa to 85kDa (Figure 3C). The exclusive presence of pS405 and pS418 in the EGF-induced 85kDa cortactin form, as well as the ability of MEK inhibition to impair the cortactin shift is consistent with results obtained from previous work [45]. Our results also identified EGF-induced Src-mediated phosphorylation of cortactin at tyrosine 421 as a necessary mediator of the cortactin shift.

Cortactin serine phosphorylation *in vivo* is independent from tyrosine phosphorylation

EGF treatment of UMSCC2 cells resulted in phosphorylation of cortactin S405/418 and cortactin pY421 (Figure 3A). A previous *in vitro* study evaluating the impact of cortactin

phosphorylation on N-WASp activation determined that S405/418 phosphorylation by ERK1/2 enables the cortactin SH3 domain to stimulate N-WASp Arp2/3 activation, while Src phosphorylation downregulates N-WASp activity and counteracts the effects of S405/418 phosphorylation [46]. This proposed “on-off switch” postulates that cortactin serine and tyrosine phosphorylation are mutually exclusive events governing the ability of cortactin to regulate N-WASp activity and downstream actin reorganization [58]. Using the available antibodies reactive against cortactin pS405 and pY421, we sought to determine if these two different classes of phosphorylation events are interdependent in any manner. Cortactin depleted SYF fibroblasts (null for the Src, Yes and Fyn kinases) were co-transfected with the temperature-sensitive vSrc construct *tsLa29*-GFP [51] to activate the Src and ERK1/2 signaling pathways, along with constructs encoding wild-type cortactin or the following Myc-tagged cortactin mutants: Y421F, Y470F, Y486F, Y421/Y470/Y486F (TPM), S405A, S418A, S405/418A (Figure 4A). A W492K cortactin mutant was also included, as this mutant abolishes the ability of the cortactin SH3 domain to interact with corresponding SH3 binding proteins [59]. After shifting to 35°C for 2 h to activate *tsLa29*-GFP, the serine and tyrosine cortactin mutants were analyzed for phosphorylation at Y421 and S405 by SDS-PAGE and Western blotting (Figure 4B). Mutations to S405 and S418 alone and in combination did not impact the ability of these constructs to be phosphorylated on Y421, as indicated by their recognition with the anti-pY421 antibody (Figure 4B). Similarly, mutations to Y421, Y470, and Y486, alone and in combination (TYM) did not affect the ability of these constructs to be phosphorylated on S405. These data indicate that cortactin is simultaneously phosphorylated at S405 and Y421 downstream of vSrc activation,

suggesting in this system that phospho-regulation of cortactin SH3 domain function is not governed in vivo by the serine-tyrosine “on-off switch” mechanism proposed from previous in vitro experimentation [46,58].

S405/418 phosphorylation is required for efficient tumor cell motility and adhesion

To evaluate the role of cortactin S405/418 phosphorylation on carcinoma cell migration, 1483 and UMSCC1 cells were treated with selumetinib and assayed for effects on motility by ECIS (Figure 5). Selumetinib treatment impaired the motility of both cell types in a dose-dependent manner, corresponding to the observed decreases in S405/418 phosphorylation (Figure 2B). Since MEK inhibition likely impaired the phosphorylation of other proteins involved in motility in addition to cortactin, we directly assessed the impact of cortactin S405/418 phosphorylation on cell migration using phosphorylation-null cortactin expression constructs. MTLn3 rat mammary adenocarcinoma cells were initially transfected with a siRNA targeted against rodent cortactin, followed by transfection with GFP-tagged human wild-type (WT), S405A, S418A and S405/418A cortactin constructs. Cortactin siRNA reduced endogenous cortactin levels to > 90%, having no impact on expression of the human GFP-labeled variants (Figure 6A). MTLn3 cells with cortactin knockdown (si) displayed a 29% reduction in motility compared to control (Ctl) (Figure 6B). Expression of wild-type human GFP-cortactin (WT) led to a 2-fold increase in motility, presumably due to increased expression of this variant over endogenous (Ctl) levels (Figure 6A). Expression of S405A, S418A or S405/418A cortactin resulted in an 49% average decrease in cell migration for each cortactin mutant, indicating that phosphorylation of

S405 and S418 are both vital in maintaining optimal carcinoma cell motility (Figure 6B). Since lamellipodia formation is required for detached cells to adhere to the ECM, we conducted ECIS assays to determine the effects of cortactin S405/418 phosphorylation on cell adhesion. MTLn3 cells lacking cortactin expression (si) exhibited a 50% decrease in cell adhesion compared to control (Ctl) cells. Expression of wild type (WT) GFP-cortactin restored adhesion to levels similar to Ctl, whereas expression of S405A, S418A or S405/418A cortactin mutants all reduced adhesion to levels 42-58% of Ctl, failing to restore adhesion to levels above cortactin si cells (Figure 6C). These results suggest that S405/418 phosphorylation is critical for carcinoma cell motility and adhesion, representing an important pro-migratory substrate targeted by the MEK-ERK1/2 pathway.

Cortactin S405/418 phosphorylation is required for carcinoma cell lamellipodia persistence

Given the localization of pS418 cortactin within lamellipodia (Figure 1B) and the effects of cortactin S405/418A expression on cell motility, we evaluated the impact of cortactin S405/418 phosphorylation on lamellipodia dynamics using live-cell imaging and kymographic analysis. Serum-starved MTLn3 cells expressing mCherry-b-actin and containing endogenous cortactin knockdown alone (si), rescued with human GFP- wild type cortactin (si+WT) or with GFP-cortactin S405/418A (si+S405,418) were stimulated with EGF for 15 min. Lamellipodia dynamics were monitored by time lapse video microscopy (Figures S1-S4) and assayed by kymography (Figure 7A). EGF-stimulated MTLn3 cells produced an initial dominant lamellipodia that reached maximal extension

between 1.5 and 3 min, and retracted to the point of origin between 5-7 min [60,61]. Control MTLn3 cells containing mCherry-b-actin displayed similar extension-retraction kinetics when assayed by kymography (Figure 7B and Figure S1). While no differences were observed in lamellipodia protrusion rates in any of the assayed cellular conditions (Figure 7A), cortactin knockdown (si) increased lamellipodia extension by an average of 5.8mm over the maximum extension length observed in control cells (Figure 7A and B). Lamellipodia formed in cortactin si cells failed to effectively retract lamellipodia, demonstrating a ~2-fold increase in average lamellipodia persistence over control levels (Figure 7A and Figure S2). These results are consistent with the observed increase in lamellipodia extension and persistence observed when MTLn3 cells contact EGF-coated bead matrices [23]. These effects are fully rescued to control levels upon expression of WT GFP-cortactin (si+WT; Figure 7A and B). Although expression of GFP-cortactin S405/418A in cortactin si cells did not impact EGF-induced lamellipodia extension, average lamellipodia persistence was reduced by 46%, from 195 sec in si+WT cells to 106 sec in si+405,418 cells (Figure 7A). The lamellipodia in si+405,418 cells displayed series of multiple short extensions and retractions, had enhanced ruffling and appeared more labile than control or si+WT cells (Figure 7B: Figures S1 and S2 compared to Figure S4). These results suggest that S405/418 phosphorylation is vital in regulating lamellipodia actin dynamics responsible for proper protrusive behavior.

Discussion

While the effects of cortactin phosphorylation at S405 and S418 by ERK1/2 have been studied at the biochemical and functional level in several systems [29,45,46,47], the spatial and temporal evaluation of S405 and S418 phosphorylation have been hampered due to the lack of suitable reagents to directly study these sites in cellular and tissue contexts. Our development of anti-pS405 and anti-p418 cortactin antibodies has allowed us to examine the localization and signaling pathways regulating these cortactin phosphorylation events. These antibodies, coupled with the use of phosphorylation-null mutant constructs, allowed us to validate and extend previous findings implicating these sites in the regulation of carcinoma cell motility and associated lamellipodia dynamics.

The localization of pS418 cortactin in carcinoma lamellipodia and invadopodia is consistent with the defined and emerging roles cortactin plays in regulating actin dynamics within these structures [26,62]. To date, all studies designed to evaluate the cellular effects of pS405/418 phosphorylation have relied on the use of phosphorylation null or phosphomimetic (S405/418D) constructs. In pancreatic tumor cells, S405/418A and S405/418D both promote lamellipodia protrusion over control levels, whereas S405/418A inhibits and S405/418D promotes cell motility [47]. While the ability of S405/418A to promote lamellipodia protrusion in these studies is unclear, the remaining results are consistent with an activating role for S405/418 phosphorylation in lamellipodia dynamics and motility. Similar results were obtained in the analysis of S405/418 on invadopodia function, with S405/418A expression impairing and S405/418D promoting ECM degradation activity [29]. Phosphorylation of cortactin S418

within lamellipodia and invadopodia (Figure 1B) supports these results. Precisely where cortactin is phosphorylated on S405/418 in carcinoma cells remains to be determined, although the phosphorylating kinases ERK1/2 and PAK1 have been localized within lamellipodia [63,64] and invadopodia [65]. This could suggest that cortactin is initially localized to lamellipodia or invadopodia, where it is subsequently phosphorylated on S405/418 when associated with the cortical actin networks within these structures. An analogous mechanism is employed for cortactin tyrosine phosphorylation within lamellipodia [40].

In HNSCC and several other tumor types, cortactin is present in the cytoplasm and is enriched at cell-cell junctions [41,66,67]. The localization of pS418 cortactin at regions of HNSCC cellular contact within tumors resembles the localization pattern of pY421 cortactin in this tumor type [68]. The staining pattern of cortactin and its tyrosine phosphorylated form is reminiscent of that found in two-dimensional epithelial monolayers, where cortactin has been shown to be essential for Arp2/3-mediated actin remodeling resultant from E-cadherin homoligation and subsequent Src activity [69,70]. While the presence of pS418 cortactin at these sites suggests additional functional roles for cortactin in E-cadherin-mediated actin regulation within tumors, whether or not cortactin S405/418 phosphorylation impacts elements of E-cadherin-based regulation of solid tumor behavior (such as tumor cell cohesion, motility or dissemination) remains to be examined.

Selumetinib inhibition of cortactin S405/S418 phosphorylation is consistent with results obtained with non-clinical MEK inhibitors [29,45], reinforcing the MEK-ERK1/2 pathway as the main signaling route responsible for phosphorylating these cortactin sites in

tumor cells. This is supported by direct phosphorylation of cortactin by ERK1/2 in vitro [45] along with our data demonstrating concomitant downregulation of active ERK1/2 resultant of selumetinib treatment. In addition to MEK, PAK1 has recently been shown to phosphorylate cortactin at S405/418, regulating N-WASp actin dynamics responsible for clathrin- and caveolin-independent endocytosis [48]. PAK1 is activated primarily by binding to active Cdc42 or Rac1 [71], although alternative modes of activation have also been described [72]. Activated PAK1 also binds and activates MEK, stimulating ERK1/2 activation [73]. Since MEK inhibition largely ablates S405/418 phosphorylation in most cell types, the impact of PAK1 activity on S405/418 phosphorylation may be context dependent, with direct PAK1 phosphorylation of cortactin S405/418 regulating actin polymerization required for endosomal trafficking, while MEK-mediated phosphorylation (activated by Raf or other MEK activators) may be primarily responsible for governing motility-based actin dynamics. In addition, the related kinase PAK3 phosphorylates cortactin at S113, an event that downregulates the ability of cortactin to bind F-actin and is important in modulating invadopodia function [29,74]. While our understanding regarding the interrelationship and regulation between PAK and MEK in governing cortactin S405/418 phosphorylation is currently incomplete, it is clear that the PAK-MEK-ERK1/2 signaling nexus impinges at multiple levels on cortactin to regulate actin dynamics involved in several membrane-based cellular processes.

Consistent with other reports [44,45], we observed the MEK-dependent EGF-induced shifting of cortactin from the 80kDa to 85kDa form by Western blotting. Direct analysis with anti-pS405 and anti-pS418 antibodies indicates that the 85kDa form is almost exclusively phosphorylated on these residues, as was determined by ³²P labeling and

tryptic peptide analysis [44,45]. The shift in cortactin M_r is not attributable to bulk addition of phosphate, since phosphatase treatment of cortactin immunoprecipitates from EGF-treated cells failed to reconvert the 85kDa form to 80kDa (data not shown). While the distinct 80kDa and 85kDa bands represent different post-translationally modified cortactin forms associated with pS405/418 phosphorylation, mutations at these sites have no effect on 80/85kDa cortactin ratios, with the S405/418A mutant displaying a similar cortactin electrophoretic pattern to wild type cortactin (Figure 4). This suggests that S405/418 cortactin phosphorylation, while associated with the shift from 80 to 85kDa, is not *necessary* for generation of the 85kDa cortactin form. This is supported by the presence of 80kDa and 85kDa cortactin forms produced in kinase-free systems [30,75] and by the existence of a single 85kDa form when analyzed by urea denaturing SDS-PAGE [75].

The lack of detailed structural data for cortactin derived by nuclear magnetic resonance spectroscopy or X-ray crystallography has hindered the field in understanding conformational changes cortactin undertakes in response to post-translational modifying events. The existence of cortactin in a “closed” versus “open” form regulated by S405/418 phosphorylation has been proposed to explain the observed 80 to 85kDa shift [45]. Support for this is derived from biochemical studies on N-WASp activation by the cortactin SH3 domain, where S405/418 phosphorylation enhances N-WASp activation and Arp2/3 actin nucleation activity [46]. These studies propose that the “closed” cortactin form undergoes an autoinhibitory conformational state where the carboxyl terminal helical proline-rich (HP) domain containing S405 and S418 is altered to render the SH3 domain inaccessible to binding N-WASp or other proteins. Phosphorylation of

S405/418 results in liberating the SH3 domain, where it in turn is capable of binding and stimulating N-WASp activation. Expression of cortactin S405/418D phosphomimetic forms in cells increases branched actin networks in actin tails associated with cytoplasmic vesicles, providing support for this model in promoting cellular actin polymerization [47]. Initial assessments of cortactin structure by rotary shadow electron microscopy revealed cortactin to exist as a rod shaped monomer 220Å in length[76]. However, a recent biophysical analysis utilizing chemical crosslinking and small angle x-ray scattering suggests that cortactin exists in a more globular form, with the carboxyl terminal HP and SH3 domains folding back onto the amino terminal actin binding region [77]. Such a structure would support a “closed” conformation, although “open” structures were not observed, nor were the effects of ERK1/2 phosphorylation evaluated. Additional evidence for an inhibitory function of the amino terminus can be inferred from the ability of the cortactin carboxyl terminal domain to promote N-WASP-dependent cell motility as effectively as wild type cortactin [78], as well as the prevalence of the 85kDa form in invasive colorectal cancer [66].

