THE FOCAL ADHESION LOCALIZATION OF P130CAS: DYNAMICS,

TARGETING MECHANISM, AND SIGNALING

By

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DEDICATION

This dissertation is dedicated to my mother, Josephine Donato, who taught me that with the power of positive thinking and a bit (and sometimes more) of stubborn perseverance, all things are possible.

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LIST OF ABBREVIATIONS

Ala alanine

AOTF acousto-optical tunable filters

Arg arganine

Asp aspartic acid

BCAR1 breast cancer antiestrogen resistance 1 gene, a.k.a. p130Cas

BCAR3 breast cancer antiestrogen resistance 3 gene, a.k.a. AND-34

BCR/ABL Breakpoint cluster region/ Abelson (gene fusion)

BSA bovine serum albumin

C3G a Ras-family guanine nucleotide exchange factor

Cas-L lymphocyte type Cas

CCH Cas-family C-terminal Homology

CHO Chinese hamster ovary (cell)

CLASP CLIP-associated protein

CLIP cytoplasmic linker protein of 170 kDa

CMS Cas-ligand with multiple SH3 domains

CoIP co-immunoprecipitation

DIC differential interference contrast

DmCas *Drosophila melanogaster* Cas

DMEM Dulbecco's modified Eagle serum

DN dominant-negative

DTT dithiothreitol

DPSS diode-pumped solid-state (laser)

ECM extracellular matrix

EDTA ethylenediaminetetraacetic acid

EGF epidermal growth factor

Efs embryonic Fyn substrate, a.k.a. Sin

EM electron microscopy

EM-CCD electron multiplying charge coupled device

ER estrogen receptor

ERK1/2 extra-cellular related kinase 1/2

FA focal adhesion

FAK focal adhesion kinase

FAT focal adhesion targeting

FBS fetal bovine serum

FGF fibroblast growth factor

FRAP fluorescence recovery after photobleaching

FRNK FAK-related non-kinase

FX focal complex

GEF guanine nucleotide exchange factor

GFP green fluorescent protein

GH growth hormone

Glu glutamic acid

GOF gain of function

GSK3-β glycogen synthase kinase 3-β

GST glutathione-S-transferase

HBS HEPES buffered saline

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HEF1 human enhancer of filamentation 1, a.k.a. Cas-L

HEPL HEF1/Efs/p130Cas-like

IB immunoblot

JNK c-Jun N-terminal kinase

KD kinase-dead

kDa kilo-Dalton

LAR leukocyte antigen related (protein tyrosine phosphatase)

LD leucine-aspartate (repeat)

Leu leucine

LIM Lin11, Isl-1, Mec-3 (-like domain)

LOF loss of function

LPA lysophosphatidic acid

LPS lipopolysaccharides

MAPK mitogen-activated protein kinase

MDCK Madin-Darby Canine Kidney (cells)

MEF mouse embryonic fibroblast

MES 2-(N-morpholino)ethanesulfonic acid

MKK4 MAP kinase kinase 4

MLCK myosin light chain kinase

MTOC microtubule organizing center

Na$_3$VO$_4$ sodium orthovanadate

NCAM neural cell adhesion molecule

NMR nuclear magnetic resonance

NSP novel SH2-containing protein

p p-value (probability of the null hypothesis being true)

p130Cas Crk-associated protein (130 kDa)

PBS phosphate buffered saline

pCas phospho-Cas

PCR polymerase chain reaction

PDGF platelet derived growth factor

PI3-K phosphatidylinositol 3-kinase

PIPES piperazine-N,N'-bis(2-ethanesulfonic acid)

PKC protein kinase C

PMA phorbol 12-myristate 13-acetate

PR proline-rich (referring to proline-rich regions of FAK)

PR-39 anti-microbial peptide

Pro proline

PTP1B protein tyrosine phosphatase 1 B

PYK2 Protein tyrosine kinase

PTP-PEST protein tyrosine phosphatase with (P)ro-(G)lu-(S)er-(T)hr sequence

pTyr phospho-tyrosine

RIPA radioimmunoprecipitation assay

RPLSPP amino acid sequence Arg-Pro-Leu-Ser-Pro-Pro

ROCK Rho-associated kinase

SFK Src-family kinase

SBD Src-binding domain

SD substrate domain

SDS-PAGE sodium doecyl sulfate - polyacrylamide gel electrophoresis

SEM standard error of the mean

SER serine-rich

Ser serine

SH2 Src homology 2

SH3 Src homology 3

SHEP1 SH2 domain-containing Eph receptor-binding protein 1

SHIP-2 Src-homology 2 containing inositol phosphatase

SHP2 SH2-containing protein tyrosine phosphatase 2

Sin Src interacting protein

siRNA single interfering RNA (ribonucleic acid)

STAT5b signal transducer and activator of transcription 5b

TBS Tris-buffered saline

Thr threonine

TIRF total internal reflection fluorescence

TRIP6 thyroid receptor interacting protein 6

Tyr tyrosine

WT wild-type

YAAP amino acid sequence Tyr-Ala-Ala-Proline

YDxP amino acid sequenceTyr-Asp-X-Pro

YxxP amino acid sequence Tyr-X-X-Pro

YFP yellow fluorescent protein

YopH *Yersinia* protein tyrosine phosphatase

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CHAPTER I

INTRODUCTION TO P130CAS IN FOCAL ADHESIONS AND CELL
MOTILITY

Principles of Cell Motility

Cell motility is a fundamental process in the development and maintenance of tissues. The coordinated movement of cells during embryogenesis is a key step in the proper generation of organs, creation of neuronal linkages, and transport of epithelial and immune cells into wounds (Ridley et al, 2003). Misregulation of cell motility, however, can lead to a variety of pathologies including vascular disease, chronic inflammation, cancer, and mental retardation (Vicente-Manzanares et al, 2005). Cell motility can be thought of as a process which occurs cyclically in three steps: 1) establishment of cortical polarity, 2) protrusion of the cell membrane to adhere to new sites, and 3) retraction at the rear of the cell (Ridley et al, 2003). Disruption of any one of these process can lead to a defect in cell motility.

Principles of cortical polarization

The polarization of a cell, or the establishment of a leading and trailing edge, is thought to be regulated by the positioning of the microtubule organizing center (MTOC) and the nucleus (Vicente-Manzanares et al, 2005), and has recently been attributed to the position of the Golgi as well (Miller et al, 2009). Though it has long been observed that the MTOC and Golgi become oriented with the leading edge in motile cells (Kupfer et al, 1982), the findings of Miller et al (2009) indicate a more active role for the Golgi in determining cortical polarity.

Another study has illustrated that the nucleus, not the MTOC, is actively repositioned to the rear of the cell during directed cell movement, presumably in an actin

1

and Cdc42-dependent manner, and that the MTOC remains stationary as the nucleus positions itself behind it (Gomes et al, 2005).

Interestingly, inhibition of Src-family kinase (SFK) activity with inhibitors PP1 and PP2 prevents MTOC reorientation and cell protrusion (Etienne-Manneville and Hall, 2001). Furthermore, integrin engagement appears to be necessary for MTOC reorientation in this context. At the same time points used for analyzing MTOC reorientation, tyrosine phosphorylation of focal adhesion (FA) proteins focal adhesion kinase (FAK), paxillin, and p130Cas were observed (Etienne-Manneville and Hall, 2001). Therefore it seems that cortical cell polarity is a complex process that likely involves the nucleus, the MTOC, and the Golgi in integrin-, microtubule-, and actin-dependent ways.

Principles of protrusion

The formation of membrane protrusions towards a stimulus is an essential step in cell motility. These protrusive areas are rich in actin filaments. The lamellipodia — membrane protrusions at the cell's leading edge — are made of branched actin networks surfing at the front of larger lamellae (Small et al, 2002). Promotion of lamellipodial protrusion is associated with the small Rho GTPases Rac and Cdc42 (Matozaki et al, 2000). Cdc42 has also been implicated in the formation of filopodia (Matozaki et al, 2000) — antenna or finger-like protrusions made up of bundled actin filaments extending beyond the lamellipodium itself (reviewed in Faix and Rottner (2006)). However, this view may be too simplistic as evidence has been presented that Cdc42 is not required for filopodial or lamellipodial formation in fibroblasts (Czuchra et al, 2005). Filopodia are often thought of as the sensory antennae of the cell, whereby the cell can sense extracellular stimuli and reorient the cell in the direction of that stimulus (Small et al, 2002).

In addition to these protrusive structures, the cell must form new sites of adhesion

from which it can exert contractile forces to propel itself forward. These forces are generated through adhesion sites at the ventral surface of the cell that connect the cell to the substratum via integrins (heterodimeric receptors responding to ECM ligands). The molecular makeup of these ECM/cell contacts will be further discussed in the section entitled "ECM/cell contacts: sites of ECM/actin linkage and pro-motile signaling". These sites, often referred to as FAs, are linked to the actin cytoskeleton and are important structures for maintaining cell shape or modulating the shape as is necessary during cell motility. Actin stress fibers, for instance, are formed of anti-parallel bundles of actin filaments that often connect FAs at the front and rear of the cell to one another and furthermore are able to exert contractile forces on the cell partially by virtue of their association with the motor protein myosin II (Kaverina et al, 2002).

Promotion of actin polymerization in membrane protrusion and the creation of contractile forces leading to FA assembly are largely associated with the small Rho GTPases (Matozaki et al, 2000). This family includes RhoA, Rac, and Cdc42. Activation of Rac, in particular, has been implicated in signaling pathways upstream of the actin nucleation factor Arp2/3 to promote actin polymerization at leading edge lamellipodia (Cotteret and Chernoff, 2002). The dynamic nature of actin polymerization through Arp2/3 drives cell motility (reviewed in Pollard and Borisy (2003)).

An important aspect of Rho GTPase signaling appears to be the antagonism of family members against one another (reviewed in Kaverina et al (2002)). Whereas Rac activation is associated with the formation of small, immature focal complexes (FXs) and forward protrusion of the lamellipodium, RhoA is associated with FA maturation and stress fiber formation (Nobes and Hall, 1995; Chrzanowska-Wodnicka and Burridge, 1996; Matozaki et al, 2000). The formation of these complexes in response to RhoA has been associated with tensile forces exerted on the cell (Chrzanowska-Wodnicka and Burridge, 1996).

The coordination of intracellular signals leading to actin polymerization and membrane protrusion is quite complex. Here, a few of the effectors of such signaling have been reviewed, yet many more effectors are known and many more are probably yet to be discovered. It is clear, however, that protrusion is an active process influenced not only by signaling molecules but also by mechanical forces exerted upon the cell and substratum. It is important to note that these processes are very dynamic and that as a protrusion extends, it forms multiple FAs at various stages of maturity. Though thinking of cell motility in three phases may be useful in elaborating our understanding of its mechanics, the elements of one process cannot be easily distinguished from the other, as is clear when the role of Cdc42 in cortical polarity and protrusion is considered. RhoA, additionally, is not exclusively an effector of protrusion or retraction.

Principles of retraction

Retraction at the rear of the cell is necessary for the cell body to move forward. A key step in this process is releasing trailing edge FAs. While protrusion would not be possible without the establishment of FAs, these trailing edge FAs must be released in a coordinated manner to assure that the cell does not lose adherence or stretch itself out beyond its breaking point (Broussard et al, 2008). Contractile forces exerted at the rear of the cell are important for releasing trailing edge FAs, though the distribution of force per area is greater at the front of the cell than at the rear (Kaverina et al, 2002). Stress fibers act as 'cables' to create tensile forces that at once cause maturation of the FAs in the front of the cell, creating strong adhesions there, but also pull at FAs located in the rear (Riveline et al, 2001). These actomyosin contractile forces are generated by RhoA and myosin IIa (Riveline et al, 2001).

Whereas disassembly of FAs at the front of the cell tends to occur by a flow of FA molecules out of the area, trailing edge FAs appear to 'slide' or alternately, to be left behind as a 'footprint' when the cell moves on (reviewed in Broussard et al

(2008)). The mechanics of trailing edge FA retraction have not been studied nearly as extensively as the disassembly of leading edge FAs, so there is still much to be learned about this process.

An important question that remains to be fully answered is how the balance of RhoA signaling is shifted to promote the retraction of FAs at the rear of the cell. In platelets and CHO cells spread on fibrinogen, a mechanism has been suggested by which calpain cleavage of β integrin cytoplasmic tails can promote RhoA activity by releasing c-Src from integrin protein complexes (Flevaris et al, 2007). This is an attractive model as it could explain the sliding mechanism previously described wherein most of the protein complexes remain intact but detachment of the FA is achieved. However, it does not easily explain why some trailing edge FAs get left behind as 'footprints'. It will be interesting to see if the same mechanism holds true for other cell types adherent to other ECM substrates.

ECM/cell contacts: sites of ECM/actin linkage and pro-motile signaling

The ability of the cell to sense the substratum and orchestrate responses is known as outside-in signaling and is achieved through integrins (Pozzi and Zent, 2003). Integrins are made up of two subunits, α and β. There are 18 α and 8 β subunits, which combine to recognize a wide range of ECM ligands from collagen to fibronectin to proteoglycans (Pozzi and Zent, 2003). When these integrins cluster together at the ECM, they form plaques known as ECM/cell contacts, which include a subset of structures including small FXs that occur exclusively at the front of the lamellipodium. These FXs can be highly dynamic, forming as small, punctate adhesions that turn over rapidly, or mature into larger, oblong FAs (Kaverina et al, 2002). These mature FAs occur throughout the cell, but especially in the lamella, lamellipodium, and at the rear of the cell (Kaverina et al, 2002). As FAs mature, they tend to grow

larger in size until they eventually disassemble. However, some FAs can become incorporated under the cell body in the form of elongated fibrillar adhesions that occur along fibronectin fibrils (Zamir et al, 1999; Volberg et al, 2001). The composition of each class of ECM/cell contact is slightly different, with small FXs containing early FA proteins such as paxillin and FAK, but having little zyxin or α-actinin content (Zaidel-Bar et al, 2003). FAs contain a wide range of proteins depending on their stage of maturation. These components will be discussed in greater detail under the subheading 'Key FA proteins'. The defining feature of fibrillar adhesions is their tensin content (Zamir et al, 1999).

ECM/cell contacts not only serve to anchor the cell to the substratum, but are also robust centers of tyrosine phosphorylation and signaling (Zamir et al, 1999). In fact, a standard method of marking FAs by immunostaining is to use an anti-phosphotyrosine antibody. To date, over 100 proteins are known to localize to FAs and are sometimes referred to as the adhesome (Zaidel-Bar et al, 2003). These proteins serve scaffolding and signaling functions, linking the actin cytoskeleton to the ECM. The overall architecture of these adhesions (from the ventral surface of the cell) starts with the ECM that acts as a ligand for the head groups of α and β integrin receptors. At the cytoplasmic tail of β integrins, FA proteins such as talin are bound. Talin and/or paxillin can then serve as scaffolds for the assembly of other FA components such as vinculin and FAK, which are furthermore bound by proteins such as α-actinin (see Fig. 1). α-actinin is known to link other FA proteins to actin filament structures, such as stress fibers. These sites are important structurally, but are in constant flux. Using spatio-temporal image correlation spectroscopy, it has been shown that elements of FAs which are in closer proximity to the substratum (such as integrins, talin, and paxillin) are less dynamic in these structures whereas the structures nearest to the actin interface such, as α-actinin and actin monomers, dissociate more rapidly (Brown et al, 2006). Signaling through FAs has been linked to cell survival, whereas loss of

signaling at FAs is linked to cell death in normal cells, but in transformed cells can lead to aberrant proliferation, cell motility, and invasion (Vuori and Ruoslahti, 1995; Vuori et al, 1996). FAs are therefore important for the maintenance of cell shape and adherence, but are also important mediators of signaling events.