In the ERK-Src “switch” model proposed for cortactin regulation, cortactin phosphorylation by Src at Y421, 470 and 486 serves to downregulate N-WASp activity promoted by S405/418 phosphorylation [46]. This model therefore suggests that serine and tyrosine phosphorylation of cortactin function in a reciprocal manner to govern N-WASp activation [58]. Our data with site-specific phosphorylation antibodies on lysates from EGF-stimulated cells indicates that S405/418 and Y421 are co-phosphorylated, and analysis of point mutant cortactin constructs does not indicate a reciprocal influence between cortactin serine and tyrosine phosphorylation events. These data suggest that

cortactin function is not exclusively regulated by a serine-tyrosine “switch” mechanism. This view is additionally reinforced by the presence of pS418 and pY421 cortactin within lamellipodia and invadopodia. While our data do not rule out scenarios where such a mechanism may be employed at the cellular level, they are consistent with biochemical and cellular evidence indicating that tyrosine phosphorylation promotes N-WASp activity through binding of the adaptor Nck1 [37,39], a component that was not present in the original assays where the “switch” mechanism was defined. The ability of cortactin to be simultaneously phosphorylated at S405/418 and Y421/470/486 may therefore provide cells with the ability to fine-tune the level of N-WASp activation and subsequent actin remodeling in response to diverse upstream stimulatory input that triggers motility and invasion.

Consistent with the mechanistic descriptions above, inhibition of carcinoma cell motility by MEK inhibition and S405/418A expression indicates that S405/418 cortactin phosphorylation is important in promoting and maintaining cell migration. While similar results were observed in wound healing assays with pancreatic cancer cells [47], our work extends these findings by evaluating the effects of pS405/418 on lamellipodia dynamics. The inability of MTLn3 cells expressing S405/418A cortactin to maintain EGF-stimulated dominant lamellipodia persistence implies that the actin networks within these cells fail to maintain proper Arp2/3 nucleation, or are more labile following lamellipodia extension. While N-WASp activation and Arp2/3-mediated actin polymerization resultant of cortactin SH3 domain binding has been shown to be important in governing motility in multiple cell types [47,78], a detailed study of EGF-induced lamellipodia protrusion in this cell type has recently shown that WAVE2 and

formin proteins, not N-WASp, are responsible for lamellipodia protrusion [14]. These results would therefore rule out a role for direct N-WASp activation by pS405/418 cortactin in MTLn3 lamellipodia extension. In addition to N-WASp, the cortactin SH3 domain interacts with several other proteins that have the potential to directly or indirectly regulate lamellipodia actin dynamics (reviewed in [62]). In particular, cortactin binds and activates the Dbl family guanine nucleotide exchange factor faciogenital dysplasia protein 1 (FGD1) [79,80], a potent activator of Cdc42 [50]. Cdc42 activity is required for localization of WAVE2 and its activator IRSp53 to the cell membrane, where it mediates lamellipodia extension [81]. FGD1 also activates the MEK-ERK1/2 pathway [50], allowing the potential of a positive feedback loop in stimulating cortactin S405/418 phosphorylation through continuous cortactin SH3-mediated FGD1 activity. FGD1 binding represents just one possible cortactin SH3 domain ligand with the capability to influence WAVE2 localization and lamellipodia dynamics. Whether such an FGD1-based regulatory circuit or other modes of potential pS405/418 cortactin regulation of WAVE2 activity exist in MTLn3 cells remains to be confirmed.

Previous studies on lamellipodia dynamics in other cells types indicate that cortactin removal decreases lamellipodia persistence, which can be rescued by re-expression of a cortactin amino terminal fragment lacking the carboxyl terminal region [20], eliminating contributions from pS405/418 in this system. These results differ from our work in MTLn3 cells, where cortactin removal results in enhanced persistence that can be rescued by re-expression of wild type cortactin. It is likely that these observed differences are due to a combination of different cell types, chemotactic cues, and analysis of dominant, initial lamellipodia versus steady-state lamellipodia dynamics [62].

Interestingly, inhibition of ERK1/2 signaling during macrophage lamellipodia extension results in decreased lamellipodia stability, with similar kymograph profiles to EGF-stimulated MTLn3 cells with S405/418A expression [64]. These studies provide supporting evidence for our observations.

Through the use of phosphorylation-specific antibodies, we have analyzed the localization of cortactin pS405 and pS418 in tumor cells and tissue, as well as the signaling pathways regulating pS405/418 phosphorylation. Through the use of these reagents, we have been able to validate and further clarify the role of pS405/418 in cortactin-based signaling. Our functional studies of carcinoma motility and lamellipodia dynamics with phosphorylation-null constructs have shed additional light on the role these phosphorylation events play in regulating lamellipodia function involved in tumor cell movement.

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Author Contributions

Conceived and designed the experiments: LCK, KEH, AGA, SAW. Performed the experiments: LCK, KEH, AGA. Acquired and analyzed the data: LCK, KEH, AGA, KHM. Contributed reagents/materials/analysis tools: KHM, SAW. Wrote the paper: LCK, KEH, AGA, SAW.

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Figure Legends

Figure 1. Specificity and validation of pS405 and pS418 phospho-specific cortactin antibodies. (A) Phospho-specific recognition of anti-cortactin pS405 and pS418 antibodies. Clarified lysates (50µg) from 1483 cells transfected with Myc-tagged wild-type cortactin (WT), Myc-cortactin S405A, Myc-cortactin S418A or Myc-cortactin S405A,S418A point mutants were immunoblotted with affinity purified anti-Cort-pS418 (left) and anti-Cort-pS405 (right) antibodies. **(B)** Localization of pS418 cortactin in areas of motile and invasive actin dynamics. UMSSC2 cells (*top row*) were serum starved for 16h prior to stimulation with 100ng/µl EGF for 1 h to induce lamellipodia formation, while UMSSC1 cells (*middle row*) were plated on FITC-conjugated gelatin coated coverslips (pseudocolored white) for 6 h to promote invadopodia formation. Cells were fixed, permeablized, and labeled with TRITC-phalloidin (Actin), anti-cortactin (Cort) and anti-cortactin-pS418 antibodies. Arrows denote localization of pS418 cortactin with total cortactin and F-actin in lamellipodia (*top*) and to invadopodia (*middle*) coinciding with areas of active matrix degradation. Bottom panels are magnified views of the indicated cellular region. Bars, 10µM. **(C)** Localization of pS418 cortactin in HNSCC tumor tissue. Serial sections from a patient with invasive HNSCC were processed for immunohistochemistry with control IgG (Ctl), pS418 cortactin (pS418), total cortactin (Cort) and phospho-ERK1/2 (pERK) antibodies. Sections were counterstained with hematoxylin. Arrowheads indicate areas of peripheral pS418 cortactin and total cortactin enrichment. Bar, 100µM.

Figure 2. Growth factor-stimulated Erk 1/2 activation mediates phosphorylation of cortactin at serine 405 and 418. (A) Growth factor-induced phosphorylation of cortactin S405 and S418. Serum starved UMSSC1 cells were stimulated with EGF (left) or FBS (right) for the indicated times. Cells were lysed and analyzed by Western blotting with anti-Cort-pS418 and anti-Cort-pS405 antibodies. Blots were stripped and reprobed with a pan-cortactin antibody to confirm equal loading (*bottom*). (B) Pharmacologic MEK inhibition inhibits cortactin S405 and S418 phosphorylation. UMSSC1 (left) and 1483 (right) cells were serum starved in the presence of the indicated selumetinib concentrations prior to stimulation with EGF for 20 min. Cortactin immunoprecipitated from cell extracts was assayed by Western blotting with anti-Cort-pS418 and anti-Cort-pS405 antibodies. Blots were stripped and reprobed with pan-cortactin antibody as in (A) (*bottom panels*). Selumetinib efficacy was verified by the blotting of lysates from selected timepoints with phospho-ERK1/2 (pERK1/2) and pan ERK1/2 antibodies (*bottom*). All blots are representative images from 3-4 independent experiments.

Figure 3. EGF-induced conversion of cortactin from 80kDa to 85kDa is impaired by Src and MEK1/2 inhibition. EGF induces the p80kDa to p85kDa shift in HNSCC cells. Serum starved UMSSC2 (A) and 1483 (B) cells were treated with 100ng/ml EGF for the indicated times. Clarified lysates were assayed by Western blotting with anti-cortactin, anti-Cort-pS418, anti-cort-pS405, anti-Cort-pY421, anti-Src-pY418, anti-pErk1/2 and total Erk1/2 antibodies as indicated. Red bars denote the position of the 85 kDa cortactin form; black bars denote the 80 kDa form. (C) Inhibition of Src and MEK1/2 kinase activity inhibits the cortactin “shift”. UMSSC2 cells were treated with vehicle

(DMSO), saracatinib, or selumetinib for 16 hrs in serum free media. Cells were stimulated with 100ng/ml EGF for 1h, lysed and analyzed by Western blot analysis with an anti-cortactin antibody.

Figure 4. Cortactin tyrosine and serine phosphorylation resultant of v-Src activation are not interdependent. (A) Schematic diagram of the cortactin point mutant constructs assayed for phosphorylation. Mutated codons are denoted on the left and displayed with the corresponding mutant amino acid at the appropriate position within cortactin in red. **(B)** Murine fibroblasts lacking endogenous Src, Yes and Fyn (SYF) were transfected with murine-specific cortactin siRNA and cultured for 48h to deplete endogenous cortactin. Cells were subsequently co-transfected with the temperature-sensitive v-Src construct La29 (*tsLa29*) and wild-type or the indicated myc-tagged human cortactin point-mutant constructs at 41°C (non-permissive temperature). TPM; triple point mutant consisting of Y-F mutations at positions 421, 470 and 486. After transfection, cells were cultured at 41°C, then shifted to 35°C (permissive temperature) for 2 h to promote v-Src activation. Recombinant cortactin proteins were assayed by immunoblotting with anti-cortactin-pY421, anti-cortactin-pS405, anti-myc, anti-cortactin, and anti-β-actin antibodies. Note that the inability of cortactin to be phosphorylated on Y421 does not impact its ability to be phosphorylated on S405, nor does lack of S405 phosphorylation impact Y421 phosphorylation.

Figure 5. Targeted inhibition of MEK1/2 inhibits HNSCC cell motility. 1483 and UMSCC1 cells (5×10^5) were starved for 24h in the presence of vehicle (DMSO) or increasing concentrations of selumetinib as indicated. Cells were assayed for motility by electric substrate impedance sensing (ECIS) following stimulation with complete

media containing the matched selumetinib concentration for 24h. Data is displayed as slope values calculated from the linear part of ECIS tracings. Bars represent mean $\pm$ SE. *, $p < 0.05$ compared to DMSO treated control cells.

Figure 6. Cortactin phosphorylation at serine 405 and 418 regulates carcinoma cell migration and adhesion. (A) Expression of GFP-cortactin constructs in MTLn3 cells. MTLn3 cells were transfected with murine-specific cortactin siRNA (Si) for 48h to silence endogenous cortactin expression. Cells were subsequently transfected with the indicated human GFP-tagged cortactin wild-type and the various Erk1/2 phosphorylation-null point mutant constructs. Following transfection, cell lysates were immunoblotted with anti-cortactin, anti-GFP and anti- β -actin antibodies. Solid arrowheads indicate the position of GFP-tagged cortactin variants; open arrowheads denote the position of endogenous cortactin. **(B)** Serine 405 and 418 phosphorylation is required for efficient carcinoma cell motility. MTLn3 cells transfected as in (A) were analyzed for cell migration by ECIS. Cell impedance versus time plots for each transfected line are shown on the *left*; slope values calculated from the linear region of each plot are displayed on the *right*. **(C)** Carcinoma cell spreading requires phosphorylation of cortactin S405 and S418. Transfected MTLn3 cells were plated, with rates of spreading were monitored by ECIS tracings over time *left*. Slope values from the linear regions are shown on the *right*. Bars represent mean $\pm$ SE for 3 independent experiments. *, $P < 0.05$ compared to control (ctl) cells.

Figure 7. Cortactin phosphorylation at serine 405 and 418 is required for lamellipodia persistence. (A) Kymographic analysis of MTLn3 lamellipodia. Serum starved MTLn3 cells (Ctl) or cells transfected with the indicated cortactin siRNA and

cortactin constructs were monitored for dominant lamellipodia formation by live cell imaging following EGF stimulation. Quantification of lamellipodia protrusion rates, length of extension, and time of lamellipodia persistence are shown for each experimental condition. ≥ 10 cells were analyzed for each group from ≥ 3 independent experiments. **(B)** Representative kymograms of each cell type. Kymograms were constructed from 1-pixel wide lines drawn from the initial leading edge and in the direction of the dominant lamellipodia. Cells were visualized by fluorescent microscopy using mCherry-b-actin as the lamellipodia marker. Images were captured every 5 sec for a period of 15 min. Black lines denote the baseline position of the leading edge prior to EGF stimulation. Bar; 5 mm.

Kelley et al., Fig 1

Kelley et al., Fig 2

Kelley et al., Fig 3

A

B

Kelley et al., Fig 4

Kelley et al., Fig 5

Kelley et al., Fig 6

A

B

Kelley et al., Fig 7

**Study 4: Transgenic Model of Oral-esophageal Tumor Cell Invasion:
Overexpression of Cortactin in Tumorigenic Mice**

Laura C. Kelley and Scott A. Weed

Abstract

Amplification of the chromosome locus 11q13 is frequently seen in squamous cell carcinomas of the head and neck (HNSCC) and results in overexpression of cortactin, a cytoskeletal protein that regulates actin dynamics. Cortactin overexpression is associated with poor prognosis in patients and increased invasion and metastasis *in vitro*. To investigate the role of cortactin in tumor progression we aimed to develop a transgenic model in which epitope-tagged murine cortactin was specifically overexpressed in the oral epithelia of C57BL/6 mice by means of the Epstein-Barr viral promoter ED-L2 (L2CortFL). Founder lines were to be crossbred with p53-mutant (Trp53R245W) mice to mimic oncogenic progression commonly seen in HNSCC. Founder lines containing the L2-Cort or L2-Trp53R245W transgenes were successfully developed. However, protein expression of the transgenic cortactin construct or overexpression of p53R245W was not detected in the founder animals. Further studies will be needed to examine whether tumors originating in the oral epithelium that overexpress cortactin are more invasive and metastatic than tumors with normal cortactin levels.

Introduction

Each year there are 36,500 newly diagnosed cases of cancer, with 7,880 deaths being directly caused by tumors originating in the oral cavity and pharynx (Altekruse SF, 1975-2007). Over ninety percent of these oral-pharyngeal cancers arise from the squamous epithelia and are classified as head and neck squamous cell carcinomas (HNSCC). The five-year survival rate of HNSCC remains less than 60%, and despite advances in diagnosis and management of the disease, these rates have not significantly improved in the past 20 years (ACS, 2010; Shiboski et al., 2000). The molecular alterations that lead to the progression of HNSCC have been extensively studied within the last five years with the goal of exploiting genetic modifications for treatment and chemoprevention (Le and Giaccia, 2003).

It is known that amplification of the gene which encodes cortactin (CTTN) is associated with poor prognosis in patients with HNSCC (Rodrigo et al., 2000). Cortactin is a multi-domain actin binding protein which was originally identified as a major target of v-Src infected cells (Wu et al., 1991). Cortactin functions as a scaffolding protein to coordinate actin dynamics driving endocytosis, intracellular trafficking, motility, and invasion through interactions with actin-related protein (Arp)2/3 complex, either directly or indirectly via N-WASp (Daly, 2004). Cortactin is overexpressed in 50% of cell lines derived from HNSCC's (Patel et al., 1996), and is believed to advance tumorigenesis by promoting cellular invasiveness and movement. It is known that upregulation of cortactin mRNA causes increased cell motility and invasion *in vitro* (Huang et al., 1998; Patel et al., 1998) and metastasis in nude mice (Li et al., 2001). However, the genetic role of cortactin overexpression in tumor progression and metastasis is not known.

HNSCC is a highly aggressive and invasive disease largely driven by overexpression of EGFR (Kalyankrishna and Grandis, 2006). Recently our laboratory found a link between CTTN copy number and enhanced tumor cell invasion and motility downstream of EGFR through comparison of HNSCC cells with and without 11q13 amplification (Rothschild et al., 2006). The increase in cell motility demonstrated by cells that overexpress cortactin is associated with increased Arp2/3 binding/activation and tyrosine phosphorylation of cortactin. Pretreatment of cells with the EGFR inhibitor, gefitinib, blocked cortactin tyrosine phosphorylation and cell motility. While these experiments indicate that cortactin overexpression directly drives HNSCC motility and invasion, validation of the role of cortactin in tumor progression can be best accomplished by the use of an *in vivo* cancer model. To this end, we have produced potential transgenic founder mice that express cortactin specifically in the oral cavity through use of the Epstein Barr virus ED-L2 tissue-specific promoter. This promoter has been used successfully to target and overexpress cyclin D1, another 11q13 gene associated with HNSCC progression, to the oral epithelia of transgenic mice in other mouse models of oral cancer (Goessel et al., 2005; Opitz et al., 2002).

Mutations in the p53 gene are found in greater than 50% of HNSCC's, and is believed to occur prior to 11q13 amplification in the current model of HNSCC progression (Boyle et al., 1993; Le and Giaccia, 2003). In addition, p53 mutations are linked to increased mRNA expression of EGFR in HNSCC (Grandis et al., 1998). Mice with dominant negative mutations of p53 have been successful in generating carcinomas in lung, breast, prostate, head and neck, and skin (Lang et al., 2004; Morris et al., 1998; Olive et al., 2004; Wijnhoven et al., 2005; Wu et al., 2002; Zhang et al.,

2000; Zhang et al., 2006; Zhang et al., 2005). Our model is based on the most common p53 mutation seen in HNSCC, an R to W mutation at codon 245 located in the transactivation domain of the encoded protein (Petitjean et al., 2007). Like cortactin, transgenic mice overexpressing the R245W p53 mutant protein will be generated using the ED-L2 promoter.

The purpose of this study is to evaluate the role of cortactin overexpression in the development of carcinomas of the oral cavity. Wild type cortactin will be specifically expressed only in the oral epithelia via the Epstein Barr virus (EBV) ED-L2 tissue specific promoter (Nakagawa et al., 1997). Founder transgenic mice will be backcrossed into a C57BL/6 background, a strain of mice that is associated with tumor progression, and then crossed with p53 R245W animals. The goal of this transgenic project is to mimic the genetic environment commonly seen in head and neck cancers and then use this model to examine and role of cortactin in disease progression. We hypothesize that overexpression of wild-type cortactin will increase carcinogenesis in the oral cavity of oncogenic mice.

Methods

Generation of L2-Cort and L2-Trp53^{R245W} mice. The FLAG-tagged cytomegalovirus (CMV)-driven cortactin expression construct pcDNA3FLAG2AB cortactin (FLAG-Cort) (Du et al., 1998) was used as the template for production of the cortactin construct. Site-directed mutagenesis was performed on Trp53 murine cDNA (p53) to produce an R to W mutation at codon 245 (Trp53R245W). FLAG-tagged wild type murine cortactin (FLAG-Cort), and (Trp53R245W) were PCR amplified and subcloned into the Epstein-

Barr virus ED-L2 vector with Mlu1 and Xho1 restriction sites. Generation of L2-Cort and L2-Trp53^{R245W} was verified by sequencing. In preparation for microinjection, excess plasmid backbone was eliminated by digestion with EcoR1 and Not1 resulting in 3.1Kb and 2.5Kb fragments for L2-Cort, and L2-Trp53^{R245W}, respectively. Fragments were given to the WVU Transgenic Rodent Facility for transgenic mouse production. Briefly, gene fragments were microinjected into the male pronucleus of single-cell embryos isolated from pregnant, superovulated FVB females. Fertilized eggs were re-implanted in the oviducts of CD-1 foster females that had been previously mated with vasectomized CD-1 males.

Genotyping. Potential founders were screened by PCR analysis from DNA isolated from tail tissue. Primers were generated to amplify a 350 Kb fragment within the L2 promoter since this nucleotide sequence is unique to the transgene and not found within the mouse genome. Primers for genotyping were produced by Integrated DNA Technologies, Inc. (Coralville, IA) and are as follows: L2-F 5'-CTTAACACACCACACAGGTAGCAA-3', L2-R 5'CTCTTAGTTTCTGGGTGTGAGAGGG-3' Internal control primers were used during genotyping to verify the integrity of the PCR reaction. The following primers amplify a 425Kb fragment of the RX gene: RX-F 5'-GGGAGTAGGGTTACTGGACTGAGGC-3', RX-R 5'CGAGTATCCCTACTGCCTGGAAATC-3'

RNA isolation and RT-PCR. Mouse tissues were harvested and stored in RNAlater (Qiagen) at 4°C overnight, and moved to -20°C for long-term storage. RNA was purified

using an RNeasy Plus kit (Qiagen). Total RNA were solubilized in RNase-free H₂O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was assured by examining OD₂₆₀/OD₂₈₀ ratio. cDNA was synthesized using SuperScript III First-Strand Synthesis Supermix (Invitrogen) according to the manufacturers' protocol. Two microliters of the cDNA reaction mixture was added to a PCR reaction with the following primer sets: P53-F 5'-CACAGTCGGATATCAGCCTAG-3' P53-R 5'-GATGGTAAGGATAGGTCGCCA-3' or Ctnn-F 5'-GGGACCTAAGCTTGGTACCATGTGGAAAGC-3' Ctnn-R 3'-GGACACCGAACTTGCCTCCGAAGCCCCGCACCG-3'.

Immunohistochemistry. Dissected mouse tongues were cryoprotected in 30% sucrose in PBS, oriented in TBS tissue freezing medium, and stored at -80°C until sectioned on a cryostat. To block nonspecific labeling, sections were immersed in PBS containing 0.6% H₂O₂ and 5% methanol for 1 hour at RT. Samples were washed in PBS and then further blocked in PBS containing 5% heat-inactivated normal serum and 0.1% Triton X-100 at RT for 1-2 hours. Primary antibodies were diluted in PBS containing 4% serum and 0.1% Triton X-100. Samples were incubated overnight in the primary antibody solution at 4°C. The antibodies used in this study were: p53 (1:1,000, #9802, Cell Signaling); p53 (1:500, AB-7, Calbiochem); Cortactin (1:1000, EP1922Y, Novus Biologicals); and DDDDK (FLAG) (1:1000, ab21536, Abcam). Slides were rinsed with PBS and incubated with appropriate biotinylated secondary antibodies (1:200, Vector Laboratories, Burlingame, CA). Slides were then rinsed with PBS or incubated with elite ABC reagent (Vector Laboratories) as described by the manufacturer (biotinylated).

Immunoreactivity was visualized with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) with 0.01% H₂O₂ in 0.5 M Tris pH 7.65. Slides were counterstained with hematoxylin and were digitally photographed on an Olympus ZX70 Provis microscope (Olympus, Center Valley, PA, USA) with a 20x/0.70 UPlanApo objective and an Optronics MicroFire 1600×1200 color CCD camera (Optronics Inc) using the StereoInvestigator imaging package (MBF Bioscience).

Immunoprecipitations. Freshly dissected mouse tissues were homogenized in 500 µl of ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES at pH 7.4, 20% glycerol, 0.1% Triton X-100, and 10 µM DTT) with a mechanical homogenizer. Clarified lysates (500µg) were immunoprecipitated with anti-cortactin (4F11) for 2 hours at 4°C before being resolved by SDS-PAGE. Cultured cells were lysed in NP40 Buffer (20 mM Hepes-KOH, pH 7.8, 50 mM KCl, 1 mM EDTA, and 1% NP40). Clarified lysates (500 µg) were incubated for 2 h at 4°C with 40 µl of EZview Red ANTI-FLAG M2 Affinity Gel (Sigma). Immune complexes were collected by centrifugation, washed twice with NP40 Buffer, separated by SDS-PAGE, and Western blotted with antibodies as described.

Cell lines, transfections and Western blotting. HNSCC cell lines UMSCC1 (Jeffery Myers, MD Anderson Cancer Center) and 1483 (David Raben, University of Colorado) were cultured as previously described (Rothschild et al., 2006). SYF^{+/+} (Src^{+/+}Yes^{-/-}Fyn^{-/-}) cells were obtained from the American Type Culture Collection. For transient transfections, 3 x 10⁶ cells were incubated with 2 µg plasmid construct or siRNA. Cell lines were transfected with the Nucleofector I device (Amaxa Biosystems). Western blotting of cell lysates was conducted as described (Rothschild et al., 2006). The following antibodies were used: β-actin (JLA-20, 1:5,000, Calbiochem); p53 (1:1,000,

#9802, Cell Signaling); p53 (1:500, AB-7, Calbiochem); Cortactin (1:1000, 4F11, Upstate); and DDDDK (FLAG) (1:1000, ab21536, Abcam).

Immunofluorescence labeling and confocal microscopy. Cells were fixed with fresh 4% formaldehyde and permeabilized with 0.4% Triton-X/PBS. Primary antibodies were diluted in 5% BSA/PBS. Antibodies used were: p53 (1:1,000, #9802, Cell Signaling); p53 (1:500, AB-7, Calbiochem); Cortactin (1:1000, 4F11, Upstate); and DDDDK (FLAG) (1:1000, ab21536, Abcam). Secondary antibodies used were Alexa Fluor 405 goat anti-rabbit and Alexa Fluor 647 goat anti-mouse (Molecular Probes). F-actin was labeled with rhodamine-conjugated phalloidin (Molecular Probes). Cells were mounted in Fluoromount-G (Southern Biotech), or ProLong® Gold antifade reagent with DAPI (Invitrogen) and imaged with a Zeiss LSM510 confocal microscope using AIM software (Carl Zeiss MicroImaging).

L2 luciferase reporter assays. 3×10^6 cells were transfected with 800 ng (low) or 3.2 μ g (high) of pXP2 or pL2-782, or with 2 μ g of pcDNA3-FLIP plasmids and 400 ng (low) or 1.6 μ g (high) of multimeric NF- κ B pGL2 luciferase vector. 1.25 μ g of the Renilla pRL-TK vector was added to all treatments. NF- κ B pGL2 vector and pRL-TK vectors were kind gifts from Christian Stehlik (Northwestern University, Chicago, IL), and pXP2 and pL2-782 were received from Anil Rustgi (University of Pennsylvania). pcDNA3-FLIP plasmids were made as described previously (Chanvorachote et al., 2005). Transfections were performed using the Nucleofector I device (Amaxa Biosystems). The following day luciferase activity was determined using a dual-

luciferase reporter assay system kit (Promega). The activity of the L2 or NF-kB reporter luciferase was standardized to that of Renilla luciferase.

Results

Experimental rationale and generation of Tg(L2-Cttn)B6 or Tg(L2-Trp53^{R245W})B6 mice. To investigate the role of cortactin in tumor progression in an animal setting, we sought to develop transgenic mice overexpressing FLAG-tagged murine cortactin specifically in the oral epithelia of C57BL/6 mice. Because we do not anticipate overexpression of cortactin alone to drive carcinogenesis, a tumorigenic background was devised in order to examine whether tumors that overexpress cortactin are more invasive and metastatic. Such a background will be supplied by the construction of transgenic mice expressing the dominant-negative mutant p53 protein (R245W) specifically in oral epithelia. Cross-breeding of mice generated will produce animals containing p53 dominant-negative driven tumors that overexpress cortactin (see Figure 1A). These mice will allow for the direct evaluation and impact of cortactin overexpression on motility and invasion in a transgenic oral cancer model. Potential founder animals were genotyped using primers specific to the L2 promoter which resulted in a ~350 Kb fragment in animals carrying the L2-Cttn or L2-Trp53^{R245W} transgenes (Figure 1A, B). To minimize the possibility of false negatives, primers that amplify a 425Kb fragment of the mouse genome were also included in all PCR genotyping reactions. One hundred and fifteen, and forty-two potential founders were genotyped for the L2-Cttn or L2-Trp53^{R245W} transgene, respectively. Two positive animals were identified in each line (Table 1). Founder animals were backcrossed with

C57BL/6 mice, a strain commonly favored for use in transgenic cancer models due to a low incidence of spontaneous tumors (Smith et al., 1973).