Key FA proteins

As there are greater than 100 proteins known to localize to FAs, I will focus this review on the most commonly used proteins for marking FAs: paxillin, talin, vinculin, zyxin, and α-actinin, as well as the non-receptor tyrosine kinases FAK and Src (see Fig. 1). Several of these proteins have been implicated in binding integrins directly including: paxillin (Schaller, 2001), FAK, talin, and vinculin (Schaller et al, 1995; Goldmann et al, 1996).

Paxillin contains several LD motifs at its N-terminus by which it is known to bind FA proteins such as vinculin, as well as having several LIM domains that are typical of zinc finger proteins (Schaller, 2001). Use of a β1-integrin peptide to identify FA proteins bound to integrin suggest that paxillin may also bind to β1-integrin tails (Schaller et al, 1995). *In vivo*, paxillin is more likely to bind to α-4 integrin (Schaller, 2001). There is evidence to suggest that the LIM domains of paxillin may serve a shuttling function, bringing paxillin from FAs to the nucleus where it is thought to engage in transcriptional regulation (Wang, 2003). However, the biological function of paxillin transcriptional regulation is poorly understood. As an FA protein, paxillin is often used as a marker of FAs throughout their lifetime, as its residence has been confirmed both in nascent and mature FAs (Zamir and Geiger, 2001). For this reason, paxillin is the most commonly used FA marker in the studies presented in this thesis.

Talin is also known to interact with a variety of FA components including integrins, vinculin, α-actinin, and actin (Goldmann et al, 1996). Its interaction with integrins is thought to be phosphorylation-dependent, and its association with FAs can be
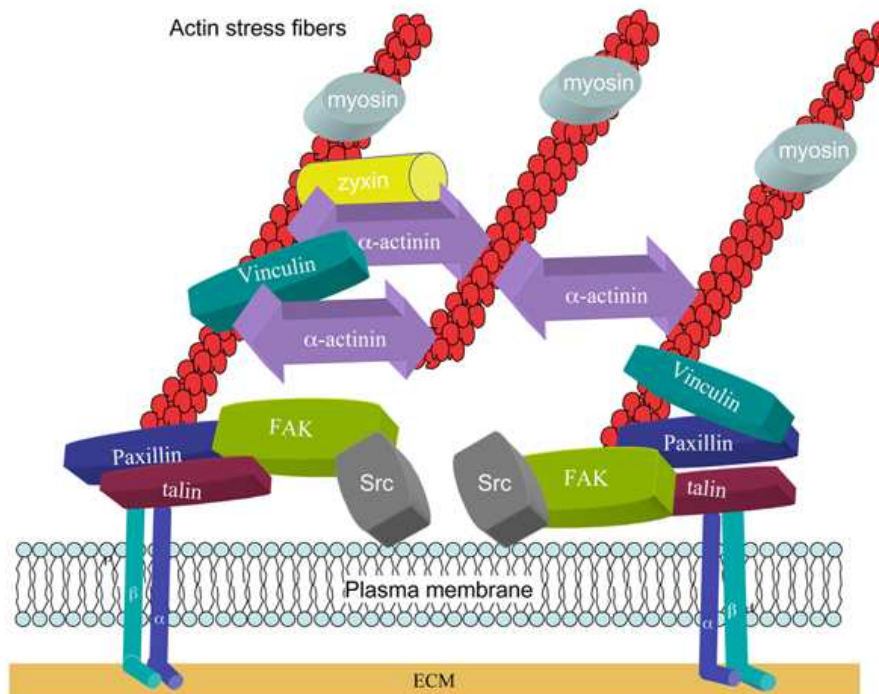
Figure 1: Molecular architecture of focal adhesions. A schematic of focal adhesion proteins discussed in this chapter is shown in relationship to their interactions with one another, integrins, and actin stress fibers (note that these interactions are not exclusive of other interactions). Integrins are indicated by the symbols α and β. Figure was drawn based on Mitra et al (2005).

disrupted by calpain cleavage (Goldmann et al, 1996). Talin plays an important role in activating integrins. The talin head group binds NPxY motifs in the β integrin cytoplasmic tail, which causes the heterodimeric complex to shift into the active conformation (Moser et al, 2009). Microinjection of talin antibody into cells results in decreased cell spreading and motility, as well as a disruption in FA and stress fiber formation (Goldmann et al, 1996).

Besides talin, vinculin also associates with paxillin, actin, and α-actinin (Goldmann et al, 1996). Vinculin is often thought of as an FA protein that bridges the gap between integrins and actin filaments. Like paxillin, it is a Src-substrate and becomes tyrosine phosphorylated at FAs (Goldmann et al, 1996). Consistent with its role as an FA protein, overexpression of vinculin reduces cell motility (Goldmann et al, 1996). In contrast, reduced expression leads to a rounded cell shape, increased motility, decreased adhesion, and less stable lamellipodia and filopodia, though formation of these structures is not inhibited (Goldmann et al, 1996).

Zyxin is a zinc finger protein with various protein-protein interaction domains (Beckerle, 1997). Like, paxillin it has been identified as an FA protein with the potential to shuttle to the nucleus and mediate transcription (Wang, 2003). Besides its three LIM domains in the C-terminus, zyxin also contains a proline-rich region in the N-terminus followed by a nuclear exit signal. Zyxin is known to interact with Vav and Ena/Vasp as well as α-actinin. The binding site of zyxin on α-actinin also happens to be the actin binding site. Consistent with binding of zyxin to the actin cross-linking protein, α-actinin, zyxin immunostaining not only reveals its localization at FAs, but also shows zyxin decorating areas of actin filaments closest to FAs (Beckerle, 1997).

The role of tyrosine kinases and their substrates in FAs

FAK, named for its prominent localization to FAs, has long been recognized as a protein tyrosine kinase that becomes autophosphorylated upon integrin engagement

(see Hanks and Polte (1997) for review). Though the targeting of FAK to FAs has been attributed primarily to its interactions with paxillin LD motifs through a C-terminal FA targeting (FAT) domain (Tachibana et al, 1995; Bertolucci et al, 2005), *in vitro* experiments with C-terminal FAK mutants also suggest that FAK may be able to bind talin (Chen et al, 1995) and β-1 integrin tails (Schaller et al, 1995).

Recent studies have shown that several FA proteins play important roles in mediating the overall disassembly of FAs, including paxillin, FAK, Src, and p130Cas (Webb et al, 2004). Mutation of tyrosine residues on some of these proteins or elimination of their protein tyrosine kinase activity led to comparable defects in paxillin turnover in FAs than when protein expression was reduced by either knock-down or knock-out (Webb et al, 2004). FAK and Src are both implicated in regulating FA disassembly (Volberg et al, 2001; Webb et al, 2004). In *FAK* -/- or Src, Fyn, Yes (*SYF*) -/- MEFs, the rate of paxillin turnover in FAs is decreased by greater than 12-fold (Webb et al, 2004). However, sustained Src tyrosine phosphorylation at FAs also appears to have negative effects on FA turnover, since increased Src activity at FAs results in unusually large peripheral adhesions and immobile cells (Siesser et al, 2008). Creation of a FAK/Src chimera wherein the Src kinase domain replaced the FAK kinase domain, resulted in excessive phosphorylation of several FA proteins including paxillin and p130Cas. The cells displayed large FAs with prominent cortical actin filament bundles joining one another at the periphery. Use of a kinase dead chimera or a Src inhibitor caused the FAs to return to 'normal' states —smaller and more diffusely spread throughout the cell body (Siesser et al, 2008). These data suggest that the influence of tyrosine kinases on FA turnover is significant.

FAK appears to be very proximal to integrins at FAs and the clustering of integrins (as demonstrated by causing integrin aggregation on antibody-coated beads) is sufficient to induce recruitment of FAK to FAs (Miyamoto et al, 1995). Clustering of integrins was also associated with a rise in tyrosine phosphorylation on these beads

(Miyamoto et al, 1995). Tyrosine phosphorylation of paxillin appears to be regulated to a large extent by FAK. Paxillin mutants that cannot bind FAK show significant reductions in tyrosine phosphorylation (Schaller, 2001). However, Src also appears to phosphorylate paxillin on tyrosine residues (Schaller, 2001). While Src localizes to many subcellular compartments, it is clear that a significant number of Src substrates can be found in FAs.

Discovery of p130Cas and its Primary Structure

P130Cas (Crk-associated substrate) is a Src substrate that functions in integrin signaling to promote cell motility, invasion, proliferation, and survival (Bouton et al, 2001; Defilippi et al, 2006). p130Cas was first recognized as a prominent tyrosine phosphorylated protein in cells transformed by retroviral oncogenes v-Crk (Mayer et al, 1988) and v-Src (Reynolds et al, 1989). The observation that p130Cas interacts directly with the Src homology 2 (SH2) domain of the v-Crk protein (Matsuda et al, 1990, 1991) contributed to the recognition of SH2 domains as phospho-tyrosine-binding modules in signal transduction.

Like FAK, p130Cas localizes to FAs and undergoes tyrosine phosphorylation in response to adhesion (Nojima et al, 1995; Petch et al, 1995; Vuori and Ruoslahti, 1995). Tyrosine phosphorylation of p130Cas occurs preferentially at FAs (Fonseca et al, 2004) and is enhanced in response to cellular stretching (Sawada et al, 2006). FAK is thought to be a mediator of p130Cas localization to FAs (Harte et al, 2000), though prior to the studies presented in this dissertation, no direct evidence for this claim was presented. Thus p130Cas is a signaling component of the FA protein complex that assembles to bring about cellular responses to integrin engagement.

Functional domains of p130Cas

The primary structure of p130Cas was first determined from a rat cDNA using degenerate primers to amplify a peptide that was affinity purified from 3Y1 cells transfected with a v-Crk plasmid (Sakai et al, 1994). An isoform coding an 874 amino acid protein was detected and analysis of the protein sequence indicated it likely function as a docking/scaffolding protein due to the observation of several protein-binding motifs, including a Src-homology 3 (SH3) domain and SH2-binding motifs that conform to consensus sites for the binding of SH2 adapter protein Crk as well as sites that conform to the SH2 domain of Src (Sakai et al, 1994) – see Fig. 2. No domains indicative of intrinsic enzymatic activity were found, however. Northern blot analysis illustrated that p130Cas is ubiquitously expressed in rat tissues, with higher expression levels seen in the brain, lung, intestine, kidney, and testis (Sakai et al, 1994).

The Src-homology 3 domain

P130Cas was independently identified in a two-hybrid screen for mouse proteins that interact with FAK (Polte and Hanks, 1995). A Src homology 3 (SH3) domain at the N- terminus of p130Cas mediates the FAK interaction, by interacting with the PR-1 and to a lesser extent the PR-2 of FAK (Polte and Hanks, 1995; Harte et al, 1996; Polte and Hanks, 1997). The interaction of p130Cas with FAK and other SH3 binding proteins will be discussed at greater length in this chapter in the section: "p130Cas binding partners".

A proline-rich region follows the SH3 domain, spanning ~ 40 amino acid residues. No known function has been attributed to this domain and therefore, this domain will not be discussed at any greater length.

Pro
74-87

SBD
R^{639}LSPPP....Y^{668}DYV

8   62   119   420   426   618   733   874

NH2- SH3   SD   SER   CCH   -COOH
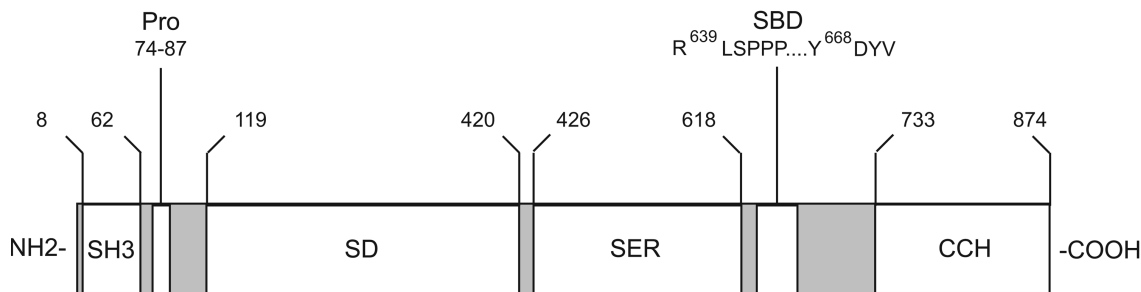
Figure 2: Illustration of p130Cas domains. The primary structure of p130Cas is represented to scale from the N-terminus to C-terminus. "SH3" corresponds to the Src-homology 3 domain, "Pro" is for proline-rich domain, "SD" is for substrate domain, "SER" is for serine-rich domain, "SBD" is for Src binding domain including the motifs for the Src SH2 and SH3 binding, and "CCH" is for the Cas-family C-terminal Homology domain. Measurements are based on information obtained from NCBI Accession Number Q61140 (p130Cas from *mus musculus*). The SD includes 15 phospho-tyrosine sites included in YxxP motifs at residues 119, 132, 169, 183, 196, 238, 253, 271, 291, 310, 331, 366, 376, 391, and 414 (Shin et al, 2004). The CCH domain is designated based on Cas-family protein sequence alignments (see Chapter 3, Fig. 9 for details.)

The Substrate Domain

The major sites of p130Cas tyrosine phosphorylation lie scattered about in the ~ 300 amino acid residue substrate domain (SD) — see Fig. 2 — located in the N-terminal half of the protein (Sakai et al, 1994, 1997). These 15 YxxP motifs (Sakai et al, 1997), most of which have been detected as *in vivo* sites of tyrosine phosphorylation (Luo et al, 2008; Rush et al, 2005) include 9 YDxP motifs that, upon tyrosine phosphorylation, best match the optimal phospho-tyrosine binding motifs for SH2 domains of the SH2/SH3 adapter proteins Crk and Nck (Cantley and Songyang, 1994). The 9 YDxP tyrosines are among 10 p130Cas SD YxxP sites known to be phosphorylated by Src *in vitro* (Shin et al, 2004), and the recruitment of Crk (Sakai et al, 1994) and Nck (Schlaepfer et al, 1997) to these sites has been documented. With their capacities to dock efficiently to sites of tyrosine phosphorylation in the p130Cas

SD, combined with their abilities to promote downstream pathways leading to actin assembly (reviewed in Feller (2001); Buday et al (2002)), adapters of the Crk and Nck families emerged as key downstream effectors of p130Cas in the regulation of cell motility and invasion. The association of p130Cas and these SH2 adapter proteins will be discussed at greater length in the section of this chapter entitled "p130Cas binding partners".