Characterization of transgenic lines. Lines were characterized phenotypically for cancer onset, transgene mRNA transcription and protein expression. One of the Tg(L2-Cttn)B6 lines (299) exhibited mRNA expression of the transgene in the tongue and esophagus (Figure 2A, B). Within the same animal mRNA expression is not detected in the small intestine or liver, validating the tissue-specific expression of the L2 promoter. Next we analyzed protein expression of the L2-Cttn transgene 299 line in the mouse tongue. Clarified lysates from homogenized tongue tissues were immunoprecipitated with anti-cortactin antibody, and then probed for cortactin expression. No differences are detectable in cortactin protein levels between the Tg(L2-Cttn)B6 mouse line and a non-transgene positive matched littermate (B6) (Figure 2C). Immunoprecipitation and western blotting with an anti-FLAG antibody were also conducted, but we were unable to detect the FLAG-tagged cortactin protein (data not shown). In addition, immunohistochemistry was conducted on frozen tissue sections of the tongue (Figure 2D) and esophagus (data not shown). Similar staining is seen in Tg(L2-Cttn)B6 and matched littermates, indicating that transgene mRNA expression did not result in increased levels of cortactin protein. Similar staining was also seen with use of several anti-FLAG antibodies. Importantly, no morphological differences were detected in the oral epithelia of 299 line compared to control animals.

Tg(L2-Trp53^{R245W})B6 animals were also characterized for transgene mRNA and protein expression. To specifically detect the L2-Trp53^{R245W} transgene from the

expression of the endogenous p53 alleles, primers were generated to complement unique DNA sequences introduced during generation of the construct. The forward primer flanks an altered Xho1 restriction site, and the reverse primer flanks the R245W mutation, resulting in specific amplification of the L2-Trp53^{R245W} transgene (Figure 3A, B). One of the Tg(L2-Trp53^{R245W})B6 lines (634, Table 1) demonstrated tissue-specific mRNA expression of the Trp53^{R245W} transgene (Figure 3C) and was further characterized for protein expression. Mutant p53 is known to accumulate in cells, unlike the wild type counterpart which is expressed at low levels (Iggo et al., 1990; Rotter, 1983). High levels of p53 in IHC patient samples are used as a predictive marker of p53 mutant status (Bartek et al., 1991; Soussi and Beroud, 2001). No differences were identified in p53 protein expression in Tg(L2-Trp53^{R245W})B6 and B6 lines by immunohistochemical analysis of mouse tongues (Figure 3D) or esophagus (data not shown). In addition, no pathological abnormalities were identified in the Tg(L2-Trp53^{R245W})B6 at ages up to 18 months.

Evaluation of L2 promoter activity in HNSCC cell lines. To determine a possible mechanism for the undetectable protein expression levels in the Tg(L2-Ctnn)B6 or Tg(L2-Trp53^{R245W})B6 mice, we evaluated the activity of the L2 promoter in HNSCC cell lines. 1483 and UMSCC1 HNSCC cell lines were transfected with luciferase reporter constructs fused to the L2 promoter (pL2-782) or a promoterless (pXP2) sequence. Fibroblast cells (SYF^{+/+}) were also transfected and used as a negative control. pL2-782 activity was higher in the 1483 and UMSCC1 cell lines than the SYF^{+/+} control (Figure 4A). Activity of the pXP2 construct was low in all three lines tested. To determine if

transfection of increasing amounts of the pL2-782 construct resulted in a corresponding increase in L2 promoter activity, lower (lo) and higher (hi) amounts (see Materials and Methods) were transfected into 1483, UMSCC1 and SYF^{+/+} cells (Figure 2B). The HNSCC lines tested display a dosage effect, with increasing L2 activities corresponding to increased levels of the reporter construct (Fig. 4B). These results suggest that the L2 promoter is active in the HNSCC lines. However, there is also a marked response to increasing amounts of the pXP2 construct in the UMSCC1 cell line, indicating that some of these increases may be non-specific. In addition, the activity levels of the L2 promoter even at high levels (~0.01 RLU) is much lower than endogenous NF-kB activity in the same cell lines (~7 RLU) (Figure 4C). While these values cannot be directly compared, these results suggest that L2 promoter activity, though specific, is very low in HNSCC cell lines.

Detection of L2 driven cortactin and p53^{R245W} protein expression in HNSCC cell lines. To determine if the low levels of L2 promoter activity led to a detectable protein product in HNSCC cell lines, 1483 cells were nontransfected, or transfected with constructs encoding FLAG-tagged cortactin behind a CMV or L2 promoter sequence (Figure 5A). Fibroblast cells (SYF^{+/+}) were used as a negative control. Anti-FLAG beads were used to immunoprecipitate FLAG-tagged cortactin from clarified lysates. CMV-driven cortactin expression led to high cortactin protein levels in 1483 and SYF^{+/+} cell lines detected by Western blotting with anti-FLAG and cortactin antibodies (Fig 5A). However, cortactin protein levels did not increase over non-transfected controls when driven by the L2 promoter construct. We also evaluated protein expression in the 1483

cells by immunofluorescence and confocal microscopy (Figure 5B). Exogenously expressed cortactin was detected in cells transfected with the CMV, but not the L2-driven cortactin constructs.

We additionally evaluated protein expression of p53 in the 1483 cell line (Figure 6). Cells were non-transfected (NT) or transfected with CMV-Trp53^{R245W} or L2-Trp53^{R245W}, and then labeled with rhodamine phalloidin (red), DAPI (blue), and two anti-p53 antibodies (a sheep monoclonal (green) and a rabbit monoclonal (purple)). Nuclear accumulation of p53 was visualized by confocal microscopy by both antibodies in cells with CMV-Trp53^{R245W}. There was no detectable increase in p53 protein levels in cells transfected with L2-Trp53^{R245W} compared to non-transfected controls.

Discussion

Transgenic animal models of tissue-specific cancers are valuable tools in gaining insight in the molecular mechanisms promoting carcinogenesis and tumor progression. Furthermore, the animal strains and the cell lines derived from them serve as potentially valuable platforms for measuring new therapies and preventative approaches. Our attempt to generate a transgenic oral cancer model to directly evaluate the impact of cortactin overexpression on cancer cell motility and invasion ultimately failed. We were able to produce Tg(L2-Cttn)B6 and Tg(L2-Trp53^{R245W})B6 mouse strains which expressed transgene mRNA, but we were unable to detect a transgene-driven protein product. In this study we created transgenic lines through pronuclear microinjection of mouse embryos with DNA fragments which results in random insertion of the transgene DNA into the mouse genome. The number of transgene inserts into the genome or the

chromosomal location at which a transgene inserts is completely random with this type of approach. Therefore, it is likely in our Tg(L2-Cttn)B6 and Tg(L2-Trp53^{R245W})B6 lines had transgene insertions in areas regulated by chromatin configurations that suppress transgene expression, leading to a undetectable protein product. Higher numbers of founder animals would be needed to potentially overcome these effects, and were not available to generate for this study due to time and financial restraints.

The L2 promoter was unable to produce measurable amounts of cortactin or p53^{R245W} protein in HNSCC cell lines. Therefore, we cannot rule out the possibility that the transgene fragments themselves are unable to support expression of cortactin or p53^{R245W} in a mouse model. However, it is also possible that these transformed cultured HNSCC cell lines have lost the expression of the tissue-specific transcription factors necessary to activate the L2 promoter. Without an oral epithelial cell line to use as a positive control, we cannot determine if this is, in fact, the case.

To date, one transgenic mouse model of cortactin has been reported (van Rossum et al., 2006). This study aimed to examine the role of cortactin in a breast cancer, in which CTTN amplification is associated with increased risk of relapse and death (Hui et al. 1998). Mammary gland-targeted overexpression of cortactin alone, or in combination with cyclin D1 overexpression, did not result in increased breast tumors compared with control mice. However, this study was limited in multiple ways that could explain why cortactin had no effect on carcinogenesis. First, the cortactin transgene was driven by the mouse mammary tumor virus and cortactin protein expression was only detected during lactation. Even if overexpression of cortactin alone could drive tumorigenesis, this window of time may be too small to show effects. Secondly, the

strain of mice in this study (FVB/N) showed a very high incidence of spontaneous mammary tumors (38%), making cortactin-driven tumorigenesis or progression difficult to unequivocally assess.

Recently, two studies have analyzed cortactin knockout MEFs derived from mice harboring floxed cortactin alleles (Cttn^{flox/flox}). Okabe and colleagues (Tanaka et al., 2009) found that cortactin depletion did not alter the F-actin architecture, the localization of actin binding proteins to actin networks, or cell motility measured by wound healing and transwell migration. The other report (Lai et al., 2009) confirmed that cortactin knockout MEFs displayed little to no alterations in Arp2/3-mediated actin networks. However, cortactin was found to be necessary for random and directional cell migration through the modulation of the Rho-GTPase CDC42. The role of cortactin depletion on epithelial cell migration or the impact of cortactin knockout on cell migration in Cttn^{flox/flox} mice was not evaluated in either of these studies.

Due to the inherent limitations of the current research examining cortactin in an animal model and the wealth of research implicating cortactin as an important mediator of human disease, additional studies are necessary and warranted to further define the role of cortactin as a promoter of tumor metastasis.

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Figure 1. Genotyping of L2 transgenic lines. **A)** Cartoon of C57BL/6 (B6) transgenic mouse lines with specific-targeting of p53 Tg(L2-Trp53^{R245W})B6, and cortactin Tg(L2-Cttn)B6 to the oral cavity. Crossing these lines will produce mice with targeted overexpression of both transgenes Tg(L2-Trp53^{R245W},L2-Cttn)B6. **B)** Schematic representation of p53 (L2-Trp53^{R245W}) and cortactin (L2-Cttn) transgenes. The red bar denotes the area of the transgene amplified in PCR genotyping analysis. **C)** Genotyping to identify potential founder animals. PCR reactions were carried out with GoTaq Green Master mix in the presence of two primer sets: 1) primers design to amplify a 351 kb fragment within the L2 promoter, and 2) Primers amplifying a 425 Kb fragment of the murine genome (internal PCR control). No DNA (lane 1), DNA from diluted transgene fragments (lane 2, 3), DNA isolated from control (lane 3, 4) or experimental mice (lanes 5-8) were added to the PCR master mix described above. Reactions were resolved on a 2% agarose gel and experimental samples were analyzed in duplicate. An animal carrying the L2-Cttn, or L2-Trp53^{R245W} transgene will display a band and 351Kb and 425 Kb (land 5, 6).

Figure 2. Cortactin mRNA and protein expression in L2-Cttn mice. **A)** Schematic representation of the flag-tagged cortactin (L2-Cttn) transgene. The yellow bar denotes the area of the transgene amplified in RT-PCR analysis. **B)** Tissue-specific expression of Cttn mRNA in the tongue and esophagus. RNA was isolated from the tissues listed and RT-PCR was performed. **C)** Mouse tongues were homogenized and clarified lysates were evaluated for cortactin protein expression. Cortactin was immunoprecipitated from Tg(L2-Cort)B6 and littermate control (B6) mice with an anti-4F11 (cortactin) antibody and then immunoblotted with anti-4F11 antibody. β -actin was used as a loading control for the total cell lysate (input). **D)** Immunohistochemistry of cortactin protein expression in Tg(L2-Cort)B6 and B6 mouse tongues using a rabbit polyclonal cortactin antibody.

Figure 3. p53 mRNA and protein expression in L2-Trp53^{R245W} mice. **A)** Schematic representation of the L2-Trp53^{R245W} transgene. The yellow bar denotes the area of the transgene amplified in RT-PCR analysis. **B)** Validation of Trp53 mutant-specific DNA amplification. Primers were generated flanking an altered Xho1 restriction site and the R245W mutation. A DNA fragment corresponding to 700 kb is only generated with the construct that contains the modified Xho1 site and R245W mutation. **C)** Tissue-specific expression of Trp53^{R245W} mRNA in the tongue and esophagus. RNA was isolated from the tissues listed and RT-PCR was performed on Tg(Trp53^{R245W})B6 mice and littermate controls (B6). **D)** Immunohistochemistry of p53 expression in Tg(Trp53^{R245W})B6 and B6 mouse tongues detected with a sheep pAb. Similar results were found with additional anti-p53 antibodies.

Table 1. Summary of Transgenic Cortactin and p53^{R245W} mouse lines.

Line	Tg(L2-Cttn)B6		Tg(Trp53 ^{R245W})B6	
Mouse ID	299	300	438	634
Generation	N5	N3	N2	N2
mRNA expression	Yes	No	No	Yes
Table abbreviations: Tg, transgenic; N, backcross generation.

Figure 4. Analysis of the L2 promoter in HNSCC cell lines. **A)** pL2-782 luciferase (green) and the promoterless pXP2 luciferase (blue) activity in HNSCC cells (1483 and UMSCC1) and fibroblasts (SYF+/+). **B)** The experiment shown in (A) was repeated with increasing amounts of the pXP2 or pL2-782 constructs. **C)** NF-kB luciferase activity in 1483, UMSCC1 and SYF+/+ cells nontransfected (NT) or cotransfected with high (hi) or low (lo) amounts of FLIP to induce activation of NF-kB. This experiment was used as a positive control for the luciferase reporter assay.

Figure 5. Analysis of L2-Cttn protein expression in HNSCC cell lines. **A)** 1483 and SYF^{+/+} cell lines were nontransfected (NT) or transfected with plasmids expressing flag-tagged cortactin from a CMV (CMV-Cttn) or L2 (L2-Cttn) promoter. Clarified cell lysates were immunoprecipitated with anti-FLAG beads and then blotted with anti-FLAG and anti-cortactin antibodies. Total cell lysates were blotted with anti-FLAG, anti-cortactin, and anti-actin antibodies. **C)** 1483 cells were nontransfected (NT) or transfected with CMV-Cttn or L2-Cttn. Cells were fixed, permeabilized and labelled with Rhodamine phalloidin (red), anti-FLAG (blue) and anti-cortactin antibodies (green).

Figure 6. Analysis of L2-Trp53^{R245W} protein expression in HNSCC cell lines. 1483 cells were nontransfected (NT) or transfected with plasmids expressing p53^{R245W} from a CMV (CMV-Cttn) or an L2 (L2-Cttn) promoter. Cells were fixed, permeablized and labelled with Rhodamine phalloidin (red), DAPI (blue), and anti-p53 sheep (green) or anti-p53 rabbit antibodies.

General Discussion

Taken together, the studies presented throughout this dissertation shed new light on pathways that control cellular invasion in HNSCC that may contribute to the metastatic process. We have demonstrated several novel findings as to how Src kinase activity regulates the metastatic phenotype of cancer cells through the formation of invadopodia. Saracatinib (AZD0530), a recently developed Src kinase inhibitor currently in phase I/II clinical trials (Kopetz et al., 2007) was reported to have anti-invasive effects in several model systems (Green et al., 2009; Koppikar et al., 2008; Nozawa et al., 2008). In Study 1 we show that the Src inhibition by saracatinib prevents the formation of invadopodia and related ECM degradation in HNSCC lines and results in decreased cell invasion and lymph node metastasis in nude mice. These results point to a model in which saracatinib exhibits anti-invasive effects in HNSCC by preventing invadopodia formation. These results have subsequently been confirmed in breast cancer models using the Src inhibitor dasatinib (Pichot et al., 2009), suggesting that blocking invadopodia formation may be a universal mechanism in suppressing invasion in Src-targeted therapies within multiple tumor types.

In Study 2 we further define how Src tyrosine kinase regulates invadopodia assembly and maturation. We demonstrate that Src activation is necessary and sufficient to target actin-associated proteins. However, regulated WT Src must be present for invadopodia to mature into functional matrix-degrading structures (see Figure 1). These results are consistent with the scarcity of activating Src mutations in human tumors (Summy and Gallick, 2003; Summy and Gallick, 2006). Therefore this study is the first to present evidence demonstrating that oncogenic forms of Src require

a WT counterpart to elicit full invasive potential, which has been previously overlooked in the field.

Figure 1 Schematic illustrating Src regulation in invadopodia maturation. Constitutively active Src is necessary and sufficient to target actin-associated proteins to, and direct the formation of pre-invadopodia, while wild-type Src is required for invadopodia maturation.

We are able to clearly demonstrate in Study 2 that WT Src directs invadopodia maturation. However, the mechanism by which this occurs is unknown. Since constitutively-active and kinase dead forms of Src do not rescue for WT Src function, it is attractive to speculate that cycles of Src activation and inactivation are necessary to temporally and spatially regulate the linking of the actin cytoskeleton to the degradative (protease activating/delivering) machinery. However, until activation/inactivation tracing, such as employed by FRET (fluorescence resonance energy transfer)-base techniques are available on a single molecule level, precise mechanistic testing will be difficult to achieve. Future studies are also needed to evaluate the targets of WT Src that are responsible for mediating preinvadopodia maturation.

It is clear that Src activation is tightly linked to phosphorylation of several cytoskeletal proteins known to localize to invadopodia sites, including cortactin (see

Study 1, and 2). Thus far, it is not known if Src is directly phosphorylating these targets to regulate invasion or if there are intermediate kinases downstream of Src responsible for transmitting the Src-generated signals responsible for these effects. We demonstrate that Src kinase activation in concert with WT Src activation/inactivation is sufficient to promote and regulate invadopodia dynamics in cell lacking other Src family kinases (Yes and Fyn; Study 2). However, we cannot rule out that other Src family members can potentially play a redundant role. Several Src family members that are overexpressed in human cancers are known to phosphorylate cortactin, including Arg and Abl kinases, so future work should aim to understand what role these proteins play in Src-mediated invasion programs. Such studies would provide further insight into the molecular mechanisms that govern cancer invasion, but from a current therapeutic standpoint, results from studies may have minimal impact since most designed Src inhibitors to date (including saracatinib) lack strict Src specificity, targeting all of the above-mentioned kinases in addition to Src (Lombardo et al., 2004; Manley et al., 2005).

Studies 1 and 2 support a model in which WT Src overexpressed in HNSCC cells primes the cell for an invasive phenotype through production of invadopodia. However, it is highly likely that Src activation is reliant upon overactivated upstream pathways commonly deregulated in HNSCC. Like Src, EGFR is commonly overexpressed in HNSCC cases (Grandis and Tweardy, 1993), but activating mutations are rare (Lee et al., 2005; Loeffler-Ragg et al., 2006). In Study 3 we demonstrate that treatment of HNSCC cells with EGF leads to phosphorylation of cortactin on Src-targeted tyrosine residues (Y421) and ERK1/2-targeted serine residues (S405, S418). Using novel

phosphorylation-specific antibodies against S405 and S418, we challenge previous reports (Martinez-Quiles et al., 2004) suggesting that cortactin serine and tyrosine phosphorylation are independent, mutually exclusive events. We show that these phosphorylation events can occur simultaneously in Src transformed cells (Study 3). In fact, serine and tyrosine phosphorylated cortactin localizes to motile and invasive structures such as lamellipodia, invadopodia, and to the invasive fronts of tumor cells *in vivo* (Studies 2 and 3). Therefore, it is highly likely that both serine and tyrosine phosphorylation of cortactin are necessary for supporting invasive tumor movement. Tyrosine (Study 1 and 2) and serine phosphorylation (Ayala et al., 2008) of cortactin is necessary for efficient invadopodia degradation. In addition, we identify S405/418 phosphorylation as an important mediator of cancer cell motility and adhesion, and a novel regulator of lamellipodia dynamics, two cellular functions necessary for efficient cell motility.

The understanding of tumor initiation and progression has advanced significantly through the use of genetically engineered mouse models (Walrath et al., 2010). However, animal models of tumor cell invasion and metastasis, the leading cause of cancer-associated death, are desperately needed to gain understanding of the molecular mechanisms that drive this process and identify potential targets of therapeutic intervention. HNSCC is an highly aggressive and invasive disease in which mouse models are particularly lacking (Lu et al., 2006). In Study 4 we attempted to create a mouse model of oral cancer to determine the role of cortactin in driving invasion and metastasis *in vivo*. For unknown reasons, the transgene constructs did not result in increased protein expression, and our approach ultimately failed. Future

studies are required to decipher how cortactin, and other motility related proteins, function *in vivo* during cancer cell invasion and metastasis.

Real-time live imaging of cancer cells is another area of emerging research that will significantly increase our knowledge of the metastatic process in live animals (Timpson et al., 2009). Capturing the movement of cancer cells away from the primary tumor, and subsequent invasion into the blood stream is a technically difficult task that requires capturing a sporadic event in an inaccessible environment. Intravital imaging of GFP-tagged breast tumor demonstrated that cells form invadopodia-like protrusions (Yamaguchi et al., 2005). As intravital imaging techniques advance, studies should focus on imaging invadopodia specific markers, such as Src and cortactin, to progress the identification and function of invadopodia *in vivo*. In addition, visual analysis of invadopodia-mediated interaction with ECM components would supply a platform for testing anti-invasive therapies that have been successful in blocking invadopodia formation and cell invasion in cell culture models. These studies would highly impact our understanding of the metastatic process and potentially impact therapeutic strategies leading to increased patient survival.

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Appendix

Actin cytoskeletal mediators of motility and invasion amplified and overexpressed in head and neck cancer

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Abstract Coordinated regulation of the actin cytoskeleton is central to cell motility, invasion and metastasis. Head and neck squamous cell carcinoma (HNSCC) is a highly invasive disease displaying frequent lymph node metastasis, compounding patient management. HNSCC progression is characterized by frequent amplification of chromosome segments 3q26-29, 8q23-24 and 11q13, events that are associated with poor patient outcome. The relative frequency of these amplification events and correlation with invasive disease raises the potential that these regions harbor actin regulatory genes important in facilitating reorganization of the actin cytoskeleton to promote tumor invasion. Identification of the actin cytoskeletal regulatory genes located within the 3q26-29, 8q23-24 and 11q13 amplicons will provide an important first step towards the comprehensive understanding of the molecular events that govern invasion and metastasis in HNSCC and other tumors containing these amplifications. We utilized Ensembl MartView to conduct a gene mining analysis within chromosome segments 3q26-29, 8q23-24 and 11q13 to identify known and predicted regulators of actin-based cell movement, tumor invasion and metastasis. All examined chromosomal regions contain genes known that regulate the actin cytoskeleton, with several (PI3-kinase

alpha, focal adhesion kinase (FAK) and cortactin) known to promote invasion in HNSCC and other carcinomas. Additional genes known to regulate motility and invasion were also identified. Amplification of chromosome 3q26-29, 8q23-24 and 11q13 therefore results in known or predicted overexpression of several key mediators that can act alone or potentially act in concert to promote actin-based cell invasion in HNSCC and other cancer types.

Keywords Actin · Gene amplification · Head and neck squamous cell carcinoma · Invasion · Metastasis · Motility

Introduction

Head and neck squamous cell carcinoma (HNSCC) exhibits a propensity for local-regional invasion and cervical lymph node metastasis. Invasive HNSCC complicates patient management contributing to the damage of surrounding soft tissue structures [1]. Patient outcome directly correlates with the degree of local and regional invasion [2]. Although it has long been recognized that invasive HNSCC is a negative prognostic indicator, the lack of a universal molecular “metastasis signature” has hampered the understanding of the signaling pathways that underlie HNSCC invasion and metastasis [3]. While recent advances in screening gene expression arrays have begun to identify candidate genes responsible for regulating motility and invasion in HNSCC [4, 5], a comprehensive understanding of the factors contributing to HNSCC invasion is still emerging.

Cancer cell motility and invasion involves a complex and integrated series of events that are primarily controlled by regulation and reorganization of the actin cytoskeleton

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[6, 7]. Regulation of actin polymerization is responsible for the formation of protrusive structures that are essential for tumor cell movement and invasion, including filopodia, lamellipodia and invadopodia. Formation and turnover of these structures require the coordinate activity of actin-binding and modifying proteins [7]. Subsequent stabilization of protrusive structures is accomplished by integrin-mediated adhesion to the extracellular matrix (ECM), linking the ECM to the actin cytoskeleton and serving the additional role of coordinating signal transduction events that regulate cell movement [8]. The proper synchronization of these of protrusion and adhesive events is required for net cell movement from the primary tumor site into surrounding tissues and vasculature [9].

Signaling pathways involved in mediating chemotactic cues from the extracellular environment that impact the actin cytoskeleton to initiate motility have been and continue to be an area of intense study. Several protein groups have been implicated in cancer cell motility and invasion, most notably receptor and non-receptor tyrosine kinases, integrin-associated proteins and Rho-family GTPases [10–13]. For protrusive events, the Rho family GTPases Cdc42 and Rac are intimately involved in mediating upstream signals that ultimately impact the terminal endpoint of these pathways, centering on activation of the Wiscott-Aldrich syndrome (WASp) superfamily of actin nucleation factors [6]. WASp proteins in turn enhance the production of free barbed ends from existing F-actin filaments at the cell cortex by activation of the actin related 2/3 (Arp 2/3) complex [14]. The resulting actin polymerization by Arp2/3 initiated at the inner face of the plasma membrane provides the internal force to push the membrane forward and begin cell movement. Actin protrusion at the cell periphery is central to the formation of filopodia and lamellipodia during cell motility, and well as invadopodia during tumor cell invasion [7]. In addition to cortical actin regulation, Rho GTPases also govern integrin containing cell-substratum adhesion turnover, polymerization and contraction of actin filaments within cytoplasmic stress fiber networks. Along with actin-based cell protrusion, the turnover of integrin containing adhesions is central to cell motility and invasion [15]. Changes in genes utilized in actin-based motility either by mutation or altered expression levels are observed in HNSCC and other carcinomas at every step in these signaling cascades, from transmembrane receptors to terminal effector proteins [16, 17].

Sequential accumulation of chromosome gains and deletions are common in HNSCC and assists to drive tumor progression. Although chromosomal deletions outnumber amplifications in HNSCC [18], amplification of select chromosomal segments has been shown to positively correlate with increased regional metastasis [19]. The cytogenetic alterations associated with HNSCC have been

reviewed elsewhere and have identified several genes with potential pro-metastatic roles [18, 20]. However, a comprehensive analysis of the most frequently amplified chromosomal segments in HNSCC to identify elevated gene numbers that participate in actin cytoskeletal remodeling during motility and invasion has not been reported. In this review we have analyzed three of the most commonly amplified chromosomal segments in HNSCC; 3q26-29, 8q23-24, and 11q13 [21] to identify genes known or predicted to regulate the actin cytoskeleton during HNSCC motility, invasion and metastasis. Data mining of the human genome to find actin-cytoskeleton associated or regulatory genes on these chromosomal segments was conducted using Ensembl MartView (<http://www.ensembl.org/Multi/martview>). The associated Gene Ontology (GO) [22] terms for the biological processes, molecular functions and cellular components of all currently recognized gene products within each chromosomal segment were exported and reviewed. Genes with GO terms associated with the actin cytoskeleton, cell motility and invasion were identified and further investigated by a literature search for evidence of amplification or protein overexpression in HNSCC or other carcinomas. Key genes with putative or demonstrated roles in HNSCC motility and invasion, known or predicted interactions between different gene products, and the how such interplay potentially contributes to HNSCC motility and invasion are presented and discussed.

Chromosome 3q26-29

Chromosome 3q26-29 has been consistently cited as the most frequently amplified region in HNSCC, with the minimal amplified region containing 3q26-27 as determined by comparative genomic hybridization (CGH) [23]. Amplification of 3q26 in HNSCC is an early aberration and correlates with the transition to invasive cancer and negative clinical outcome [19, 24]. Amplification of 3q24-29 is seen more commonly in human papilloma virus (HPV) positive HNSCC tumor specimens than HPV negative samples [25]. There are 448 genes in this region, 17 of these genes were found to be associated with cell motility or the cytoskeleton. Five genes in this region are well characterized with respect to cell migration in HNSCC or other tumor types and are discussed in detail.