The Serine-rich Domain

In addition to these sites of tyrosine phosphorylation, p130Cas also has a serine-rich (SER) domain — see Fig. 2 — whose phosphorylation has been associated with its ability to bind the 14-3-3 protein (Garcia-Guzman et al, 1999), which will be discussed at greater length in this chapter under the heading of "p130Cas binding partners." NMR studies have shown that the SER domain folds into a four helical bundle similarly to the FAK FAT domain, but also has novel features indicative of protein-protein binding characteristics near the C-terminal end of the domain (Briknarova et al, 2005). Significant serine phosphorylation of p130Cas has been documented by phosphopeptide mapping when fibroblasts are subjected to fibronectin replating for 3 hours (Schlaepfer et al, 1997).

Interestingly, an increase in serine phosphorylation of p130Cas (and concomitant loss of tyrosine phosphorylation) has also been documented when cells are undergoing mitosis, a process shared by FA proteins FAK and paxillin (Yamakita et al, 1999). Interestingly, recovery of tyrosine phosphorylation after mitosis occurs in a similar time course for p130Cas, FAK, and paxillin, with the highest levels of tyrosine phosphorylation occurring first on FAK, then paxillin, and last on p130Cas (Yamakita et al 1999). This coordination of tyrosine phosphorylation may be indicative of the dynamics with which these proteins repopulate FAs after mitosis. It would therefore be interesting to see whether this time course is a general feature of how these FA

proteins become tyrosine phosphorylated or if this time course only occurs as a result of exit from mitosis.

Recently, a mechanism has been suggested by which AND-34/BCAR3 can promote serine phosphorylation of p130Cas in breast cancer cells (Makkinje et al, 2009). Serine-phosphorylation of p130Cas appears to be elevated in estrogen receptor (ER) positive breast cancer cells in comparison to ER negative breast cancer cells (Makkinje et al, 2009). Serine phosphorylation of p130Cas does not require direct binding of AND-34 to p130Cas, but appears to be reliant on an SH2 and serine-rich region in AND-34 for the interaction, which induces p130Cas to run as a slower migrating band during SDS-PAGE (Makkinje et al, 2009). Serine phosphorylation of p130Cas occurs independently of Src and FAK phosphorylation, although abrogating the FAK/p130Cas interaction leads to a reduction in the amount of p130Cas that is serine phosphorylated without completely abrogating its phosphorylation (Makkinje et al, 2009). Cell adhesion and the integrity of the actin cytoskeleton, however, is necessary to induce serine phosphorylation of p130Cas (Makkinje et al, 2009). The kinase responsible for this phosphorylation has not yet been identified, but might generate insight into the mechanisms by which p130Cas can induce anti-estrogen resistance in breast cancer (see section in this chapter entitled "The roles of p130Cas in cancer" for more details).

The Src-binding Domain

In addition to the FAK/Src bridge recruiting Src to p130Cas, two motifs near the p130Cas C-terminus have been characterized as the Src-binding domain (Fig. 2). At least one SH2-binding site in p130Cas was predicted to bind the Src SH2 domain (Sakai et al, 1994; Nakamoto et al, 1995). The contribution that the SH2-binding site makes in the recruitment of Src to p130Cas is controversial, however. The YDYV

motif in the SBD contributes negligibly to the interaction with Src while the polypro-line region of p130Cas is a more likely mediator of Src binding, through the Src SH3 domain (Burnham et al, 2000; Pellicena and Miller, 2001; Ruest et al, 2001). This polyproline region stands alone from the aforementioned proline-rich region and starts near the C-terminus at residue 639 – see Fig. 2. The sequence of this motif is RPLPSPP. The binding of Src to p130Cas through the SBD has also been linked to enhanced c-Src activity (Burnham et al, 1999, 2000; Pellicena and Miller, 2001). The p130Cas SBD interaction can compete with the inhibitory intramolecular inter-actions of Src to bring the kinase into its open and active conformation and this may be primarily achieved through the Src SH3 interaction with p130Cas polyprolines (Burnham et al, 2000; Pellicena and Miller, 2001).

The Cas-family C-terminal Homology Domain

Though it is one of the most conserved regions amongst p130Cas family members, the functions of the Cas-family C-terminal Homology Domain (CCH) are poorly un-derstood. This region, which includes the last 141 amino acid residues of p130Cas, has been noted as an interaction domain for NSP family proteins (see section entitled "CCH domain interacting proteins"), but has also been attributed with a number of functions ranging from transcriptional regulation to FA targeting (see Bouton et al (2001) for a review). To better understand the importance of this domain, the greater Cas-family must first be discussed.

Cas-family members

p130Cas is the founding member of the Cas family of proteins that, in mammals, also includes HEF-1/Cas-L/NEDD9, Efs/Sin (reviewed in(O'Neill et al, 2000), and

HEPL (Singh et al, 2008). While p130Cas is expressed ubiquitously, HEF-1/Cas-L/NEDD9, Efs/Sin, and HEPL have restricted tissue distributions. Additionally, a p130Cas homolog has been found in *Drosophila melanogaster*, which shows a surprising level of domain conservation, especially in the N- and C-terminal domains (Huang et al, 2007). Fig. 3 illustrates the many common features of these Cas-famioly members, as well as demonstrating the variability. Notably, the number of and consensus motifs of the SD domains vary amongst family members. Unlike most of the Cas-family members, HEF-1 lacks the RPLSPP motif for binding the Src SH3 domain (**?**). While the SH3 domain is one of the most conserved domains amongst most of the Cas-family members, HEPL displays the least primary sequence conservation of this domain (Singh et al, 2008).

HEF-1/Cas-L/NEDD9

Human enhancer of filamentation 1 (HEF1) was identified by screening a HeLa cDNA expression library for genes that induced pseudohyphal growth in *Saccharomyces cerevisiae* (Law et al, 1996). The HEF1 C-terminus was once thought to contain a divergent helix-loop-helix motif with similarities to transcription factors such as MyoD (Law et al, 1999). Recent data from this and other research groups now suggest that this area is not in fact a helix-loop-helix motif and may instead fold as either a β-sheet turn α-helix (Singh et al, 2008) or as helices 2 and 3 of a four α-helical bundle (Garron et al, 2009).

HEF1 is processed into a variety of different isoforms post-translationally (Law et al, 1998, 2000). Tyrosine phosphorylation of HEF1 results in a shift of the original band (105 kDa) to a slower migrating band at 115 kDa. The protein can furthermore be processed into smaller fragments by caspase cleavage *in vivo*. The appearance of a 55 kDa band (consisting of the N-terminal half of the protein) during mitosis has been correlated with a role for HEF1 in the cell cycle where this fragment associates with

the mitotic spindle (Law et al, 1998), whereas the appearance of a 28 kDa fragment (that consists mostly of the conserved C-terminus) has been linked to a role for HEF1 in mediating apoptosis (Law et al, 2000). Accumulation of the 28 kDa product was also correlated with JNK phosphorylation, which was found to colocalize with a GFP fusion of this C-terminal HEF1 product in FAs.

Considering the conservation of HEF1 and p130Cas domains, it is not surprising that HEF1 was found to coimmunoprecipitate (CoIP) with FAK and its localization to FAs was confirmed by immunostaining (which also revealed some nuclear and cytoplasmic localization) (Law et al, 1996). Though Law et al (1996) did not clearly demonstrate an association between HEF1 and v-Crk, an association between HEF1 and Crk1 was later shown in cells wherein leukomogenesis was induced by BCR/ABL (de Jong et al, 1997). Indeed, HEF1 was independently identified in lymphocytes as Cas-L (lymphocyte-type Cas), a protein implicated in integrin signaling (Minegishi et al, 1996). It was also identified in melanocytes as a gene conferring metastatic properties in melanomas and was renamed NEDD9 in that context (Kim et al, 2006). Therefore, HEF1 appears to share some structural features and signaling functions with p130Cas, yet also exerts some divergent functions.

Efs/Sin

Expression of Efs, "Embryonic fyn substrate", is primarily restricted to embryonic tissues such as the embryo and placenta, but also to a lesser extent in skeletal muscle, brain, and the brainstem (Ishino et al, 1995). Efs was discovered by screening a cDNA expression library with the SH3 domain of Fyn, though it was also found to interact with Yes (both are SFKs). Further study revealed that the protein spans 560 amino acids and runs as an 83 kDa band (despite a predicted mass of only 59 kDa) (Ishino et al, 1995).