Protein kinase C ι

Protein kinase C (PKC) is a multigene family of phospholipid-dependent serine/threonine kinases consisting of at least 11 isoforms that are expressed in virtually all cells and tissue types and are involved in regulation of cellular

proliferation, differentiation and apoptosis [26]. The PKC enzyme family is divided into three subgroups: Calcium-dependent, novel, and the atypical PKCs. Protein kinase C ι (PKC ι) is an atypical PKC isozyme, which is not dependent on calcium or diacylglycerol for its activation [27]. PKC ι is activated by c-Src and is an upstream activator of the Rac1/Pak/MEK/ERK signaling cascade, which is crucial to cell motility [28]. The activation of PKC ι has been linked to nicotine, as has its phosphorylation of calpain 1 and 2, contributing to enhanced cellular migration and invasion in lung cancer [28]. Although there is no current evidence directly linking it to HNSCC, PKC ι is overexpressed in non-small cell lung cancer and is a prognostic indicator of poor survival independent of tumor stage [29]. In ovarian carcinoma PKC ι is overexpressed more than other PKC isozymes [30] and its expression correlates with decreased survival time [31]. Based on these data, the role of PKC ι in HNSCC cases with 3q26-29 amplification warrants additional investigation.

Phospholipase D1

Intracellular signaling by phospholipase D (PLD1) has been of interest as a therapeutic target for inflammation and tumor metastasis for decades [32]. PLD1 is a phospholipase that generates second messenger lipid products following the binding of a variety of cell surface ligands [33]. PLD1 catalyzes the hydrolysis of phosphatidylcholine to choline and phosphatidic acid. Phosphatidic acid (PA) in turn is hydrolyzed to produce diacylglycerol (DAG) and lysophosphatidic acid (LPA). LPA is involved in physiological activities including wound-healing, inflammation, oncogenesis and metastasis [32]. LPA is a potent inducer of cell motility by regulating the activation levels of a number of Rho GTPases (described in further detail below with regards to autotaxin). PLD1 regulates actin stress fiber and integrin-containing focal adhesion organization in fibroblasts during cell movement [34]. PLD1 activates the tyrosine kinase c-Src and mitogen activated protein kinase (MAPK), two kinases central to the regulation of tumor cell growth and invasion [35]. PLD1 is overexpressed in breast cancer and is required for tumor cell invasion [36, 37]. Data from the Human Protein Atlas (<http://www.proteinatlas.org/index.php>) indicates that PLD1 is overexpressed in HNSCC, and future studies will be valuable in evaluating its role in regulating HNSCC growth and invasion.

Phosphoinositide-3-kinase α subunit (PIK3CA)

Phosphoinositide 3-kinases (PI3Ks) constitute a family of lipid kinases that play a central role in many cellular functions associated with malignant behavior, including

control of cell cycle progression, invasion, and survival [38]. Class I PI3Ks typically contain a 110 kDa catalytic subunit and an 85 kDa regulatory subunit [39]. PIK3CA encodes the class I p110 α catalytic subunit [40] and is amplified and overexpressed in HNSCC, correlating with increased vascular invasion [41]. PIK3CA is a master regulator for activating Cdc42, Rac and Rho, reorganizing the actin cytoskeleton to result in the formation of filopodia, lamellipodia and stress fibers utilized during cell migration [42]. In particular, PI3K catalytic activity is required for EGF-stimulated actin nucleation during lamellipodia extension in breast cancer cells [43] and accelerates heregulin-induced breast cancer invasion [44]. The prevalence of PIK3CA amplification in HNSCC and its association with the transition from dysplasia to invasive carcinoma [45] make it a prime candidate for anti-tumor and anti-invasive therapeutic strategies.

Claudin-1

Claudin proteins comprise a growing protein family that are essential for the formation of tight junctions (TJs) in epithelial and endothelial cells [46]. TJs are critical for cell–cell adhesion, functioning to physically fuse the membrane of adjacent cells together, creating a paracellular seal that regulates the control of solutes between cells and establishes a diffusion barrier for integral membrane proteins in the lipid bilayer [46]. TJs are indirectly linked to the actin cytoskeleton through a series of adaptor and signaling proteins. Like all claudins, claudin-1 can form homotypic or heterotypic interactions with other claudin members as TJs are formed, with the precise combination of claudin types determining the strength of the junction [46]. Maintenance of cell–cell adhesion provides resistance to the epithelial to mesenchymal (EMT) transition during neoplastic transformation, and is often associated with altered claudin expression [47]. Accordingly, loss of claudin-1 expression is correlated with higher-grade colorectal carcinoma than cases with normal claudin-1 levels [48]. However, many claudin members, including claudin-1, display increased expression in HNSCC and other cancer types [49], suggesting other functions for claudin proteins besides TJ biogenesis and homeostasis. In HNSCC, claudin-1 overexpression enhances the activity and expression of matrix metalloproteinases MT1-MMP and MMP2, resulting in elevated cleavage of the extracellular matrix protein laminin-5 and increased tumor cell invasion [50]. While the precise mechanism of how claudin-1 regulates MMP activity is currently unclear, the evaluation of claudin-1 expression on clinical outcome in HNSCC cases with 3q26-27 amplification may validate the role of claudin-1 as a pro-invasive biomarker in select HNSCC cases.

ACK1

Activated Cdc42-associated kinase 1 (ACK1) is a dual specificity tyrosine and serine/threonine kinase that is amplified and overexpressed in HNSCC [51, 52]. ACK1 was initially identified as an interacting protein for GTP-bound Cdc42 [53], suggesting a role in actin cytoskeletal regulation. In support of this, ACK1 mediates signaling events that lead to actin cytoskeletal reorganization. ACK1 indirectly regulates Rho family protein activity by phosphorylation and activation of the Rho family guanine nucleotide exchange factor Dbl [54]. ACK1 also regulates actin cytoskeletal architecture by directly phosphorylating p130^{CAS} [55], a protein linked to activation of Rac and promotion of cell motility and invasion [56]. In addition, ACK1 phosphorylates WASp on tyrosine and serine residues, resulting in enhanced stimulation of Arp2/3-mediated actin nucleation activity in cell extracts [57]. These data collectively indicate that ACK1 utilizes multiple signaling pathways in regulating actin cytoskeletal assembly, indicating that carcinoma motility, invasion and metastasis may be enhanced in tumors containing amplification and overexpression of ACK1. Direct evidence for ACK1 amplification and overexpression playing a role in tumor invasion has been recently documented in esophageal and lung carcinoma, cancers that correlate with adverse environmental exposure. ACK1 amplification and overexpression in these tumors corresponds with poor patient outcome, as well as stimulating invasion and metastasis in animal model systems [52]. In addition, ACK1 is activated downstream of EGFR [58, 59], suggesting that overexpression of EGFR in HNSCC may serve to additionally potentiate signaling pathways in tumors with ACK1 overexpression. Along with its role in regulating tumor cell motility and metastasis, ACK1 has recently been shown to promote tumorigenesis in prostate carcinoma by phosphorylating the tumor suppressor WW domain containing oxidoreductase (Wwox), targeting it for polyubiquitination and degradation [60]. The multiple roles of ACK1 in tumor progression may make it a viable candidate for novel therapeutic development.

Chromosome 8q23-24

Amplification of the 8q region is common in HNSCC, and has been cited as the second most important early chromosomal event in HNSCC progression [23, 61, 62]. The most commonly amplified subregion is 8q23-24 [62]. The oncogenic transcription factor c-myc is located on 8q24, and HNSCC cases with 8q24 amplification demonstrate increased c-myc expression that is associated with poor survival [18, 63]. There are 273 genes within the 8q23-24

region, with 14 associated with actin cytoskeletal regulation, cell motility or invasion. Four of these are either known or have the potential to regulate HNSCC motility and invasion and are described below. Table 1 contains additional relevant genes found in this region

Autotaxin

Autotaxin (ATX) is an extracellular glycoprotein that is a potent stimulator of tumor cell motility and invasion in a variety of normal and tumor cell lines [64, 65]. ATX functions as an extracellular nucleotide pyrophosphatase and phosphodiesterase (NPP) [66, 67]. The phosphodiesterase activity of ATX is required for mediating cell movement [67], and it was later discovered that ATX is identical to lysophospholipase D, a serum enzyme responsible for producing LPA in serum [68]. LPA produced by ATX stimulates cell movement by activating Rac1 and RhoA and associated actin structures through engagement of the LPA₁ and LPA₂ G-protein coupled receptors [69], creating a potent autocrine loop for maintaining cell migration. ATX is also a stimulator of angiogenesis, a factor contributing to its ability to enhance tumor aggressiveness [70]. ATX overexpression has been documented in non-small cell lung cancer [71] and its overexpression correlates with increased invasive potential in breast carcinoma [72]. While the known data and characteristics of ATX make it an attractive candidate as a mediator of HNSCC invasion and metastasis in cases with 8q23-24 amplification, or overexpression of ATX in HNSCC has yet to be confirmed.

MIM1

Missing in metastasis 1 (MIM1, MTSS1) was initially named due to the absence of the gene product in metastatic bladder and prostate cancer cells [73], suggesting that MIM1 functions as a tumor suppressor. Evaluation of MIM1 function by structure-function studies indicates that it serves as a scaffold protein that regulates actin dynamics and gene transcription [74]. MIM1 binds G- and F-actin and serves to coordinate actin assembly and lamellipodia formation through activation of Rac [75]. MIM1 also interacts with cortactin, enhancing the ability of cortactin to facilitate Arp2/3 mediated actin assembly while inhibiting Arp2/3 activation by N-WASp [76](see below). Binding of MIM1 to cortactin enhances lamellipodia formation and fibroblast motility initiated by PDGF [75, 76]. PDGF-mediated Src activation leads to tyrosine phosphorylation of MIM1, an event essential for the formation of pro-migratory membranous dorsal waves [77]. Although

Table 1 Amplified and overexpressed genes that impact actin organization and cell motility in HNSCC

CR	Band	Gene name	Protein name(s)	Actin cytoskeletal/cellular functions	Gene product o/x in HNSCC [reference]
3	q26.2	PRKCI	Protein kinase C iota	Serine/threonine kinase, signal transduction cascades impacting actin cytoskeleton organization in motility and invasion	Unknown
3	q26.31	PLD1	Phospholipase D1	Phospholipase activity, second messenger generation, produces LPA responsible for activating Rho GTPases and regulating actin cytoskeletal dynamics during cell motility	Yes (HPA)
3	q26.32	PIK3CA	Phosphoinositide-3-kinase p110 alpha catalytic subunit	Lipid kinase activity, regulator of Rho GTPase signaling, controls actin organization downstream of EGFR in tumor invasion	Yes [41, 45]
3	q28	CLDN1	Claudin 1	Tight junction structural protein, regulates cell–cell adhesion, indirectly linked to the actin cytoskeleton. Overexpression implicated in regulating MMP activity during invasion	Yes [50] (HPA)
3	q29	TNK2	TNK2, ACK, ACK1	Tyrosine and serine/threonine kinase activity activated by EGFR, regulated by Cdc42, stimulates WASp-mediated Arp2/3 nucleation, involved in cell invasion	Yes [51, 52]
8	q24.12	ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2, autotaxin	Extracellular LPA production, activation of G-protein coupled receptors leading to RhoA and Rac1 activity, regulates cell invasion	Unknown
8	q24.13	MTSS1	Metastasis suppressor 1, missing in metastasis	Scaffolding protein, binds actin monomers, regulates cortactin activity and Arp2/3-induced actin nucleation, activated by Src, regulates leading edge formation and cell motility	Unknown
8	q24.21	DDEF1	Development and differentiation enhancing factor 1, AMAP1, ASAP1, centaurin beta 4	ADP ribosylation factor, GTPase activating protein for Arf family members, scaffolding protein that binds FAK and cortactin, involved in lamellipodia formation, focal adhesion turnover and invadopodia formation	Unknown
8	q24.3	PTK2	Focal adhesion kinase 1, FAK, pp125FAK	Protein-tyrosine kinase, regulates integrin-mediated cell–substratum adhesion turnover and focal adhesion assembly, influences Rho family GTPase and cytoskeletal remodeling, controls cell motility and invasion	Yes [87, 88] (HPA)
8	q24.3	SHARPIN	SHANK-associated RH domain interacting protein, hSIPL1	Adaptor protein, interacts with SHANK2, function unknown	Unknown
11	q13.1	CDC42EP2	CDC42 effector protein 2, BORG1, CEP2	GTP-Rho binding protein, involved in actin filament organization and polymerization, regulates lamellipodia formation	Yes [109]
11	q13.1	CFL1	Cofilin-1	Bind F- and G-actin, severs actin filaments, regulates actin polymerization and depolymerization at the leading edge	Yes [119]
11	q13.1	FOSL1	FOS-related antigen 1, FRA-1	Transcription factor, regulates CD44 and c-MET expression, regulates cell migration by MAPK-induced uPAR regulation of Rac and Arp2/3	Yes [125]
11	q13.2	RAB1B	Ras-related protein Rab-1B	Small GTP binding protein, regulates vesicle transport	Unknown
11	q13.2	RIN1	Ras and Rab interactor 1	Ras binding, regulates motility by activation of Abl and Arg kinases	Yes [135]
11	q13.2	SPTBN2	Spectrin beta chain, brain 2	F-actin actin binding and cross linking protein, prevents actin depolymerization, found in lamellipodia	Unknown
11	q13.2	RHOD	Rho-related GTP-binding protein RhoD	Rho-family GTPase, regulates endosomal vesicle motility	Unknown
11	q13.2	SSH3	Protein Phosphatase Slingshot homolog 3	Protein serine/threonine phosphatase. Regulates cofilin activation	Unknown

Table 1 continued

CR	Band	Gene name	Protein name(s)	Actin cytoskeletal/cellular functions	Gene product o/x in HNSCC [reference]
11	q13.2	CORO1B	Coronin-1B	F-actin binding, downregulates Arp2/3 activation, inhibits WASp activation, targets slingshot to lamellipodia, coordinates actin polymerization during motility	Unknown
11	q13.3	FGF4	Fibroblast growth factor 4	Growth factor and ligand for FGFR signaling	Yes [109], No [103]
11	q13.3	FGF3	Fibroblast growth factor 3, INT-2	Growth factor and ligand for FGFR signaling	No [103]
11	q13.3	CTTN	Cortactin, EMS1, Src8, Amplexin	F-actin binding, activates Arp2/3 complex, activates N-WASP, regulates MMP2/9 secretion, governs lamellipodia and invadopodia dynamics	Yes [149, 150]
11	q13.3	SHANK2	SH3 and multiple ankyrin repeat domains 2, CortBP1	Adaptor/scaffolding protein, links neurotransmitter receptors and ion transporters to the actin cytoskeleton via cortactin binding	Yes [163]
11	q13.5	PAK1	Serine/threonine-protein kinase PAK1, p21 activated kinase 1, Alpha-PAK	Protein serine/threonine kinase. Activated by Rac1/Cdc42 and PI3K, signal transduction pathways involved in cofilin, filamin, cortactin and Arp2/3 activity, regulates cell-substratum adhesion turnover and leading edge formation, controls tumor cell invasion	Yes [177]

Key actin cytoskeletal regulatory genes from chromosome (CR) 3q26-29, 8q23-24 and 11q13 are listed, along with the encoded protein products and known cellular functions. Gene expression information with regards to HNSCC is found in the indicated references; HPA refers to the Human Protein Atlas (<http://www.proteinatlas.org/index.php>), which contains immunohistochemical verification of protein expression of the indicated proteins in HNSCC and other tumor sites

these studies suggest MIM1 expression enhances motility in cell model systems, MIM1 expression levels vary widely in different carcinomas [74]. MIM1 expression is down-regulated in prostate, bladder and gastric cancers but it is upregulated in basal cell carcinomas [78–80], making the precise function of MIM1 expression in cancers unclear. While data on MIM1 amplification and expression in HNSCC is currently lacking, its presence on chromosome 8q24 suggests that MIM1 overexpression may play a potential role in enhancing HNSCC invasion in cases with coamplification of 8q24 and the cortactin locus on 11q13.

FAK

Focal adhesion kinase (FAK, PTK2) is a non-receptor tyrosine kinase that has been intensively studied with regards to its role in cell motility and tumor cell invasion [81, 82]. FAK was initially identified as a v-Src substrate that localizes to integrin-containing focal adhesions in cell culture [83, 84]. Integrin or growth factor receptor activation leads to FAK activation, resulting in autophosphorylation at tyrosine 397. Phosphorylation at tyrosine 397 creates a binding site for the SH2 phosphotyrosine binding domain of activated Src and related tyrosine kinases. The activated Src/FAK nexus in turn is responsible for the downstream phosphorylation of the cytoskeletal associated proteins paxillin and p130 Crk Associated Substrate (CAS), stimulating cell migration [81]. FAK-null cells contain increased numbers of focal adhesion complexes and are defective in motility, indicating that one role FAK plays during motility is to regulate efficient adhesion complex turnover [85]. It is now clear that FAK exerts many of its effects on motility through the kinase-independent association with a multitude of signaling and effector proteins. Of note is the ability of FAK to influence activation of Cdc42, Rac and Rho, assisting in regulating the proper spatial and temporal regulation of the actin cytoskeleton during cell movement [82]. FAK also plays an active role in tumor invasion and invadopodia formation through signaling pathways involving Src that also activate Rac and Jun N-terminal Kinase (JNK), leading to the increased expression of MMP2 and MMP9 [86]. FAK overexpression been well documented in HNSCC and other tumor types, corresponding to increased invasive and metastatic potential [2, 87]. While the FAK locus at 8q24.3 is amplified in HNSCC cases, FAK gene amplification does not completely correspond with FAK protein overexpression [88]. FAK overexpression occurs early in HNSCC and is present at all stages in HNSCC progression, statistically correlating with lymph node metastases [88]. In HNSCC, disease free survival seems to be impacted by FAK overexpression [88], suggesting that FAK may be a valid therapeutic candidate. FAK activation and tumor cell invasion is

downregulated in HNSCC cell lines treated with the Src kinase inhibitor dasatinib [89], indicating that FAK activity is indirectly impacted as a result of Src inhibitor therapy in HNSCC. Effective disruption of FAK signaling by direct targeting of FAK may be complex, given the kinase-dependent and -independent roles FAK plays in cell motility and invasion [90]. However, recent studies demonstrating impaired migration in tumor cells treated with novel small molecular inhibitors of FAK kinase activity [91, 92] may validate the further evaluation of these and related compounds in clinical settings as anti-invasive and metastatic agents.

DDEF1

Development and differentiation enhancing factor 1 (DDEF1, known as AMAP1, ASAP1 or centaurin beta4) is a member of the centaurin family of ADP-ribosylation factor GTPase-activating proteins that regulate the actin cytoskeleton in cell motility and cancer cell invasion [93–95]. Activation of PI3-kinase or Src phosphorylation enhances the ability of DDEF1 to downregulate activated Arf proteins by the hydrolysis of bound GTP to GDP [96, 97]. Arf proteins are members of the Ras superfamily of small GTP-binding proteins that function in regulating vesicle trafficking and cytoskeletal regulation [98]. DDEF1 localizes to the cell periphery within focal adhesions, where it regulates growth factor induced lamellipodia formation [93]. DDEF1 also interacts directly with FAK and is involved in the regulation of focal adhesion turnover during migration [99]. Overexpression of DDEF1 in breast cancer cells corresponds to an invasive phenotype and leads to invadopodia formation [100]. DDEF1 expression is essential for invadopodia formation, where it forms a trimeric complex with cortactin and paxillin that is required for extracellular matrix degradation [100]. The presence of DDEF1 in this complex may regulate endocytosis of degraded matrix components by localized activation of Arf6 in cooperation with the membrane deforming Bar domain of DDEF1 [94]. Targeted disruption of the cortactin/DDEF1 binding interface with either cell permeable peptides or UCS15A, a small molecule inhibitor that competitively binds to the DDEF1 binding site of cortactin, inhibits breast cancer invasion and metastasis [101]. These studies indicate that DDEF1 expression plays a critical role in tumor cell invasion, particularly in cases where it is overexpressed. Amplification of the DDEF1 locus on 8q24.21 corresponds with DDEF1 overexpression, increased motility and higher tumor grade in uveal melanoma [102]. The location of DDEF1 on 8q24 as well as its important role in regulating the actin cytoskeleton and tumor cell invasion suggest that it likely plays a critical role in HNSCC invasion.

Chromosome 11q13

Amplification of the 11q13 region has been well recognized as a major event driving HNSCC invasion and metastasis, occurring in 30–50% of all HNSCC cases [18, 19, 103]. The presence of 11q13 amplification correlates with decreased disease free survival [104]. The 11q13 amplicon contains 353 genes, with the cell cycle regulatory protein cyclin D1 the best-characterized amplified gene product with regards to regulation of HNSCC progression [18]. In addition to cyclin D1, the 11q13 amplicon contains several well-characterized genes known to regulate actin-based motility and invasion that are overexpressed in HNSCC and other tumor types. Fourteen of these genes are shown in Table 1; nine are discussed in detail.

CDC42EP2

Cdc42 effector protein 2 (BORG1, CEP2) is a member of a class of effector proteins initially identified by direct interaction with activated Cdc42 [105, 106]. Ectopic expression of CDC42EP in fibroblasts induces long, finger-like filopodia (pseudopodia) and accumulates into leading edge lamellipodia [105, 106]. CDC42EP2 expression in cultured keratinocytes reduces the thickness and organization of the actin stress fiber network [106], consistent with changes observed during motility. The changes in F-actin architecture are attributed to the direct binding of CDC42EP2 to members of the septin GTPase protein family [107]. Septins self-polymerize to form multimeric filaments that further organize into ring-like structures responsible for binding F-actin bundles at the cell periphery and for the completion of cytokinesis [108]. CDC42EP2 binding to septins disrupts septin organization [107], providing a mechanism for the observed reduction of stress fiber thickness and organization in cells with increased CDC42EP2 expression. While overexpression occurs in HNSCC [109], a clear role for CDC42EP2 in cell movement or tumor invasion has yet to be established.

Calpain 1

Calpain 1 (μ -calpain, CAPN1) is a member of a 16-gene superfamily of cysteine proteases responsible for regulating cell motility and other cellular processes by select cleavage of adhesion and cytoskeletal protein substrates [110, 111]. Calpain 1 and the related calpain 2 have been the best characterized members with respect to cell movement, where the role of calpain 2 has been well established in regulating motility through the cleavage of several components of focal adhesions and the actin cytoskeleton. Calpain 2 substrates of note include that are involved in adhesion and/or motility include FAK, cortactin, paxillin,

spectrin and talin, with cleavage resulting in the disassembly focal adhesion complexes, loss of adhesion to the substratum, remodeling of the actin cytoskeleton and increased cellular motility [111]. Studies utilizing knockout cells or RNA interference indicate that calpain 1 either does not cleave these substrates or is compensated for by other calpain forms [112, 113], casting some doubt on the participation of calpain 1 in cell migration. However, calpain 1 has been shown to be required for lamellipodia production during the spreading of bovine aortic endothelial cells by activating Rac and Rho to regulate the production of initial integrin focal contacts [114, 115]. Other studies demonstrate inhibited cell spreading when calpain 1 is downregulated [112], making the issue difficult to interpret. In cancer models, a role for calpain 1 in regulating tumor cell invasion is better established. Calpain 1 has been shown to be critical for breast and colon cancer cell motility and invasion in the absence of calpain 2 expression [116, 117]. Additional evidence to support a role for calpain 1 in tumor metastasis comes from work in basal cell skin carcinoma, a rarely metastatic cancer that is devoid of calpain 1 whereas metastatic cases exhibit strong calpain 1 overexpression [118]. While there is no evidence to date that calpain 1 plays a role in HNSCC, association of calpain 1 expression in invasive and metastatic tumors combined with its amplification on 11q13.1 suggest that calpain 1 overexpression may play an important role in regulating HNSCC invasion.

Cofilin 1

Cofilin 1 is a member of a family of well-characterized actin-binding proteins that bind G- and F-actin. A two-fold overexpression of cofilin has been documented in oral squamous cell carcinoma and is in accordance with other cancer types that display increased invasive capacity [7, 119]. Cofilin functions in cell motility and tumor cell invasion by promoting lamellipodia and invadopodia formation [120, 121]. Mechanistically, cofilin functions by severing existing actin filaments in response to EGF and other growth factor stimuli [120]. The severing activity of cofilin has a dual effect on actin dynamics to promote cortical actin-based protrusions. Severing increases the number of available barbed (+) ends allowing for rapid actin polymerization at the cell cortex independent of Arp2/3 complex activity. Severing also serves to enhance the rate of F-actin depolymerization, allowing G-actin monomers to be utilized in a subsequent round of polymerization [7]. Cofilin is regulated by phosphorylation on serine 3 by LIM and TES family kinases, which inactivates cofilin's severing activity [122]. Dephosphorylation of serine 3 by the phosphatases slingshot, chronophin, type 1, 2A and 2B phosphatase activates cofilin [122]. While this

cycle of cofilin regulation is well established, evidence to date indicates that cofilin activation in invasive carcinoma cells is not coupled to dephosphorylation but to hydrolysis of bound inhibitory phosphatidylinositol-4,5-bisphosphate (PIP2) by the enzyme phospholipase C (PLC) [123, 124]. The two independent methods of cofilin regulation have been reconciled to support the presence of a rapidly activated cofilin pool (by the PIP2/PLC cycle) that contributes to membrane protrusion and a phosphorylation-regulated pool that recycles cofilin or confines it to specific subcellular compartments [7]. The overexpression of cofilin in oral carcinomas makes it likely that cofilin dynamics play a central role in regulating the actin cytoskeleton during HNSCC motility and invasion.

FOSL1

The Fos related antigen-1 (*fosl1*, more commonly known as Fra-1) is a transcription factor of the Fos family overexpressed in HNSCC and other human carcinomas [125, 126]. Fra-1 is a component of the activator protein-1 (AP-1) transcription complex, a semi-variable family of nuclear dimeric complexes comprised of either homodimers of the Jun family of transcription factors, or heterodimers of the Jun and Fos family [127]. Enhanced activation of AP-1 is required for tumor promotion, interacting with various cofactors to regulate the expression of a wide variety of genes involved in proliferation, angiogenesis, apoptosis, migration and invasion [126]. Fra-1 expression alone is sufficient for cellular transformation and anchorage independent growth [128], indicating that this component of the AP-1 complex plays a significant role in cancer. Identified motility and invasion genes regulated by Fra-1 include the matrix metalloproteinase MMP-9 and the pro-invasive transmembrane receptors CD44 and c-MET [129, 130]. Expression and stabilization of Fra-1 is regulated by MAPK [131, 132], a terminal kinase of the Ras pathway displaying elevated activity resultant of EGFR overexpression. In colorectal carcinoma cells, upregulation of Fra-1 by MAPK activity results in decreased activation of RhoA [133], a small GTPase responsible for regulating cell-substrate adhesion by governing focal adhesion and stress fiber formation. Reduced RhoA activation by MAPK is required for subsequent MAPK-induced expression of the urokinase plasminogen activator receptor (uPAR), which in turn promotes cell motility through increased activation of Rac and Arp2/3 complex [133, 134]. Downregulation of Fra-1 expression suppresses motility and invasion [133]. The dependence of Fra-1 on MAPK activity suggest that therapeutic targeting of MAPK activating enzymes and other components of the EGFR-Ras pathway may be valid strategies for reducing Fra-1 levels in HNSCC and other carcinomas.

RIN1

The RIN1 gene is amplified to varying degrees in 50% of cell lines derived from OSCC patients with 11q13 amplification [135]. The RIN1 protein is an effector molecule that binds to activated H-Ras and regulates pathways involved in endocytosis and cell migration [136]. With regards to migration, RIN1 expression in fibroblasts and epithelial cells induces changes in actin cytoskeletal organization through the binding and activation of Abl and Arg tyrosine kinases [137]. Activation of Abl and Arg as a result of RIN1 expression increases cell membrane protrusion and enhances cell motility [137]. Mechanistically, RIN1 expression may enhance cell motility through Abl/Arg mediated phosphorylation of the adaptor protein CRK [137], which when phosphorylated increases binding to the CRK associated substrate CAS [138]. The CAS/CRK complex subsequently induces motility through activation of Rac leading to Arp2/3-induced lamellipodia formation [139]. A second potential mechanism where RIN1 may promote cell migration is through Abl/Arg-mediated phosphorylation of cortactin [140], an actin binding and Arp2/3 activating protein known to promote HNSCC motility and invasion (see below). The utilization of either of these pathways downstream of RIN1 to promote motility in HNSCC or other cancer types has yet to be validated.