Figure 3: Protein sequence alignment of Cas-family members. Protein Sequence alignments were performed by ClustalW on UniProt website with sequences of p130Cas, Mouse Uniprot ID Q61140; HEF1, Mouse Uniprot ID O35177; EFS, Mouse Uniprot ID Q64355; DmCAS, Drosophila Uniprot ID Q7KVE5; HEPL, Mouse Uniprot ID Q08EC4. Conserved domains are indicated by color: the SH3 domain (green), YxxP motifs in the SD (yellow), features of the SBD (purple), and the CCH domain (cyan).

```
p130Cas  ----------------------------------MTVPNVLAKALYDNVAESPDELSPRKGDIMTVLERD-TQGLDGWMLCSLHGRQGIVPGNRLKILVGMYDKKPAGPGPGPPATPPQPQ   86
HEF1     -------------------------MWARNLMARALYDNVPECAEELAFRKGDILTVIEQN-TGGLEGWMLCSLHGRQGIVPGNRVKLLIGPVQETPGHEQPTP-----                78
EFS      -------------------MAIATSAQLARALYDNTAESPQELSFRRGDLRVIQREGAGGLDGWCLCSLHGQQGIVPANRVKLLPAGPAPKPSLCPASP---------                  81
DmCAS    MLDKTTSSSVSVSTSCCSSNAINADLDYDVPVSRRILIKSKLYAKAIVDNYADTSDELPFKKGDILTVLEQD-TEGFQGWMLCSLRNRQGLCPGNRLRVLNYDSGCFSPSPASS----      114
HEPL     ----------------------MRGTSIREGAPKTLLARALYDNHADCSDELAFSRGDILTIVEQN-VPESEGWWRCLLHGRQGLAPANRIQVLRETPADRPCPLLPRGPD---           88

p130Cas  PSLPQGVHAPVPPASQYSPMLPTAYQPQSDNVYLVPTPSKTQOGLYQAPGPNPQFQSPPAKQTSTFSKQTPHHSFPSPATDLYQVPPGPGSPAQDIYQVPPSAGIGHDIYQVPPSLDTRGWEG  209
HEF1     --------GPMHQTFGQ--QKLYQVPNS-----------QAASRDTIYQVPPSYQN---NIGGTNG                                                            146
EFS      -------TQPGSSCPTP----                                                                                                         112
DmCAS    ----------------ERGCEEQEYVVIPPARP----CSA                                                                                      172
HEPL     --------PVPSLAASTATLDSS-----ICSAEIYENGSVISAVSSNSNGGASSGLGSESVSRVSRRSW                                                        157

p130Cas  TKPPAKVVVPTRVGQGYVYEAAQTEQ-DEYDTPRHLLAPGPQDIYDVPPVRGLLPNQYGQEVYDTPPMAVK--GPNGRDPLLDVYDVPPSVEKGLLSSSHHSVYDVPPSVSKDVPDGPLLREE  329
HEF1     PLLSKKVITPVRTGHGYVYEYPSRYQKDVYDVP----PSHSTQGYDIPPSSVKGP-----VFSVPVGEIKPQGVYDIPTPQGVYAIPPSACRDEAG-LREKEYDFPPPMKQDGKPDTRP-EG  258
EFS      SGLPARSCSPS--------------------SDSIYKVPRVAAHRLVFVGDTLGRLAASAALRAQVGAAGTMLAQTLRA... 186
DmCAS    HASPTKVITPQRHGDVIYNYSPGSATGSGARAAP--STPQQQIYSNQSIYQNFAMMSSPRQNASGSEFETYDIPKPATPVPLNYDSPRSGLPKASNSTTSSSLGTRFETLKSVAASIAEE    293
HEPL     LPRPTRASLPTLPSQVYDVFVQRQGFSTLERLEKQQFYDIPTSSQKALLHSSTSQGRDVTLAPTMAFRQGGGYNPLSSPQKSERIHDTPVLLEKADVRNVSMTSFTKDSGSRAIPGSSAVHTG  280

p130Cas  TYDVPPAFAKPKPFDPTRHPLILAAPPPDSPAAEDVYDVPPPAPDLYDVPPGLRRPG--PGTLYDVPR--------ERVLPEVA--------DGSVVDDGYVAVPP-----AEREAPTDGKRLSASS  435
HEF1     VYDIPPTSTKTAGKD--------LHIKFPCDAPGGVEPMARRHQSFSLHHAPSQLGQSGDTQSDAYDVPRGVQFLEVPTETSEK-----ANPEERDGVYDVPLHNPADAKGSRDVVDGINRLSFSS  371
EFS      PYDVP--------------LALKPPAELERDPEWEGGREPGPPLYAAPSNLKRAS-ALLNIYEAPE---ELLANG---ESRDADEGIYDVPLLG------PEPPSPEPPVASSS              273
DmCAS    SYDVPRPLILGS--------NQLQOQMSQMTPSSASSLLTSDSLSLSFSSSNRSSLANMPDYDIPR------RNPLP---VRRQQSGLTYDFPLPP------LQEQVPRVLCTP             385
HEPL     AVALSPQLGNTVQRKNSLPEEPTYAFPTSRDPLPSDAGGSYKVPSRFLIPRVEQQNT--MPNIYDTPKAMQGVSHNAPKAMQGVSLAGKELERGREAPENSPWISGQTSFLSPDSDRLSVASS    401

p130Cas  TGSTRSSQSASSLEVVVP--------GREPLELEVAVESLARLQQGVSTTVAHLLDLVGSASGPGGWRGTSEPQEPPAQDLKAAVAAVHGAVHELLEFARGAVSNATHTSDRTLHAKLSRQL  549
HEF1     TGSTRSNMSTSSTSSKESSLSASPSQDKRLRLDPDTAIEKLYRLQQTLEMGVCSLMSLVTTDWRCYGY--------MERHINEIRTAVDKVELFLREYLHFAKGALANASCLPELVLHNKMREL  488
EFS      TDLDTVAQLPTRSS----------------PPQHRPRLPSTESLSRRPLPALPVS----                                                                   312
DmCAS    STVTTTKELPLELSS--------------ALETLAKLQLQTTAAINRLLSFVVPNWRTRAQ----LEPAIMDLKLAALRLRTTLHDLAEFGEGALGNATRSEDRNLALKLRPLV            481
HEPL     DSRASVVSSCSSISMDSSSGSSSEDSVKELWMDVDFAKETAVSLQHKVASSAAGLLLFVSRTWRFKDS---LETNIHRIRRAADHVEESVREFLDFAQGVGGTACNLTDSYLQARIRDQL     518

p130Cas  QKMEDVYQTLVVHGQVLDSGRGSPGFTPEDLDRLVACSRAVPEDAKQLASFLHGNASLLFRRTKAPGPGEGSSLHPN------------PTDKASSIQSRPLPSPPKFTSQ           650
HEF1     QRVEDSHQILSQTSHDLNECSWSLNIIAINRKPQNKCDDLDRFVMVAKTVPDDAKQLTTTISTYAETLFRADPANSHLKNGPNSIMNSSEYTHPGSQMQPLHPGDYKAQVHSKPLP-PSLSKDQ  610
EFS      -------------------------------------------EAPAPSPAPSPAPGRKGSIQDRPLPPPPCLPG                                                  344
DmCAS    RALRDANKLIHDSSESLDAQG----------------AWSIDQLARSDEKHGCRPPDALDQLMVCAQTLTEDVRSTTSFIQGNASLLFK---------RQLTVGHNG                    563
HEPL     QTISSSYQTLLDAKGSLDRCNWSLEVLVTDKVQNSLDDLERFVATARIVPEDVKRFTSIVIANGKLLFKQNCEKGEMDLKCERCIRP---------PQRETES                      612

p130Cas  DSPDGQYENS-EGG----------WMEDYDYVHLQGKEEFEKTQKELLERG--NIMRQGKQLELQLKQFERLEQEVSRPIDHDLANWTPAQPLVPGRTGGLGPSDRQLLLFYLEQC        755
HEF1     PPDCGSSDGS-ERS----------WMDDYDYVHLQGKEFERQQKELLEKE--NIMKQSKAQLEHHQLSQFQLLEQEITKPVENDISKWKPSQSL-PTTNNSVGAQDRQLLCFYYDQC       714
EFS      YGGLKPEGDPECREVANDPAGPHNEYEGIPMAEEYDYVHLKGVD------------TAQGSRPLDKAFPVDPELLERGLAERKEALS--------PEEPLVLSTGDLQLLHFYAGQC       442
DmCAS    GSGTGVLEGSRGSAE----------WLEDYDYVAFESKDAAARKNEALREAIPADLKTQFDTVIRTAESSAMGAGSGSIPTTPTART-PTT----PCKSPEMTDKDKKLVRYYAQQI       666
HEPL     YQESSPFDRQ-----PTTEHSFELARKNR--------------VNVCWQQSPNLQEKGKPTMEGKSNRNPDFHGMSPP----------PLTSPSPSGQNTERKIHLSKHS            693

p130Cas  EANLTTLTDAVDAFFTAVATNQPPKIFVAHSKFVILSAHKLVFIGDTLSRQAKAADVRSQVTHYSNLLCDLLRGIVATTKAAALQYPSPSAAQDMVDRVKELGHSTQQFRRVLGQLAAA*    874
HEF1     ETHFISLLNAIDALFSCVSSAQPPRIFVAHSKFVILSAHKLVFIGDTLTRQVAAQDIRNKVRNSSNQLCEQLKTIVMATKMAALHYPSTTALQEMVHQVTDLSRNAQLFKRSLLEMATF*    833
EFS      QSHYSALQAAVAALVASTQANQPPCLFVPHGKRVVVAAHRLVFVGDTLGRLAASAALRAQVGAAGTMLAQTLRATVLAVKGAALGYPSDTAVQEMARCVAEILAGQALRFTTLLAGLLP*    560
DmCAS    STHMGNLMQAIDSFLETVENQPPKFFIAYGKFVVVSAHNLVTIGDNVYRNISKEALREKILRCTNALSDALKTCVLKSKKAAAHFPSGSAVQEMVDSVVHINNLARDLKSVMLQAVHLSTAAGVGA*  793
HEPL     RLYFGALFKAISVFASSLSNGQPPEVFITQSKLVITVGQKLV---DTLLCSETQEKDERNEILCGSSHLCGLLKDLALATKSAVIQYPSPSALSLLQSEVERLEHHSRKFRDTLE*        804
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Efs was separately identified as Sin, "Src interacting or signal integrating protein", in a screen to identify proteins that interacted with the Src SH3 domain (Alexandropoulos and Baltimore, 1996). Expression of a short sequence of Sin including a polyproline region led to an increase in c-Src activation and increased phosphorylation of the enolase substrate (Alexandropoulos and Baltimore, 1996). The ability of Sin to promote c-Src activation was further increased when a Sin expression construct was expanded to include the SH3 domain, suggesting that like p130Cas, Sin interactions with Src may be enhanced through binding FAK family proteins (Alexandropoulos and Baltimore, 1996). Crk binding to Sin appears to be Src-dependent (Alexandropoulos and Baltimore, 1996) and has been implicated in neurite outgrowth as a result of EGF stimulation in PC12 cells (Yang et al, 2002). The effects of Sin on neurite outgrowth through EGF were found to be dependent upon receptor protein tyrosine phosphatase-α and the Sin SD (Yang et al, 2002). In T lymphocytes, Sin has been shown to modulate T-cell receptor signaling by sequestering signaling components such as PLCγ (Xing et al, 2004). Small variations in the Efs/Sin SD and its restricted expression may further differentiate its signaling capacities from p130Cas.

HEPL

HEPL (HEF1-Efs-p130Cas-like) was recently identified in a BLAST search for proteins that have similar primary structure features to Cas-family members (Singh et al, 2008). HEPL exhibits a similar distribution in tissues to HEF-1 (especially in the lung and spleen) and mRNA levels of HEPL were most readily detectable in ovarian and leukemia cell lines (Singh et al, 2008). The HEPL protein consists of 786 amino acids and like other Cas-family members and secondary structure alignment of HEPL, Efs, HEF1, and p130Cas C-terminal sequences suggests a structure with three α-helices and one β-sheet occurring between the first and third helices (Singh et al, 2008).

Despite lower conservation of the SH3 domain sequence, yeast two-hybrid confirmed interaction of the HEPL SH3 domain with FAK. HEPL localization to FAs was also observed. Depletion of HEPL was correlated with a reduction in cell spreading and cell motility, though these effects appear to be intermediate to those seen in the siRNA scramble control and the HEF1 siRNA population. No significant effects on proliferation or apoptosis were observed in cell populations treated with HEPL siRNA (Singh et al, 2008). This is the first and currently only study on HEPL. Clearly, greater research is needed to elucidate the functions of this protein.

DmCAS

DmCas was found in a bioinformatic search for genes in *Drosophila melanogaster* with similarities to p130Cas (Huang et al, 2007). Sequencing of the cDNA from a candidate gene was predictive of a 793 amino acid protein with a primary structure similar to Cas family members including an SH3 domain (see Fig. 3). Study of *Drosophila larvae* revealed that DmCAS is highly expressed in the nervous system and loss-of-function (LOF) mutants as well as gain-of-function (GOF) mutants exhibit neuronal defasciculation defects, wherein the motor neurons fail to branch out from the fascicle (Huang et al, 2007). These defects mimic α1-integrin LOF and GOF mutants. It will be interesting to see whether DmCAS will exhibit similar signaling functions to mammalian Cas-family members.

p130Cas acts as a docking site for multiple binding proteins

Though p130Cas has no intrinsic enzymatic activity, it exerts influence on a myriad of signaling pathways by virtue of its protein docking properties. Known binding partners of p130Cas are discussed in this section, with those binding the N-terminus of the protein discussed first (see Table 1).

Table 1: Known p130Cas binding partners and interaction domains

Cas Domain	Binding Partner	Site Bound	References
SH3	FAK PYK2 FRNK PTP1B C3G CMS PR-39 PTP-PEST	PR	(Polte and Hanks, 1995, 1997) (Astier et al, 1997) (Harte et al, 1996) (Liu et al, 1996) (Kirsch et al, 1998) (Kirsch et al, 1999) (Chan and Gallo, 1998) (Garton et al, 1997)
SD	Crk Nck SHIP-2	SH2	(Sakai et al, 1994) (Schlaepfer et al, 1997) (Prasad et al, 2001)
SER	14-3-3	K49	(Garcia-Guzman et al, 1999)
SBD	SFKs Neph PI3-K	SH2,3 SH3 SH3	(Nakamoto et al, 1995, 1997) (Donaldson et al, 2000) (Li et al, 2000)
CCH	AND-34 Chat Ajuba	GEF GEF Pre-LIM	(Gotoh et al, 2000) (Sakakibara and Hattori, 2000) (Pratt et al, 2005)

SH3 domain interactions

At its N-terminus, p130Cas has an SH3 domain known to associate with FAK (Polte and Hanks, 1995), the FAK family member protein tyrosine kinase 2 — PYK2 (Astier et al, 1997), guanine nucleotide exchange factor C3G (Kirsch et al, 1998), Cas-ligand with multiple SH3 domains — CMS (Kirsch et al, 1999), antimicrobial peptide PR-39 (Chan and Gallo, 1998), and the protein tyrosine phosphatases PTP1B (Liu et al, 1996) and PTP-PEST (Garton et al, 1997). The interaction between p130Cas and FAK was determined by yeast two-hybrid screening using a C-terminal truncation of FAK as bait (Polte and Hanks, 1995). Mutation of a PR region in the bait to Ala residues abrogated the interaction (Polte and Hanks, 1995). The interaction between p130Cas and FAK was later shown to occur primarily through the PR-1 and partly through PR-2 region of FAK (Polte and Hanks, 1997). Despite the direct interaction of the p130Cas SH3 domain with FAK, tyrosine phosphorylation of p130Cas is attributed

to SFKs (Hamasaki et al, 1996; Vuori et al, 1996; Sakai et al, 1997). Nevertheless, FAK serves an important role in the efficient tyrosine phosphorylation of p130Cas by acting as a scaffold to recruit Src to p130Cas by virtue of the Src SH2 domain interaction with the FAK autophosphorylation site Y397 (Ruest et al, 2001).

PYK2 was found in association with p130Cas by CoIP after simulating integrin ligation using a β-1 integrin monoclonal antibody (Astier et al, 1997). The interaction was found to be dependent on cytoskeletal integrity, as cytochalasin D treatment abrogated the ability of the proteins to bind one another (Astier et al, 1997).

Association of C3G with p130Cas binding partner CrkII was previously documented (Vuori et al, 1996), yet p130Cas can also bind C3G directly through its SH3 domain (Kirsch et al, 1998). Mapping studies of C3G revealed that the binding site for the p130Cas interaction is in fact a different motif than the one needed for the C3G-CrkII association (Kirsch et al, 1998). Mutation of C3G amino acid residues 287 and 289 abrogated the association (Kirsch et al, 1998). Like PTP1B and PTP-PEST, these studies confirm that the p130Cas SH3 domain preferential binds to PPxP motifs (Kirsch et al, 1998). C3G activity has been shown in relationship to Rap1 (Gotoh et al, 1995) and R-Ras (Gotoh et al, 1997). Further studies are needed to understand how C3G influences aspects of p130Cas signaling.

CMS, like C3G, was identified by yeast two-hybrid with the p130Cas SH3 domain as bait (Kirsch et al, 1999). CMS contains three SH3 domains followed by a proline-rich region through which the p130Cas SH3 domain is thought to bind (Kirsch et al, 1999). IP experiments with a transfected p130Cas SH3 domain or full-length protein confirmed the interaction of CMS with p130Cas in HEK-293 cells (Kirsch et al, 1999). By immunostaining experiments, Kirsch et al (1999) found that CMS localizes primarily to the cytoplasm in a punctate manner (which was not seen when a truncated version of the protein expressing only the SH3 domain or lacking the C-terminal

coiled-coil domain were expressed). Upon stimulation with phorbol 12-myristate 13-acetate (PMA), however, CMS colocalized with actin in membrane ruffles similarly to p130Cas (Kirsch et al, 1999). Kirsch et al (1999) noted that p130Cas and CMS colocalized in membrane ruffles as 'data not shown'.

PR-39 is an antimicrobial peptide (endogenous, non-synthetic) that has been shown to have anti-LPA properties and stimulates wound-healing (Chan and Gallo, 1998). An interaction between the p130Cas SH3 domain and PR-39 was found when full-length p130Cas expressed in endothelial cells was CoIPed with PR-39 (Chan and Gallo, 1998). Fractionation experiments showed that this interaction was most robust in the cytoskeletal fraction (Chan and Gallo, 1998). These data suggest another mechanism by which p130Cas may regulate microbial pathogenesis by altering the actin cytoskeleton (see the subheading "Pathogenesis" in this chapter for more details).

PTP1B, a protein tyrosine phosphatase, associates with p130Cas by virtue of one of its two proline-rich regions (located around residues 300-320), which fits the consensus sequence for the p130Cas SH3 domain (Liu et al, 1996). Mutation of the first two residues in this sequence (PPRP) abrogated the ability of p130Cas to CoIP with PTP1B (Liu et al, 1996). Liu et al (1996) concluded that the ability of PTP1B to associate with p130Cas was not dependent on p130Cas tyrosine phosphorylation (since p130Cas and PTP1B still CoIPed in non-transformed cells wherein p130Cas tyrosine phosphorylation was not detected). They did, however, find that overexpression of PTP1B in cells that were v-Crk transformed resulted in a reduction of p130Cas phosphorylation (Liu et al, 1996). These data make PTP1B an interesting candidate for the phosphatase that regulates the absence of p130Cas phosphorylation in the cytoplasm. It would be interesting to see if the association of p130Cas with PTP1B truly occurs in the absence of its tyrosine phosphorylation (it is now known that in order to detect p130Cas phosphorylation in non-transformed cells by immunoblotting, sodium vanadate treatment must be used to inhibit phosphatase activity — see Fonseca et al

(2004)). Use of λ phosphatase to treat cell lysates prior to IP might offer such insight, for example.

Garton et al (1996) demonstrated that p130Cas and PTP-PEST interact via the p130Cas SH3 domain to further enhance the ability of PTP-PEST to dephosphorylate the protein. Previous studies showed that PTP-PEST dephosphorylates p130Cas on tyrosine residues, though dephosphorylation of p130Cas on tyrosine by the PTP-PEST catalytic domain was slightly less efficient in comparison to the PTP-1B catalytic domain (Garton et al, 1996). IP experiments demonstrated a dose-dependent interaction between the p130Cas SH3 domain and the C-terminal region of PTP-PEST (Garton et al, 1996). PTP-PEST contains two proline-rich regions at the C-terminus, yet inclusion of the first proline-rich region alone was sufficient for the p130Cas interaction (Garton et al, 1996). While interaction of PTP-PEST with p130Cas through the catalytic domain appears to be pTyr- dependent (pervanadate treatment increases the amount of protein CoIPed), the interaction of the p130Cas SH3 domain with PTP-PEST is not affected by pTyr content (Garton et al, 1996). While CoIP experiments with full-length PTP-PEST and p130Cas were unsuccessful (presumably because the interaction between PTP-PEST and p130Cas is so transient), use of a PTP-PEST mutant D199A (which has much lower catalytic activity) allowed the investigators to detect p130Cas and PTP-PEST in complex (Garton et al, 1996).

These results present an interesting mechanism by which the SH3 domain of p130Cas can regulate its own tyrosine phosphorylation and dephosphorylation. Since the SH3 domain is also the recruitment site for FAK, it would be interesting to study the dynamics of FAK and PTP-PEST interaction with p130Cas in live cells with FRET probes. In this manner, it would be possible to see whether p130Cas recruits PTP-PEST to FAs where it exchanges its interaction with FAK for PTP-PEST or whether the PTP-PEST interaction occurs only in the cytoplasm.

Substrate domain (SD) interactions

When p130Cas is phosphorylated at the SD by Src and its family members (Nakamoto et al, 1995), it is not only able to recruit Crk and Nck, but also inositol phosphatase SHIP-2 (Prasad et al, 2001). The association of p130Cas with Crk and Nck has been linked to Rac activation and cell motility (Chodniewicz and Klemke, 2004). P130Cas can bind CrkII via 10 of its 15 YxxP motifs (Shin et al, 2004). Association of p130Cas with Crk is implicated in variety of signaling pathways. These aspects will be further elaborated in the section of this chapter entitled "p130Cas signaling paradigms". Nck1 associates with the p130Cas SD in response to fibronectin replating (Schlaepfer et al, 1997). Interestingly, the sites indicated for Nck association are within the first 6 YxxP motifs of the SD (Schlaepfer et al, 1997), whereas the Crk binding sites are indicated in the last 10 YxxP sites (Shin et al, 2004). Nck SH2 binding of p130Cas after cells are stimulated with platelet-derived growth factor (PDGF) has also been noted and is linked to cell motility (Rivera et al, 2006).

The Src-Homology 2 containing inositol phosphatase-2 SHIP-2 (or inositol polyphosphate phosphatase-like 1 (INPPL1)) interaction with tyrosine phosphorylated p130Cas is dependent upon its SH2 domain (Prasad et al, 2001). The interaction is furthermore regulated by cellular adhesion as evidenced by the lower amount of protein CoIPed when HeLa cells are trypsinized versus adherent or replated on fibronectin (Prasad et al, 2001). Upon replating, a faster migrating SDS-PAGE band of p130Cas was found in association with SHIP-2 in whole cell lysates from HeLa cells that was abrogated when cells were treated with protease but not caspase inhibitors (Prasad et al, 2001). SHIP-2 was furthermore found to colocalize with paxillin in FAs, which was dependent on the SH2 domain and cell spreading was reduced in cells expressing a mutant form of SHIP-2 wherein the catalytic domain was mutated (Prasad

et al, 2001). These data suggest that SHIP-2 may associate with multiple species of the p130Cas protein to modulate separate processes. Perhaps the catalytic domain mediates a role for p130Cas in membrane ruffles and cell spreading, whereas the SH2 interaction is more important for p130Cas signaling in FAs. Alternately, the SH2 interaction with p130Cas may serve as a negative feedback mechanism by which phosphorylated p130Cas can be recognized by SHIP-2 at FAs and can then be shuttled into membrane ruffles for eventual targeting to the proteasome.

Though protein motifs mediating the interaction of p130Cas with the Ras effector protein Grb2 are not known, it appears that the association of p130Cas and Grb2 is pTyr-dependent in cells treated with epidermal growth factor (EGF) (Wang et al, 2000). Grb2 IP in EGF, but not fibroblast growth factor (FGF) treated cells, resulted in p130Cas CoIP (Wang et al, 2000). Expression of a receptor protein tyrosine phosphatase, LAR, resulted in dissociation of p130Cas and Grb2 and also resulted in reduced appearance of a pTyr band in the 130 kDa range (Wang et al, 2000).

Serine-rich domain interactions

The p130Cas SER domain has been shown to interact with 14-3-3ζ protein both *in vitro* and *in vivo* (Garcia-Guzman et al, 1999). Expression of a mutant form of 14-3-3 wherein the Lys 49 residue was mutated to Asp, calf alkaline phosphatase treatment of cell lysates, or SER domain deletion all resulted in dissociation of these proteins (Garcia-Guzman et al, 1999). The binding of p130Cas with 14-3-3 was increased in cells that were allowed to adhere to fibronectin in comparison to those plated on polylysine or left in suspension (Garcia-Guzman et al, 1999). Immunostaining of p130Cas and 14-3-3 demonstrated strong colocalization in membrane ruffles of spreading cells as well as in the cytosol of well-spread cells (Garcia-Guzman et al, 1999). Slight FA localization of 14-3-3 was also seen by immunostaining using vinculin as an FA marker (Garcia-Guzman et al, 1999). These results are interesting since the

function of p130Cas serine phosphorylation is still poorly understood.

14-3-3 proteins are known to coordinate the formation of a number of different protein complexes. It would be interesting to know what other proteins constitute the p130Cas/14-3-3 complex and whether or not other 14-3-3 isoforms similarly interact with p130Cas. In particular, it would be interesting to know whether or not 14-3-3 is important in bringing AND-34 into complex with p130Cas for serine phosphorylation, since the interaction of p130Cas and AND-34 after serine phosphorylation has been shown to occur indirectly (Makkinje et al, 2009).

Src-binding domain (SBD) interactions

Several other proteins are known to bind to the Src-binding domain (SBD). These proteins include: the SFKs Src, Fyn, Yes, Lck (Nakamoto et al, 1995), Lyn and Hck (Manie et al, 1997), as well as Nephrocystin (Donaldson et al, 2000) and PI3-K (Li et al, 2000). The RPLPSPP motif is known to bind the SH3 domain of Src, whereas the the YDYV motif at residue 668 is able to bind the Src SH2 domain (Nakamoto et al, 1997).

In kidney epithelial cells, studies by Donaldson et al (2000) showed that p130Cas localizes to cell-cell contacts, colocalizing with E-cadherin. CoIP experiments showed a direct interaction between p130Cas and the SH3 domain of Nephrocystin (Donaldson et al, 2000). In MDCK cells, p130Cas localizes prominently to FAs instead of cell-cell adhesions, leading the authors to suggest that in subconfluent cells, p130Cas localizes to FAs, establishing contact with the ECM. As the cells then become more confluent and begin to contact one another, p130Cas may be involved in integrin-mediated orientation of apical-basal cell polarity (Donaldson et al, 2000).

As a result of adenovirus entry, p130Cas becomes heavily tyrosine phosphorylated and becomes associated with the p85 subunit of PI3-K (Li et al, 2000). The SBD

appears to mediate the interaction of p130Cas and PI3-K, as site-directed mutagenesis of the RPLPP motif in the p130Cas SBD is sufficient to abrogate the interaction. This mechanism appears to be regulated by integrin signaling through the actin cytoskeleton (Li et al, 2000; Armulik et al, 2004).

CCH domain interacting proteins

NSP family member Chat (also known as NSP3 or SHEP1) has been shown to interact with p130Cas through its conserved C-terminus (Sakakibara and Hattori, 2000). Chat localizes with p130Cas along actin filaments and membrane ruffles, as ascertained by immunostaining (Sakakibara and Hattori, 2000). Another NSP family member, AND-34 (also known as BCAR3 or NSP2) localizes with p130Cas in lamellipodia and membrane ruffles (Riggins et al, 2003). Mapping studies of the p130Cas/AND-34 interaction revealed a necessity for the C-terminal domain of p130Cas (Gotoh et al, 2000). Additional studies showed that mutation of residue 791 in p130Cas disrupts AND-34 binding to p130Cas (demonstrated by CoIP), suggesting that AND-34 binds directly to this residue (Gotoh et al, 2000). The importance of this residue in the p130Cas/AND-34 interaction has been futher supported by the observation that AND-34 binding to HEF-1 occurs through the same binding motif (Garron et al, 2009). Mutation of this residue does not, however, disrupt the ability of HEF-1 to localize to FAs (Garron et al, 2009). Indeed, it was previously shown that even though AND-34 colocalizes at lamellipodia and membrane ruffles with myc-tagged p130Cas, FA staining is not observed for AND-34 (Riggins et al, 2003).

NSP1 is also thought to associate with p130Cas through the C-terminal domain, since the GEF domain (by which Chat and AND-34 bind p130Cas) is well-conserved amongst family members (Alexandropoulos and Regelmann, 2009). However, mapping studies to confirm these interactions between p130Cas and NSP1 have not yet

been published. Despite this limitation, it is known that p130Cas associates with NSP1 (Lu et al, 1999).

In addition to these NSP family members, a zyxin family member, Ajuba, has also been identified as a CCH-interacting protein (Pratt et al, 2005). Evidence for the direct interaction between p130Cas and Ajuba was shown through mapping studies wherein a p130Cas mutant lacking the C-terminal domain was not able to associate with Ajuba in comparison to WT (Pratt et al, 2005). The Pre-LIM region of Ajuba was identified as the interacting domain through another series of CoIPs and competitive binding experiments (Pratt et al, 2005). Though not indicated as an integral protein for the association of p130Cas with FAs, Ajuba may play an important role in localizing p130Cas to lamellipodia and membrane ruffles (Pratt et al, 2005). Immunocytochemistry experiments with *Ajuba* -/- cells reveal normal FA formation but greatly reduced lamellipodial extension and membrane ruffling (40% less than WT). Cell motility and p130Cas localization defects were reversible in *Ajuba* -/- cells by exogenous expression of Rac1, indicating that Rac1 activation downstream of p130Cas may be important for this particular pathway and that Ajuba may signal upstream of Rac1. *Ajuba* -/- cells co-transfected with WT Ajuba and a p130Cas mutant lacking the C-terminal domain were unable to stimulate lamellipodial extension in comparison to cells expressing WT p130Cas (Pratt et al, 2005).

p130Cas functional roles and signaling paradigms

By virtue of its docking properties, p130Cas regulates a large variety of signaling pathways and cellular functions. Here, I will present a review of known p130Cas signaling paradigms and various cellular functions that these affect. The major mode of p130Cas signaling is through the phosphorylation of tyrosine residues in its SD, which occurs as a result of a number of stimuli such as: integrin engagement (Nojima et al,

1995; Petch et al, 1995; Vuori and Ruoslahti, 1995), stimulation by growth factors such as EGF, PDGF, and insulin, antigen stimulation such as LPA and lymphocyte function-associated antigen-1 (Petruzzelli et al, 1996; Casamassima and Rozengurt, 1997, 1998), neuropeptides such as bombesin (Casamassima and Rozengurt, 1997; Rozengurt, 1998), glucose stimulation in pancreatic β cells (Lee et al, 2004), and signaling from the urokinase-type plasminogen activator receptor (Smith et al, 2008). Central to many of p130Cas signaling paradigms is the association of p130Cas with Crk (Chodniewicz and Klemke, 2004), which is summarized in Fig. 4.
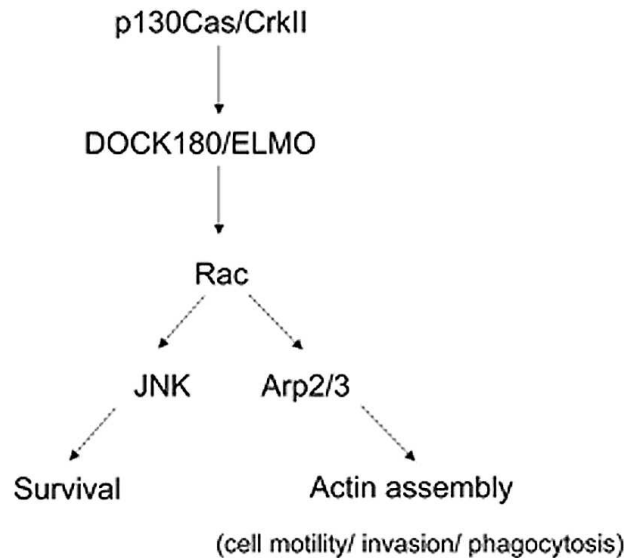

Figure 4: The coupling of p130Cas to CrkII is central to several p130Cas signaling paradigms. Signaling through p130Cas/CrkII leads to actin assembly pathways involved in cell survival as well as cell motility, invasion of cancer cells, and phagocytosis of pathogens. Figure is partially based on Chodniewicz and Klemke (2004).

Functional roles of p130Cas in the heart and vasculature

Cas -/- mice die of severe cardiac abnormalities at embryonic day 12.5 wherein the heart consists of a thin myocardium and dilated blood vessels (Honda et al, 1998). Electron microscopy revealed that the myofibrils and Z-discs in the myocardium of

Cas -/- mice show extensive disorganization in comparison to WT littermates (Honda et al, 1998). *Cas* -/- MEFs analyzed for their actin organization by phalloidin staining showed a reduction in stress fiber formation, although FA formation was documented as indicated by vinculin immunostaining (Honda et al, 1998). p130Cas and FAK have been further implicated in heart development as they are known to localize to Z-lines in the myocardium wherein disassociation of p130Cas and FAK can cause sarcomeric disorganization (Kovacic-Milivojevic et al, 2001, 2002). Arterial contraction in response to serotonin is also regulated in part by p130Cas (Ogden et al, 2006). Arterial contraction leads to p130Cas tyrosine phosphorylation in a Src- and actin-dependent manner (Ogden et al, 2006). Use of a serotonin inhibitor reduces p130Cas tyrosine phosphorylation and arterial contraction is reduced by p130Cas knock-down (Ogden et al, 2006).