Slingshot homolog 3

Slingshot homolog 3 (Slingshot 3, SSH3L or hSSH3L) is a member of a phosphatase family that serves to regulate cofilin activation [122]. Slingshot phosphatases selectively dephosphorylate cofilin on serine 3 resulting in increased cofilin severing activity and actin depolymerization [141]. While Slingshot-mediated dephosphorylation counteracts the ability of LIMK and TESK kinases from inhibiting cofilin, as mentioned above it appears that it is not the primary mechanism of cofilin regulation utilized during cell movement. However, LIMK activity is important in restricting cofilin activity within lamellipodia [123] and thus elevated slingshot expression could result in increased activated cofilin levels within the lamellipodial cofilin pool, potentially accelerating cortical actin remodeling. Slingshot 3 is unique in that it does not bind actin filaments, as do other Slingshot proteins, and it displays the weakest cofilin dephosphorylation activity of all family members [142]. The weak activity of Slingshot 3 towards cofilin may be beneficial in cases where cofilin and Slingshot 3 are overexpressed, allowing equilibrium in the activation state of cofilin to be maintained. The proper balance in regulating cofilin phosphorylation and activation is critical for cell movement and invasion, as evidenced by impaired motility when cofilin is overexpressed at

abnormally high levels or when constitutive active LIMK is expressed [7]. The modest overexpression of cofilin coupled with Slingshot 3 overexpression in HNSCC may therefore provide the ideal dichotomy for maximizing cofilin activity levels to stimulate motility and invasion.

Coronin 1B

Coronin 1B (coronin-2, coronin_{SE}) is a member of a seven gene superfamily of actin-binding proteins [143] originally identified in *Dictyostelium*, so named due to its localization in crown-like structures on the dorsal cell surface [144]. Coronin 1B localizes to leading edge lamellipodia, where it forms homotrimeric complexes that bundle F-actin [143, 145]. Coronin 1B and related coronins bind Arp2/3 complex and inhibit its activation by WASp family proteins [146, 147]. This binding is regulated by phorbol ester-mediated activation of PKC isoforms that phosphorylate coronin on serine residue two, downregulating the binding of coronin 1B to Arp2/3 complex [147]. Coronin 1B therefore provides additional regulatory control of Arp2/3-driven actin polymerization in lamellipodia during cell movement. In agreement with this, depletion of coronin 1B in fibroblast cell lines inhibits motility and alters lamellipodia actin architecture [148]. Coronin 1B also binds to and is dephosphorylated by the phosphatase Slingshot homologue 1L (SSH1L), and is responsible for targeting SSH1L to lamellipodia where it can dephosphorylate and activate the F-actin severing activity of cofilin [148]. In this capacity coronin 1B serves as a molecular bridge with the ability to accurately coordinate and regulate actin polymerization and depolymerization events within lamellipodia, resulting in optimal lamellipodia dynamics and cell movement. While direct evidence for the involvement of coronin 1B overexpression in HNSCC or any cancer is currently lacking, it is likely that future studies will evaluate the role of coronin 1B and the impact of its overexpression on tumor cell movement.

Cortactin

Cortactin (EMS1, amplaxin, SRC8) is an adaptor protein that is overexpressed at high levels in roughly 30% of HNSCC cases [149]. Cortactin gene amplification and overexpression in HNSCC is associated with poor clinical outcome [150], increased motility and cellular invasion [151]. Cortactin is found in lamellipodia and invadopodia, and functions by binding to F-actin and the Arp2/3 complex, stimulating actin nucleation and subsequent polymerization [152]. Interactions with the commonly amplified 8q23-24 gene products MIM1 and DDEF1 [76, 101] likely contribute to the ability of cortactin to stimulate Arp2/3 activity and/or regulate cellular invasion in cases

where both amplicons are present. In addition to regulating actin polymerization within lamellipodia, cortactin also stabilizes the actin networks formed by Arp2/3-driven polymerization, an activity congruent with the requirement for cortactin to maintain lamellipodial persistence [153, 154]. While cortactin is not necessary for cells to produce lamellipodia, it is absolutely required for the formation of functional (matrix degrading) invadopodia [155, 156]. Cortactin is essential for the establishment of the actin core within invadopodia, where its recruitment precedes the localization of the matrix metalloproteinase MT1-MMP to sites of matrix degradation [155]. Recently, cortactin has been shown to be required for the secretion of MMP-2 and MMP-9 in HNSCC, further expanding the role of cortactin in cancer invasion [156]. Cortactin activity is regulated in part by phosphorylation resulting from the activation of EGFR and other receptor tyrosine kinases. Downstream activation of Src, MAPK and additional cytoplasmic kinases leads to direct cortactin tyrosine and serine phosphorylation at multiple sites [157]. Tyrosine phosphorylation of cortactin is required for efficient migration in HNSCC and other carcinomas, and is also associated with matrix degradation activity at invadopodia and distant metastasis in animal models [157–159]. Serine phosphorylation by MAPK has been proposed to regulate the ability of cortactin to activate N-WASp [160], indicating that Src and MAPK pathways converge to govern the ability of cortactin to regulate actin polymerization. Collectively, studies to date point to cortactin as playing a central role in HNSCC motility and invasion, suggesting that therapeutic intervention targeting phosphorylation pathways or binding partners may be warranted means of controlling local-regional and metastatic spread.

Shank2

Shank2 is a member of a multigene family responsible for encoding large scaffolding proteins intimately associated with the inner face of the plasma membrane. Shank2 and related proteins have been best characterized at the post-synaptic density in neurons, where they have been shown to functionally sequester neurotransmitter receptors into multiprotein complexes, linking them to a wide variety of cytoplasmic signaling proteins [161]. Expression of Shank2 and related isoforms are found in epithelial cells, where they bind and regulate the function of the Na⁺/H⁺ exchanger 3 to assist in maintaining salt and water homeostasis [162]. In oral squamous cell carcinomas Shank2 is often coamplified and overexpressed with cortactin [163] and the two protein products directly interact [164]. This suggests that Shank2 provides a molecular link between transmembrane receptors and the actin cytoskeleton through cortactin. Shank2 also interacts with sharpin,

a protein of unknown function whose gene is also present and amplified within the 11q13 region [165]. While the functional significance of Shank2 overexpression in HNSCC is currently unclear, an intriguing possibility is that Shank2 may participate in enhancing cellular invasion given its association with cortactin and dynamin2 [166], proteins that are required for invadopodia protrusion and extracellular matrix degradation [167]. Whether Shank2 serves as a scaffold for coordinating invadopodia function in this regard in HNSCC or any cancer type has yet to be resolved.

PAK1

p21-activated kinase 1 (PAK1) is a serine/threonine kinase that regulates signaling pathways governing motility and invasion in a variety of human tumor types [168]. Identified as the first effector protein to interact with activated Cdc42 and Rac [169], PAK1 kinase activity is stimulated by Cdc42/Rac binding as well as through other activation mechanisms [168]. One Cdc42/Rac independent mechanism of note is the direct binding of PI3 kinase to PAK1, resulting in increased PAK1 activity and phosphorylation of downstream substrates [170]. PAK1 participates in cell migration by driving lamellipodia formation and regulating cortical actin cytoskeletal organization [171, 172]. The ability of PAK1 to influence the cortical actin network is accomplished by the direct phosphorylation of numerous actin regulator proteins. Phosphorylation of LIM kinase by PAK1 promotes LIM kinase activity, leading to cofilin inactivation and increased lamellipodia protrusion [173]. Elevated expression and/or activation of either PAK1 or LIM kinase leads to enhanced tumor cell invasion and metastasis in breast and prostate cancers [174], demonstrating the importance of this PAK1 pathway in cancer progression. PAK1 also phosphorylates the p41 subunit of the Arp2/3 complex, resulting in direct stimulation of actin nucleation and cell migration independent of other nucleation promoting proteins [175]. The actin bundling protein filamin is also a PAK1 target, where PAK1-mediated phosphorylation of filamin is required for lamellipodia extension [176]. Finally, PAK1 phosphorylates cortactin, an activity thought to influence the ability of cortactin to bind F-actin [177]. These studies suggest that PAK1 serves as a master regulator of cortical actin dynamics leading to cell migration and tumor cell invasion. While PAK1 overexpression in HNSCC has been demonstrated and functions downstream of EGFR activity to promote HNSCC invasion [178], the PAK1 gene on 11q13.5 is not amplified in OSCC cases that demonstrate amplification of the 11q13 genes cortactin, cyclin D1 and SHANK2 [163]. These data suggests that gene amplification may not be responsible for PAK1 overexpression in HNSCC.

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DOCTORAL DISSERTATION **Dynamic Src Tyrosine Kinase Signaling Orchestrates Invadopodia Biogenesis in Head and Neck Cancer: Novel Insights into the Original Oncogene**

Dissertation Defense Date: August 27, 2010

Laboratory techniques learned and utilized included cell culture, cell migration and invasion assays, immunofluorescence, immunohistochemistry, western blot analysis, immunoprecipitation, fluorescent substrate cleavage assays, luciferase gene reporter assays, gelatin zymography, confocal microscopy and live cell imaging, PCR, reverse transcriptase PRC, molecular cloning techniques (including sub-cloning, site directed mutagenesis, and plasmid preparation), cell transfection and nucleofection, cell lentivirus infections, transgenic animal models, mouse colony management (including backcrossing, husbandry, and genotyping), mouse surgical techniques, RNA isolation from animal tissue.

PUBLICATIONS **Kelley LC, Ammer AG, Hayes KE, Weed SA. Cortactin phosphorylation by extracellular-related kinase is required for carcinoma cell migration and lamellipodia persistence. PLoS ONE. In revision.**

Kelley LC, Ammer AG, Hayes KE, Martin KH, Machida K, Jia L, Mayer B, Weed SA. Oncogenic Src requires a wild-type counterpart to regulate invadopodia maturation. Journal of Cell Science. Accepted Jul 22, 2010.

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ABSTRACTS

West Virginia University Van Lier Research Convocation (Morgantown, WV April 2010) **Oncogenic Src requires a wild-type counterpart to regulate invadopodia maturation. Kelley LC, Machida K, Mayer B, and Weed SA.**

American Society for Cell Biology (ASCB) (San Diego, CA December 2009) **Regulated Src Kinase Activity Directs Invadopodia Maturation. Kelley LC, Weed SA.**

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Meeting on Invadopodia, Podosomes and Focal Adhesions in Tissue Invasion (Hyeres, France September 2009) **Invadosome Maturation: Src completes the cycle. Kelley LC, Ammer AG, and Weed SA.**

American Association for Cancer Research (AACR) (Denver, CO April 2009) **The Src inhibitor AZD0530 suppresses head and neck squamous cell carcinoma invasion by disrupting invadopodia activity.** Ammer AG, **Kelley LC**, Hayes KE, Evans JV, Lopez-Skinner LA, Martin KH, Rothschild BL, Frederick B, Raben D, Green TP, Weed SA.

West Virginia University Van Lier Research Convocation (Morgantown, WV April 2009) **Role of Cortactin and Src Kinase in Invadopodia Maturation. Kelley LC, Ammer AG, and Weed SA.** *This abstract was also selected for an oral presentation*

The Cancer Biology Training Consortium (CABTRAC) Annual Meeting (Park City, UT October 2008) **Role of Cortactin and Src Kinase in Invadopodia Maturation. Kelley LC and Weed SA.**

4th Annual West Virginia COBRE/INBRE Conference (Morgantown, WV October 2008) **Role of Cortactin and Src Kinase in Invadopodia Maturation. Kelley LC and Weed SA.**

American Association for Cancer Research (AACR) (Las Angeles, CA April 2007) **The novel Src/Abl kinase inhibitor AZD0530 inhibits proliferation, invasion and invadopodia formation in head and neck squamous cell carcinoma** Lopez-Skinner LA, **Kelley LC**, Ammer AG, Rothschild BL, Frederick B, Raben D, Green TP, Flynn D, Weed SA

American Society for Cell Biology (ASCB) (San Diego, CA December 2006) **Cortactin is required for lamellipodia retraction in MTLn3 Breast Cancer Cells.** Ammer AG, **Kelley LC**, and Weed SA.

American Society for Cell Biology (ASCB) (San Diego, CA December 2006) **Role of Cortactin in Invadopodia Formation Downstream of Src.** **Kelley LC**, Ammer AG, and Weed SA.

2nd Annual West Virginia COBRE/INBRE Conference (Roanoke, WV November 2006) **Cortactin is required for Invadopodia Formation Downstream of Activated Src Kinase.** **Kelley LC**, Ammer AG, and Weed SA.

American Association for Cancer Research (AACR) (Washington DC, April 2006) **Cortactin is Required for Invadopodia Formation Downstream of Activated Src Kinase.** **Kelley LC**, Ammer AG, and Weed SA.

American Association for Cancer Research (AACR) (Washington DC, April 2006) **Cortactin Regulates Lamellipodial Dynamics in Response to EGF Stimulation.** Ammer AG, **Kelley LC**, and Weed SA.

West Virginia University Van Liere Research Convocation (Morgantown, WV April 2006) **Role of Cortactin in Invadopodia Formation Downstream of Src Kinase.** **Kelley LC**, and Weed SA.

52nd annual meeting for the American College of Sports Medicine (ACSM) (Nashville, TN, May 2005) **Effect of Chronic Exposure to Stretch-Shortening Cycles on Apoptotic Markers in Skeletal Muscle of Aged Rats.** **Kelley LC**, Siu PM, Geronilla KB, Cutlip RG, Alway SE

PROFESSIONAL COURSES	14th Annual Short Course on Experimental Genetic of the Laboratory Mouse in Cancer Research (August 21-September 1, 2005) The Jackson Laboratory, Bar Harbor, ME
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AWARDS	<div>West Virginia University Van Liere Research Day 2010 First Place Poster Presentation (\$500)</div> <div>West Virginia University Van Liere Research Day 2009 Second Place Oral Presentation (\$400, + \$500 travel award)</div> <div>Selected for a \$250 Travel Award from WVU School of Medicine to attend AACR Conference 2006</div>
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	West Virginia University Van Liere Research Day First Place Poster Presentation (\$500)	2006
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