Functional roles of p130Cas in the brain

In addition to finding heart defects in *Cas* -/- mouse embryos, Honda et al (1998) observed a decrease in brain size. Later studies showed that WT mice exhibit elevated levels of p130Cas in the cerebellum around 7 days postpartum (Huang et al, 2006). Coincident with these elevated levels of protein are elevated levels of p130Cas tyrosine phosphorylation (Huang et al, 2006). These findings are perhaps not surprising, since p130Cas is a major Src substrate and Src activity is elevated in the cerebellum of developing rats at levels 6-10 times higher than is usually seen in fibroblasts (Cartwright et al, 1988). This trend of elevated activity is seen even though the expression of Src remains relatively constant throughout cerebellar development — 1-2 times higher than fibroblasts (Cartwright et al, 1988).

Knockdown of p130Cas with siRNA in cerebellar granule cells results in significant defects in growth cone elongation (Huang et al, 2006). Similar defects in growth cone elongation were seen when cells were transfected with p130Cas SD deletion mutants,

but not when p130Cas constructs containing SH3 or SBD mutations were expressed (Huang et al, 2006). The importance of p130Cas tyrosine phosphorylation was further suggested by the observation that Src, p130Cas, and Crk colocalize with one another in the growth cone (Huang et al, 2006). NCAM and N-cadherin were found to interact with p130Cas in the growth cone by both coimmunoprecipitation and immunostaining, furthermore linking p130Cas signaling to cell adhesion as both NCAM and N- cadherin have been extensively identified as neuronal adhesion molecules (reviewed in Gerrow and El-Husseini (2006)).

Survival and Apoptosis

Association of p130Cas with its binding partners has been linked to survival signaling whereas abrogating those interactions has been shown to induce apoptosis. Binding of p130Cas to FAK, for instance, is associated with pro-survival signaling in fibroblasts and cells plated on fibronectin (Almeida et al, 2000; Zouq et al, 2009). Similarly, p130Cas has been implicated as a major mediator connecting several pathways through Crk to the Rac/JNK pathway (Dolfi et al, 1998), but only in the context of integrin signaling and not in the context of EGF stimulation or v-Src expression. However, in response to reactive oxygen species, Src appears to be essential in mediating a p130Cas/Crk/JNK pathway of survival (Yoshizumi et al, 2000). Central to all of these paradigms is the activation of the JNK pathway. Interestingly, growth hormone (GH) stimulation of CHO cells leads to the activation of the JNK pathway, which is concomitant with the formation of large protein complexes including p130Cas (Zhu et al, 1998). The p130Cas protein complexes that formed in response to GH contained not only FAK, but also CrkII, paxillin, tensin, C3G, the p85 subunit of PI3-K, Grb-2, and Sos (Zhu et al, 1998). It would therefore be interesting to dissect whether some of the proteins brought into complex with p130Cas during GH stimulation are similarly involved in the aforementioned survival pathways.

In the absence of serum, the binding of the p130Cas SH3 domain to the PR regions of FAK is necessary to support survival (Almeida et al, 2000). Expression of p130Cas constructs lacking either the SH3 or SD domains results in a dramatic rise in apoptosis (Almeida et al, 2000). Therefore, the interaction of p130Cas with FAK as well as p130Cas phosphorylation are important in mediating p130Cas/FAK-mediated signaling pathways of survival (Almeida et al, 2000). Using a variety of inhibitors, DN expression constructs, and constitutively active signaling proteins, Almeida et al (2000) concluded that fibronectin-mediated survival signaling through FAK/Cas results in activation of the Ras/Rac1/Pak1/MKK4/JNK pathway wherein p-JNK is detected at FAs with FAK and is also detected in the nucleus.

The mechanisms by which FAK can promote survival in cells appears to be cell and substrate dependent and can be linked to whether or not FAK is found in a protein complex with p130Cas or paxillin (Zouq et al, 2009). Under conditions that would normally promote an apoptotic state known as anoikis, wherein cells die due to a loss of attachment to the ECM (Frisch and Ruoslahti, 1997), FAK/p130Cas scaffolding can promote survival in fibroblasts, but not epithelial cells (Zouq et al, 2009). Expression of the SH3 domain acts as a DN in these fibroblasts, inhibiting p130Cas phosphorylation and significantly increasing apoptosis when cells were not allowed to attach to the substratum and serum was removed (Zouq et al, 2009). The authors further supported the p130Cas/Crk/Rac1/Pak1/MKK4/JNK pathway previously suggested as the mechanism leading to cell survival through FAK/p130Cas (Almeida et al, 2000; Yoshizumi et al, 2000). Interestingly, this pathway appears to be activated only in response to fibronectin. In the presence of collagen, FAK mediates survival signaling independently of p130Cas (Almeida et al, 2000).

In an interesting twist, a 31-kDa fragment containing the conserved C-terminus of p130Cas was found to localize to the nucleus where it may act as a transcriptional

repressor of cell cycle progression (Kim et al, 2003). This fragment occurs as a result of caspase cleavage and is furthermore able to induce anoikis (Kim et al, 2003). Kim et al (2003) suggest that, once cleaved, p130Cas may be able to further signal in a pro-apoptotic pathway by the localization of the 31-kDa fragment to the nucleus, as determined by analyzing colocalization of GFP-tagged p130Cas constructs with Hoechst stain. A GST fusion of residues 835-908 in the p130Cas C-terminus (those residues that would constitute the helix-loop-helix previously suggested by Law et al (1999)) was able to pull down E47, a splice variant of E2A (Kim et al, 2003). Strangely, the authors used a longer splice variant of p130Cas for their studies that has only been documented in rats (mouse and human p130Cas is only 874 amino acid residues in length). The authors concluded that this 31-kDa fragment of p130Cas dimerizes with E47, thereby allowing entry into the nucleus, and promoting apoptosis (Kim et al, 2003). A p130Cas fragment of a similar size is seen by immunoblotting when endothelial cell monolayers are treated with lipopolysaccharides (LPS) and subsequently undergo apoptosis due to ECM detachment (Bannerman et al, 1998). This event has also been shown to be caspase dependent and treatment of endothelial cell monolayers with caspase inhibitors during LPS treatment prevents apoptosis (Bannerman et al, 1998).

Pathogenesis

In order to mediate phagocytosis of bacteria, the cell must engage the actin cytoskeleton to engulf the bacteria. Some bacteria have evolved mechanisms to prevent this uptake and ultimately their destruction. Bacteria of the genus *Yersinia*, such as *Y. pseudotuberculosis*, employ a protein tyrosine phosphatase, which can enter host cells and dephosphorylate its endogenous substrates (Black and Bliska, 1997). A catalytically inactive form of such a phosphatase, YopH, has been useful to probe for potential substrates including p130Cas (Black and Bliska, 1997). YopHC403S

(catalytically inactive YopH) localizes to FAs in immnostaining experiments where 4G10 (pTyr antibody), paxillin, or vinculin were used as markers (Black and Bliska, 1997). While expression of WT YopH induced dephosphorylation of a band in the 130 kDa range as detected by immonoblotting for pTyr; YopHC403S transfected cells displayed robust phosphorylation of this band (Black and Bliska, 1997). YopHC403S transfection also led to the distribution of this phosphorylation into the insoluble fraction (Black and Bliska, 1997). By immonoblotting experiments Black and Bliska (1997) further demonstrated that this hyperphosphorylated protein was p130Cas (using Cas antibodies) and that YopHC403S and p130Cas bind one another (illustrated by CoIP experiments).

Black and Bliska (1997) showed that an increased amount of p130Cas becomes localized to FAs when YopHC403S is expressed in comparison to uninfected cells. Note that uninfected cells did, however, exhibit some FA localization of p130Cas and that the uninfected cells shown in the example were less well-spread than the infected counterparts. Expression of WT YopH led to rapid dephosphorylation of p130Cas on tyrosine residues (Black and Bliska, 1997). These data, therefore, suggest that *Yersinia* can employ YopH to dephosphorylate p130Cas and that this has a negative effect on the ability of p130Cas to be targeted to FAs. Further explanation of how targeting p130Cas for dephosphorylation benefits *Yersinia* was provided when authors discovered that p130Cas/Crk signaling mediates the uptake of *Yersinia* by epithelial cells (Weidow et al, 2000).

Cancer

Enhanced cell migration, invasion, cell survival, and/or proliferation have been correlated to p130Cas expression and SD phosphorylation in a large number of cancer types (see Tikhmyanova et al (2010) and Table. 2 for a review of p130Cas involvement in various cancers). Within the context of breast cancer, however, interest in

Table 2: Implications for p130Cas signaling in various cancer types

Cancer Type	Process implicated	References
Breast	Invasion	(Alexander et al, 2008)
	Cell proliferation and invasion	(Wendt et al, 2009)
	Cell proliferation	(Cabodi et al, 2004)
		(Cabodi et al, 2006)
	Cell survival	(Soni et al, 2009)
	Cell migration and invasion	(Cunningham-Edmondson and Hanks, 2009)
	Tamoxifen-resistance	(van der Flier et al, 2000)
		(Brinkman et al, 2000)
		(Riggins et al, 2006)
Colon	Survival	(Beinke et al, 2003)
		(Casanova et al, 2006)
	Proliferation	(Yu et al, 2004)
Liver	Invasion/poor prognosis	(Guo et al, 2008)
Lung	Cell migration	(Achiwa and Lazo, 2007)
	Survival	(Beinke et al, 2003)
Melanoma	Invasion	(Nakashima et al, 2007)
	Invasion and proliferation	(Hamamura et al, 2005, 2008)
Multiple myeloma	Cell migration	(Podar et al, 2004)
Neuroblastoma	Invasion	(Frankel et al, 2008)
Stomach	Anchorage independent growth	(Noguchi et al, 2001)

p130Cas signaling and function has been the most extensive. One of the most common and effective treatments for breast cancer is an anti-estrogen drug called tamoxifen. However, patients can become resistant to this treatment (anti-estrogen resistant), requiring a change in their treatment regimens (Dorssers et al, 2001). Using retroviral insertion mutagenesis of a human estrogen-dependent breast cancer cell line ZR-75-1, Brinkman et al (2000) were able to confer anti-estrogen resistance to cells and map the integration sites of genes involved in tamoxifen resistance, including the human homolog of p130Cas. This discovery prompted the authors to rename the p130Cas

homolog: BCAR1 for "breast cancer anti-estrogen resistance gene 1". Studies addressing how BCAR1/p130Cas confers antiestrogen resistance have demonstrated a role for p130Cas in promoting cell proliferation and survival in breast cancer cells (Cabodi et al, 2004, 2006; Riggins et al, 2006; Soni et al, 2009). As is the case with many p130Cas signaling paradigms, SD tyrosine phosphorylation has been implicated in these processes (Soni et al, 2009; Cunningham-Edmondson and Hanks, 2009).

Culturing cells in tamoxifen-containing media demonstrated that BCAR1 overexpressing cell lines had a proliferative advantage over control cells (that did not express significant levels of BCAR1) despite the fact that ER regulated gene PS2 was similarly affected in BCAR1 overexpressers and control cells (Brinkman et al, 2000). These results suggest that tamoxifen resistance through BCAR1 occurs independently of ER-regulated gene expression. Further investigation with nearly 1000 breast tumor samples demonstrated that patients who express BCAR1 at higher levels become resistant to treatment with tamoxifen and have a greater chance of disease recurrence (van der Flier et al, 2000). Another research group showed that p130Cas is associated with ER-α in a non-genomic manner (Cabodi et al, 2004). Instead, p130Cas appears to associate with ER-α in a multi-molecular complex including Src and PI3-K upon estradiol treatment (Cabodi et al, 2004). The interaction between p130Cas and ER-α appears to be mediated by c-Src since p130Cas was not detected by CoIP with ER-α when c-Src is first IPed from lysates and thereby excluded from the CoIP (Cabodi et al, 2004). Kinase activity assays confirmed that overexpression of p130Cas in an estrogen receptor-positive breast cancer cell line after estradiol treatment increases c-Src activity, as well as ERK1/2 phosphorylation and cyclin D1 expression (Cabodi et al, 2004). Hence, it appears that p130Cas expression in estrogen positive breast cancer cells leads to formation of a protein complex with ER-α and c-Src to increase Src activity resulting in ERK1/2 phosphorylation and cyclin D1 expression. Since p130Cas overexpression is associated with tamoxifen resistance, these studies raise

questions as to whether or not tamoxifen may also induce these protein complex formations. This seems likely since studies have shown that tamoxifen treatment actually promotes phosphorylation of p130Cas, as well as FAK and Src (Cowell et al, 2006).

Neu/p130Cas transgenic mice display elevated activation of Src, ERK, and cyclin D1 in breast tissue during various stages of development and overexpression of p130Cas in the context of Her2/Neu positive breast epithelium leads to greater tumor burden and poorer chances of survival in those mice (Cabodi et al, 2006). Additionally, p130Cas may interact with EGFR to facilitate anti-estrogen resistance through STAT5b (Riggins et al, 2006). These results indicate the possibility of using Erb inhibitors as adjuvant treatments for anti-estrogen resistant breast cancer treatment. Indeed, overexpression of p130Cas may lead to other drug resistance problems as well, as adriamycin treatment (which promotes apoptosis) is greatly hampered by p130Cas overexpression, whereas use of single interfering RNA (siRNA) to knockdown p130Cas expression improves the ability of adriamycin to promote apoptosis (Ta et al, 2008). This mechanism appears to be Src, and not EGFR-dependent (Ta et al, 2008).

Independently of anti-estrogen resistance, p130Cas SD tyrosine phosphorylation has been implicated in the promotion of cell motility and invasion (Klemke et al, 1998; Huang et al, 2002; Shin et al, 2004; Brabek et al, 2005; Rivera et al, 2006; Cunningham-Edmondson and Hanks, 2009), while other studies have linked p130Cas coupling to Crk in cell survival during invasion (Cho and Klemke, 2000). Conversely, the disassociation of p130Cas and Crk in carcinoma cells, due to phosphorylation of Crk on Y221, has been implicated in promoting apoptosis (Kain et al, 2003).

While p130Cas does not appear to promote transformation by aberrant proliferation (Brabek et al, 2004) in Src-transformed cells, p130Cas does promote formation of invasive structures called podosomes as well as promoting proteolysis of the ECM by metalloproteases (Brabek et al, 2005). Mutation of SD tyrosines to phenylalanines

reduces the ability of Src transformation to induce invasion and podosome formation similarly to *Cas* -/- parental controls (Brabek et al, 2005). Mutation of the p130Cas SH3 and SBD domains has intermediate effects on invasion and podosome formation, whereas the SH3 mutant shows lower metalloprotease activity (Brabek et al, 2005). A previous study had found that using a C-terminal construct of p130Cas containing the SBD did not inhibit Src-transformation (Burnham et al, 1999). However, this study used cells that expressed endogenous p130Cas, possibly accounting for the discrepancies between the two studies.

Cell motility

p130Cas has long been associated with promotile signaling (Honda et al, 1998; Cary et al, 1998). DOCK180/ELMO has been implicated as a major GEF involved in Rac activation downstream of the p130Cas/Crk complex (Kiyokawa et al, 1998a,b). Using phagocytosis to model cytoskeletal rearrangement, Reddien and Horvitz (2000) demonstrated that Rac lies downstream of Crk and DOCK 180. Rac has furthermore been implicated in this promotile signaling by pseudopodia isolation wherein membrane protrusions that contained higher levels of p130Cas/Crk complexes also exhibited higher levels of Rac GTP (Cho and Klemke, 2000).

Not only do *Cas* -/- MEFs have a disrupted actin cytoskeleton as evidenced by phalloidin staining, but they are also defective in their ability to promote cell motility (Klemke et al, 1998) and adopt a well-spread morphology (Honda et al, 1999). The inability of knock-out cells to promote efficient cell motility (Honda et al, 1999; Shin et al, 2004) is attributed to a loss in SD phosphorylation as evidenced by the inability of p130Cas SD point mutants to rescue motility defects (Shin et al, 2004). Similarly, expression of a DN p130Cas whose SD has been deleted, suppresses motility in cells expressing endogenous p130Cas (Klemke et al, 1998). The interactions between SD pTyrs and SH2-adapter proteins Crk (Klemke et al, 1998; Chodniewicz and Klemke,

2004; Shin et al, 2004) and Nck (Rivera et al, 2006) have been identified as critical mechanisms driving p130Cas-mediated cell motility.

While tyrosine phosphorylation of p130Cas is necessary to enhance cell motility, the inability to dephosphorylate p130Cas at FAs may be equally detrimental. A role for the protein tyrosine phosphatase SHP-2 has been suggested in regulating cell motility in response to fibronectin and vitronectin (Inagaki et al, 2000). Expression of a catalytically inactive form of SHP-2 in Rat-1 fibroblasts leads to the formation of larger, more stable FAs, increased cellular adhesion and cell spreading, as well as enhanced tyrosine phosphorylation of both p130Cas and paxillin. Additionally, CHO cells expressing a mutant SHP-2, but not the WT demonstrated reduced motility rates after insulin stimulation (Inagaki et al, 2000).

It seems plausible that tyrosine phosphorylation of FA proteins such as p130Cas and cell motility correlate along a bell-shaped curve where excessive phosphorylation causes the cell to be too adherent due to the formation of prominent, stable FAs and too little phosphorylation inhibits cell motility due to a loss of branched actin filament assembly.

Rationale of the current study and presentation of the hypothesis

Since p130Cas appears to be phosphorylated at SD tyrosines primarily when it is localized to FAs, the localization of p130Cas to these sites appears critical to its ability to promote cell motility. Observation that the p130Cas CCH domain is the most highly conserved area of the protein suggests an important role for this domain. Further observations that this domain has some sequence similarity to the FAK FAT domain is suggestive of this domain having an FA targeting function (Arold et al, 2002). The research in this dissertation aims to answer the following questions: 1)

What contributions do the conserved N- and C-terminal domains make to the targeting of p130Cas to FAs and 2) What are the dynamics of p130Cas localization to FAs? Additionally, preliminary data on the structural features and alternate subcellular localizations of p130Cas will be presented.

CHAPTER II

THE MECHANISM OF P130CAS TARGETING TO FOCAL ADHESIONS

Introduction

A full understanding of p130Cas function requires knowing the mechanism by which it targets to FAs. Prior attempts to do so have led to contradictory conclusions about the importance of the SH3, SBD, and conserved C-terminus of p130Cas in mediating such targeting (Nakamoto et al, 1997; Harte et al, 2000). The possible role of the C-terminal region of p130Cas as an FA targeting domain is intriguing as there has been some speculation about the tertiary structure of this domain. Arold et al (2002) observed that this region has some sequence similarity to the FAK FAT domain, which folds into a bundle of four α-helices. To determine what the domain requirements of p130Cas are to regulate its FA targeting, I used microscopy techniques to visualize and quantify the FA localization of fluorophore-tagged p130Cas variants in live and fixed cells. In addition to elucidating these aspects of p130Cas FA targeting, the data further demonstrate that p130Cas FA localization plays an important role in mediating integrin signaling and cell motility. The importance of FAK in mediating the localization of p130Cas to FAs is also illustrated.

Materials and Methods

Cells and cell culture

Cas -/- MEFs were kindly provided by Hisamaru Hirai (University of Tokyo). *Paxillin* -/- MEFs were a gift from Sheila Thomas (UVA School of Medicine, Charlottesville, VA). MEFs were grown in Dulbecco's Modified Eagle Medium (DMEM)

also supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 1% antibiotic/antimicotic (Mediatech, Manassas, VA), 5 μg/ml plasmocin (InvivoGen, San Diego, CA), and 1% non-essential amino acids (Invitrogen, Carlsbad, CA). TetFAK cells were previously described (Owen et al, 1999) and were maintained in the media mentioned above, which was furthermore supplemented with 5 μg of tetracycline (Calbiochem, La Jolla, CA) per ml of media to repress FAK expression. FAK expression was induced by placing cells in complete media without tetracycline for 24 hours. Phoenix Ecotropic (E) viral packaging cells (a gift from Gary Nolan, Stanford University) were maintained in DMEM, supplemented with 10% heat-inactivated FBS, 1% antibiotic/antimicotic, and 1% non-essential amino acids.

Antibodies

Monoclonal mouse antibodies to total p130Cas and paxillin were obtained from BD Transduction Laboratories (San Jose, CA). Phospho-specific pCas polyclonal antibodies against p130Cas SD tyrosines 165, 249, and 410 were previously described by Fonseca et al (2004). Mouse monoclonal antibody to β-actin was obtained from Sigma-Aldrich (St. Louis, MO). Cy3-conjugated AffiniPure donkey anti-mouse IgG, Texas Red dye-conugated AffiniPure donkey anti-rabbit IgG, and horseradish peroxidase-conjugated goat secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Whole cell lysates were prepared from *Cas* -/- MEFs or TetFAK cells expressing various Venus-tagged p130Cas variants by scraping on ice for 10 minutes in modified radioimmunoprecipitation assay (RIPA) buffer (0.15 M NaCl, 50 mM Tris-HCl (pH 7.4), 1% Nonidet P40, 0.1% SDS, 1% deoxycholic acid, 5 mM EDTA, 50 mM NaF) supplemented w/ 100 μM Na$_3$VO$_4$ and 1% aprotinin. Whole cell lysates were sheared through 26 gauge needles to separate proteins from the membrane fraction.

For pCas immunoblotting, cells were treated 6 hours with 500 μM Na$_3$VO$_4$ (phosphatase inhibitor) before lysing in modified RIPA buffer. All lysates were boiled in 2X Laemmli buffer containing 250 μM Dithiothreitol (DTT) and were separated by 7% SDS-PAGE using 20 μg protein/lane. Nitrocellulose membranes were blocked for 1 hour in Tris-buffered saline (TBS) containing 5% milk. Blots were visualized by enhanced chemiluminescence using Western Lightning Luminol and Oxidizing Reagent (PerkinElmer Life Sciences, Inc., Boston, MA) using 1 minute incubations of membranes prior to developing Polaroid films. Tyrosine phosphorylation of p130Cas SD was quantified from scanned blots using ImageJ software to measure pixel intensities to compare pCas band intensity to total p130Cas (adjusted to actin loading).

Plasmid construction and protein expression of p130Cas variants

Standard PCR-mediated mutagenesis strategies were used to create p130Cas fusion proteins. pCS2-Venus-Cas-ATAA was engineered by changing the stop codon in pCS2-Venus to ATAA by PCR amplification resulting in a shift in the open reading frame (after the Venus-YFP coding region) so that the EcoR1 site could be used to insert a p130Cas coding sequence that would then be expressed with an N-terminal Venus-YFP tag. The PCR product was ligated into pGEM-T-Easy cloning vector. Positive clones were cut with BamH1/EcoR1 and the insert was placed into BamH1/EcoR1-cut pCS2-Venus. The p130Cas sequence was then ligated into the altered pCS2-Venus vector.

For C-terminal Venus yellow fluorescent protein (YFP) fusions, mouse p130Cas cDNAs were generated without a STOP codon and BamH1 restriction enzyme sites were introduced at the end of p130Cas coding regions. The WT sequence was PCR amplified from pSK-CAS2003 with sense (5′-AGC GCG CAA TTA ACC CCA CTA AAG-3′) and antisense (5′-GGT CCT CTG GGA TCC GGC AGC AGC-3′) oligonucleotide primers. CASΔSH3 cDNA was generated from pGEX-CASΔSH3 using PCR

amplification with sense (5′-AAC GCC GGA TTC GCG-3′) and antisense (5′-GGT CCT CTG GGA TCC GGC AGC AGC-3′) oligonucleotide primers. CASΔCCH cDNA was generated by PCR amplification from pSK-CAS2003 using sense (5′- GCA ATT AAC CCT CAC TAA AGG GCG AAC CGT-3′) and antisense (5′-CCG GCC GGA TCC CAG GGG CTG GGG CTG GTG T-3′) oligonucleotide primers to exclude the last 141 amino acids of p130Cas. All sequences were ligated into pGEM-T-Easy to create pGEM-WT-CAS-gga, pGEM-CAS-ΔSH3, and pGEM-CASΔCCH, respectively. To obtain pGEM-CAS-ΔSH3/ΔCCH, pGEM-CAS-ΔSH3 and pGEM-CASΔCCH plasmids were cut with Sph1 and Sal1 restriction enzymes. The Sph1-Sal1 fragment containing the SH3 deletion was ligated with the Sal1-Sph1 fragment containing the CCH mutation and the pGEM backbone fragment (Sph1-Sph1) of pGEM-CAS-ΔCCH.

PGEM-CAS plasmids were digested with BamH1 and ligated into pCS2-Venus vector (Nagai et al, 2002) using the BamH1 restriction digest sites in the vector poly-linker region such that Venus-YFP was fused to the C-terminus of p130Cas. To create LZRS-MS-IRES-zeo-Cas-Venus plasmids, the pCS2-Cas Venus plasmid was cut by EcoRI restriction digestion. The p130Cas-Venus inserts were then ligated into EcoR1-cut LZRS-MS-IRES-zeo plasmid (a gift from Al Reynolds, Vanderbilt University), which was previously dephosphorylated by treatment with calf intestinal phosphatase.

Stable expression of untagged p130Cas variants co-expressing green fluorescent protein (GFP) from a separate transcript was achieved through retroviral transduction from LZRS-MS-IRES-GFP (Ireton et al, 2002) plasmids. LZRS-Cas-WT(GFP) and LZRS-Cas-ΔSH3(GFP) were previously described (Shin et al, 2004; Fonseca et al, 2004). To engineer p130Cas lacking the CCH domain with a stop codon before the invariant proline, a T3 forward primer and the following oligonucleotide primer were used to amplify the sequence from pSK-Cas-2003 with flanking EcoR1 sequences: 5′-

TGA AGG CCC GAA TTC CCC TGT CCG GCC CTA CAC CAG - 3′. The same reverse primer was used to engineer the SH3/CCH mutant cDNA, using the following forward oligonucleotide primer to amplify the sequence from pGEX-3X-Cas-ΔSH3: 5′-AAC GCG GAT TCC CCG TCG CG -3′. These products were ligated into pGEM-T Easy and positive clones were digested with EcoR1 for ligation into EcoR1-cut LZRS-MS-IRES-GFP (previously dephosphorylated by calf intestinal phosphatase).

Plasmid mCherry-C1-paxilllin was generated to provide an FA marker for use in live cell studies. The coding region of paxillin-α was subcloned into plasmid mCherry-C1 (provided by Maria Nemethova, Vienna, Austria) from pGEX-2T-paxillin-α (provided by Hajime Yano, Osaka, Japan), such that paxillin is expressed with an N-terminal mCherry tag. In order to do so, pGEX-2T-paxillin-α was cut with BglII and EcoRI enzymes and the smaller fragment was ligated into mCherry-C1 vector cut with BamH1 and EcoR1. Plasmid mCherry-C1-zyxin was a gift from Irina Kaverina (Vanderbilt University, Nashville, TN). All plasmid sequences were confirmed before use.

Stable expression of p130Cas variants in *Cas* -/-, TetFAK, and *paxillin* -/- MEFs was achieved by retroviral infection using viral supernatants of Phoenix ecotropic (E) packaging cells transfected with LZRS-Cas-Venus plasmids or LZRS-Cas(GFP) plasmids. Phoenix E cells were transfected by calcium phosphate transfection of 60 mm culture dishes of cells plated at $\sim$60% confluence. Five to ten minutes prior to transfection, cells were treated with 25 μM chloroquine. The following transfection mixture was made:

8 μg DNA

62 μl of 2 M CaCl$_2$

sterile dH20 to 500 μl volume

then 500 μl 2X HBS (50 mM HEPES, 10 mM KCl, 12 mM dextrose, 280 mM NaCl,

1.5 mM Na$_2$PO$_4$, pH 7.7)

The mixture was pipetted vigorously to produce bubbles for 15 seconds and then applied to the dish containing the chloroquine-treated Phoenix E cells. Transfection proceeded for 7 hours at 37°C after which the media was replaced and cells were allowed to recover for 48 hours .

Forty-eight hours after transfection, Phoenix E cells were shifted to the permissive temperature (33°C) to produce virus. After overnight incubation, viral supernatants were taken from the cells, which were subsequently spun down and filtered through a 0.45 μm syringe filter. Phoenix E cells were shifted back to 37°C and then subjected to puromycin selection with 5 μg puromycin per ml of media (2 weeks after selection, more viral supernatants were obtained). Before viral supernatants were added to *Cas* -/- MEFs, polybrene was added to the filtered supernatants at a concentration of 4 μg/ml. Once viral supernatants were added to cells, they were shifted to 33°C for 6 hours to promote infection. Three rounds of infection with the viral supernatants were performed. For p130Cas(ΔSH3) tagged and untagged variants, it was necessary to select three clones of higher expressers to produce a clonal population that expressed at levels equivalent to sorted cell populations (prior attempts at achieving equivalent expression using cell sorting failed due to a rapid loss of expression). *Paxillin* -/- cells expressing p130CasVenus(WT) were analyzed after infection prior to sorting. All other p130CasVenus and untagged p130Cas cell populations were further selected by fluorescence activated cell sorting (FACS) for YFP and GFP, respectively, to obtain a population in the lower half of the fluorescence ranges.

After infection and sorting for low levels of Venus-YFP, cells were periodically maintained in media containing 10 μg/ml zeocin (Invitrogen) to assure continued expression from the bicistronic transcript that includes the bleomycin resistance gene expressed from the internal ribosome entry site. Transient expression of mCherry-paxillin and mCherry-zyxin was achieved by transfection with Lipofectamine LTX according to manufacturer's instructions (Invitrogen, Carlsbad, CA) using 12 μl of

reagent to 3 μg of DNA in OptiMEM media (Invitrogen, Carlsbad, CA). Cells were incubated for 16 hours in transfection media after which cells were placed in normal DMEM supplemented as indicated above in "Cells and Cell Culture". For Venus-p130Cas(WT), Lipofectamine 2000 reagent was used instead and the incubation time was lessened to 7 hours.

Wide-field fluorescence microscopy

Cas -/- MEFs transiently transfected with pCS2-Venus-Cas-ATAA were plated on glass coverslips coated with 10 μg/ml fibronectin. Live cells were mounted for microscopy using PBS as an interface between coverslips and glass slides. To reveal p130Cas tyrosine phosphorylation by immunostaining experiments, cells were grown on glass coverslips coated with 10 μg/ml fibronectin and fixed in 2% paraformaldehyde dissolved in a buffer containing 20 mM PIPES (pH 7.1), 127mM NaCl, 5mM KCl, 1.1mM NaH_2PO_4, 0.4 mM KH_2PO_4, 2mM $MgCl_2$, 5.5 mM glucose, and 1mM EGTA. Cells were permeabilized for 10 minutes in 0.4% Triton-X in phosphate buffered saline (PBS). After blocking for 1 hour with 1% bovine serum albumin (BSA) in PBS, the coverslips were placed in a primary antibody solution with phosphoCas-Y165 antibody with detection by Texas Red dye-conjugated secondary antibody. Coverslips were kept in the dark once secondary antibody was added to prevent fluorophore degradation. The coverslips were mounted in Vectashield Mounting Media (Vector Labs, Burlingame, CA).

Images of live fluorescent cells were captured by QED Capture software (Media Cybernetics, Silver Spring, MD) through a 60X/1.40 oil immersion lens, whereas as immunostained cells were imaged a through a 100X/1.40 oil immersion lens on an upright Nikon Eclipse 80i microscope (Nikon, Melville, NY) equipped with Nikon Epifluorescence D-FL attachments. Fluorophores were excited by an EXFO X-Cite

120 fluorescence illumination system (EXFO, Quebec, Canada) using Nikon C-FL G-23/C TRITC and C-FL B-2E/C FITC filter cubes. After capture, TIFF images were imported into Adobe Photoshop CS (Adobe, San Jose, CA) and the autocontrast function was used to adjust the images.

TIRF imaging of FAs in live cells

Cas -/- MEFs expressing p130CasVenus variants were transiently transfected with plasmid mCherry-C1-paxillin, and then grown to confluence on coverslips coated with 1 μg/ml fibronectin (from human plasma, Sigma-Aldrich, St. Louis, MO). Two hours prior to analysis, scratch wounds were prepared by creating denuded areas using a pipette tip. Media was aspirated from these cells to remove cellular debris and fresh media was applied, which was furthermore supplemented with 10mM HEPES to maintain media pH when cells were placed outside of the incubator on the microscope stage. Shortly before microscopy analysis, wounded coverslips were mounted into a chamber containing this same media, which was furthermore covered with a clean coverslip to prevent debris from entering the chamber. The temperature of this chamber was maintained by a heater (at 37°C). Cells migrating into the wound and expressing both fluorophores to appropriate levels were chosen for analysis of dynamic FAs using total internal reflection fluorescence (TIRF) microscopy, which was performed on a Nikon ECLIPSE TE2000-E inverted microscope equipped with a Perfect Focus System and a TIRF 100X/1.49 NA oil-immersion lens. Fluorophores were excited with an 18 mW argon laser (Melles Griot) and 10 mW DPSS laser 85YCA010 (Melles Griot, Albuquerque, NM). A custom-made double-dichroic TIRF mirror and emission filters (Chroma Technology, Rockingham, VT) in a Ludl filter wheel (Ludl, Hawthorne, NY) were used to make two-color movies at 25 second intervals between frames for a total of 180 frames.

Images were captured by a back-illuminated EM-CCD camera Cascade 512B

(Photometrics) driven by IPLab (Scanalytics, Fairfax, VA) software. Images were imported into ImageJ (NIH – open source software), where file type was converted to 8-bit grayscale. Background (non-cellular) was zeroed on each image by adjusting the lower limit of the display range using the brightness/contrast function in ImageJ.

Analysis of p130Cas localization to FAs in fixed cells

Cells expressing p130CasVenus variants were fixed, immunostained for paxillin as an FA marker, and visualized by confocal spinning disk fluorescence microscopy. Cells growing at low density on coverslips coated with 10 μg/ml fibronectin (from human plasma, Sigma-Aldrich, St. Louis, MO) were fixed for 30 minutes in 2% paraformaldehyde (in a buffer containing 20 mM PIPES (pH 7.1), 127mM NaCl, 5mM KCl, 1.1mM NaH_2PO_4, 0.4 mM KH_2PO_4, 2mM $MgCl_2$, 5.5 mM glucose, and 1mM EGTA), and then permeabilized for 10 minutes in 0.5% Triton-X in PBS. After blocking for 1 hour with 1% BSA in PBS, the coverslips were immunostained for paxillin with detection by Cy3-conjugated secondary antibody. Coverslips were kept in the dark once secondary antibody was added to prevent fluorophore degradation. The coverslips were mounted in Prolong Gold Mounting Media (Invitrogen).

Confocal images were captured at a single 0.2 μm slice depth (at the most appropriate ventral plane for visualizing FAs) with a Yokogawa QLC-100/CSU-10 spinning disk head attached to a Nikon TE2000E microscope using a 100X/1.4 oil Plan Apo objective lens (Nikon), and a back-illuminated EM-CCD camera Cascade 512B driven by IPLab software. Two-color excitation was achieved by a krypton-argon laser (75 mW 488/568 from Melles Griot with AOTF control). Custom-made double dichroic mirror and filters (Chroma Technology) were used in a Ludl filter wheel. Images were processed to remove non-cellular background as described above. These slices were then inverted in order to locate areas of robust paxillin staining for analysis. From these robust areas of staining, 50 FAs (10 each from 5 separate cells) were selected

for each experimental population. Masks of the selected FA sites were created based on paxillin-delimited boundaries. The masks were then superimposed onto the corresponding images in the green emission channel to obtain measurements of the mean Venus-YFP fluorescence within the FAs. Non-FA-associated Venus-YFP fluorescence was subtracted from these values, based on measurements of single, adjacent cellular areas using the same FA masks. Calculations were performed in Microsoft Excel and statistical significance was analyzed using Excel T-Test function to perform an unpaired, two-tailed, Student's t-test.

Cell motility assay

Scratch wounds were created, as described above, in confluent cell monolayers on coverslips coated with 1 μg/ml fibronectin and analyzed by differential interference contrast (DIC) microscopy. The wounded monolayers were then placed in complete DMEM further supplemented with 10 mM HEPES, mounted into a 37°C heating chamber on an inverted Nikon ECLIPSE TE2000-E2 microscope, and visualized through a 20X Plan Apo DIC objective lens. Frames were captured every 5 minutes for 8 hours on a CoolSnapHQ camera (Photometrics) using IPLab software. Sixty representative cells at wound margins (12 each from 5 independent wounds per experimental population) were followed using the ImageJ manual tracking function. The mean total distances traveled per hour were calculated, starting at 2 hours after wounding. The average total distance traveled per hour was calculated after 5 and 8 hours migration after wounding (meaning that each movement was tracked from frame to frame and summed, then divided by the total number of hours migrated from hour 2). Statistical significance was determined using an unpaired, two-tailed, Student's t-test.

Development and characterization of fluorophore-tagged p130Cas

In order to study p130Cas localization in live and fixed cells, it was desirable to create a fluorescent fusion protein with good photo- and thermo-stable properties. Since the discovery of green fluorescent protein (GFP), many fluorescent proteins have been discovered and the properties of GFP have also been altered by mutagenesis strategies to alter the properties of the protein. Due to such advances, a yellow fluorescent protein (YFP) called Venus, with superior properties for live cell imaging was identified (Nagai et al, 2002). Initial experiments with an N-terminal Venus-YFP fusion of the WT p130Cas protein, Venus-p130Cas(WT), resulted in an aberrant localization of p130Cas wherein the protein was primarily localized to the nucleus and appeared to be excluded from FAs (Fig. 5). For this reason, the N-terminal fusion protein was found unsuitable for further analysis.
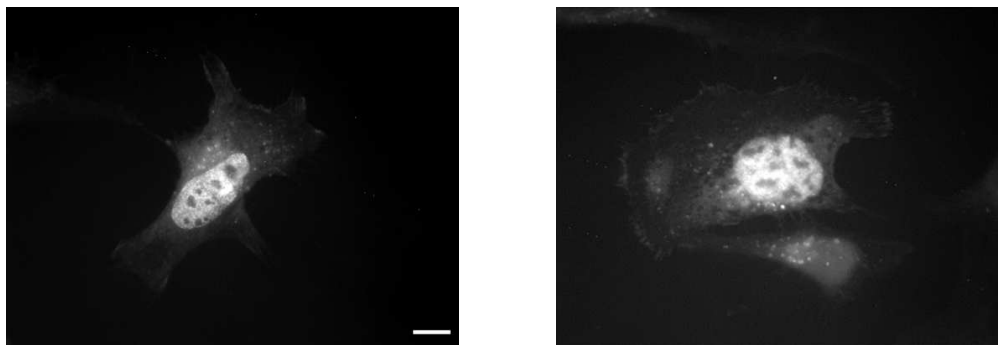

Figure 5: Fusing Venus-YFP to the N-terminus of p130Cas results in an aberrant subcellular localization. A plasmid expressing p130Cas with an N-terminal Venus-YFP tag was transiently transfected into *Cas* -/- MEFs. Live cells plated on the coverslip were gently pressed against a glass slide in the presence of PBS. Microscopy was performed using a 60X lens on a wide-field fluorescence microscope. Scale bar is 10 μm.

Placing the Venus-YFP tag on the C-terminal end of the protein eliminated the aberrant localization to the nucleus. By wide-field fluorescence microscopy, p130Cas-Venus(WT) could be seen localizing to structures that appeared to be FAs and in

a large cytoplasmic pool (see Fig. 6), consistent with previous studies of p130Cas localization (Nakamoto et al, 1997; Harte et al, 2000; Fonseca et al, 2004; Ballestrem et al, 2006).
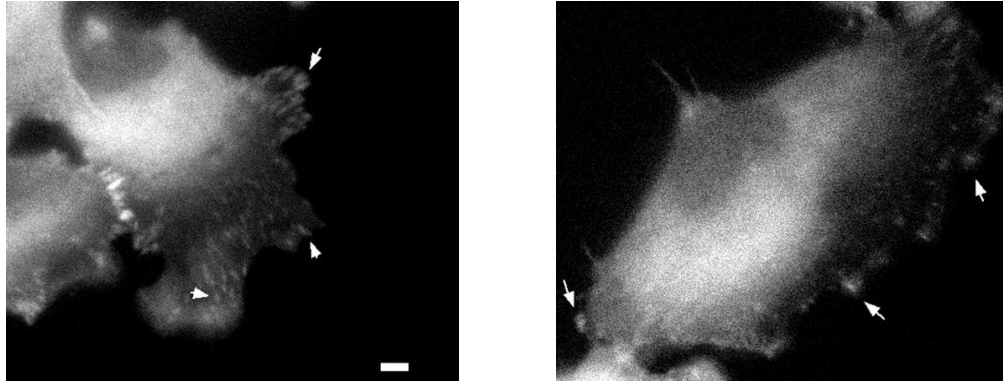

Figure 6: Using a C-terminal Venus-YFP tag results in normal subcellular localization of p130Cas. *Cas* -/- MEFs expressing p130Cas fused to a C-terminal Venus-YFP tag were plated on fibronectin-coated coverslips and visualized by live wide-field fluorescence microscopy using a 100X objective lens. Areas with FA-like appearance are indicated with arrows. Scale bar is 10 μm.

Further studies with p130CasVenus(WT), wherein mCherry-paxillin was used as a marker of FAs, illustrated that the protein localized to FXs (Fig. 7) as well as larger FAs at the leading and trailing edge of cells. In order to visualize these structures, I employed total internal reflection fluorescence (TIRF) microscopy, since the large cytoplasmic pool of p130Cas often obscured such sites in as is seen by wide-field fluorescence imaging.

Expression of p130Cas variants tagged with fluorophore Venus-YFP

To study the mechanism p130Cas localization to FAs, mouse p130Cas variants tagged at their C-terminal ends with the fluorophore Venus-YFP were stably expressed to similar levels in *Cas* -/- mouse embryo fibroblasts (MEFs) using a retroviral vector. Cells were sorted for low levels of YFP expression using fluorescence activated cell sorting (FACS). C-terminally tagged p130Cas variants included WT,
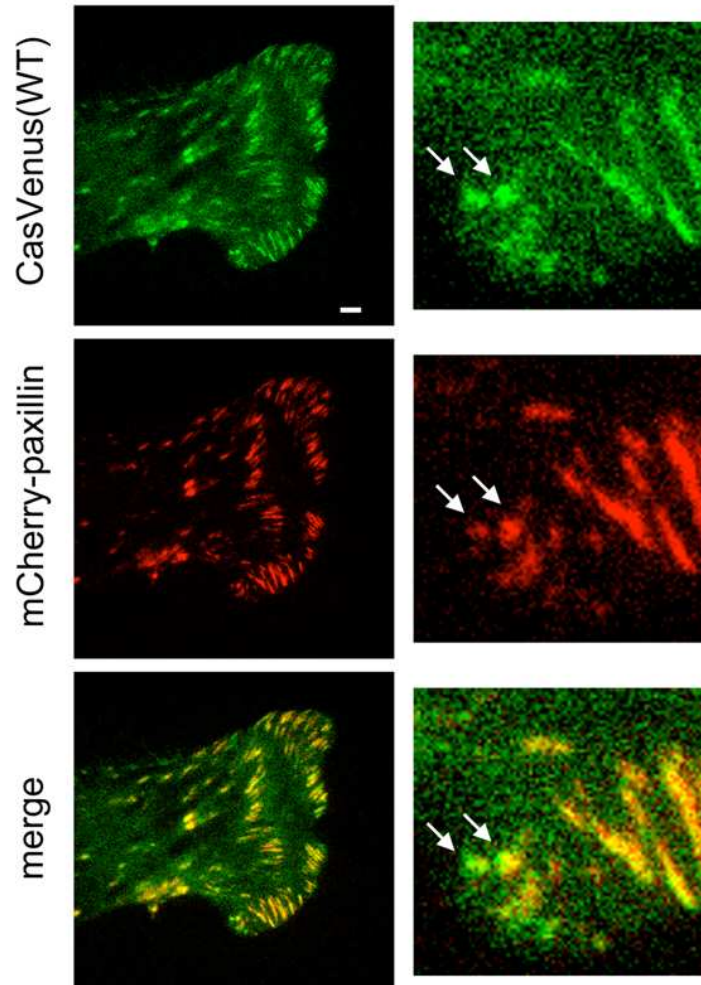
Figure 7: p130CasVenus(WT) localizes to FXs. *Cas* -/- MEFs expressing p130CasVenus(WT) plated on fibronectin-coated coverslips were transiently transfected with mCherry-paxillin to mark FAs and FXs and were then visualized by live TIRF microscopy. Arrows indicate FXs. Scale bar is 10 μm.
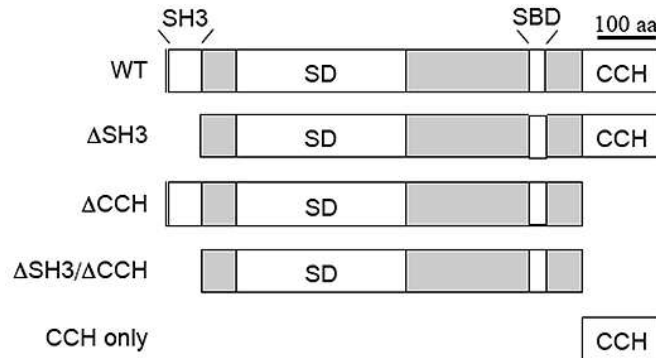
ΔSH3, ΔCCH, ΔSH3/ΔCCH, and CCH only (Fig. 8).

Figure 8: p130Cas deletion mutants. Indicated are positions of the N-terminal SH3 domain, the substrate domain (SD), the Src-binding domain (SBD), and the Cas-family C-terminal homology (CCH) domain. To study subcellular localization, the variants were expressed with Venus-YFP fused to their C-termini.

Based on sequence conservation among members of the Cas family, including a distant relative from Drosophila, the Cas-family C-terminal Homology (CCH) domain was defined as the 141 amino acid residues at the C-terminus, beginning with an invariant proline (Fig. 9).

Within the p130Cas CCH domain are three 25-30 amino acid stretches strongly predicted to adopt alpha-helical secondary structure (Fig. 9). While these predicted helical regions correspond to three of the four helices of the FAK FAT domain, β-strand content is predicted for a highly conserved stretch in the p130Cas CCH domain that would correspond to the other FAK FAT helix (Fig. 9).

Immunoblotting of total cell lysates confirmed that p130CasVenus variants are expressed to roughly equivalent levels and have the expected SDS-PAGE mobilities (Fig. 10, top panel).

The signaling capacities of the variants was assessed by immunoblotting with a mixture of three pCas phospho-specific antibodies that recognize phosphorylated YxxP tyrosines in the p130Cas SD (Fonseca et al, 2004). To better enable detection

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                9998888760089999999999999999999999999998865057887358744773589973452542030
                -----------HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH------EEEE----EEEEEE--EEEE----
p130Cas  PGRTGGLGPSDRQLLLFYLEQCEANLTTLTDAVDAFFTAVATNQPPKIFVAHSKFVILSAHKLVFIGDTL
mHEF1    PTTNNSVGAQDRQLLCFYYDQCETHFISLLNAIDALFSCVSSAQPPRIFVAHSKFVILSAHKLVFIGDTL
EFS      PEEPLVLSTGDLQLLHFYAGQCQSHYSALQAAVAALVASTQANQPPCLFVPHGKRVVVAAHRLVFVGDTL
DmCAS    PCKSPEMTDKDKKLVRYYAQQISTHMGNLMQAIDSFLETVEKNQPPKFFIAYGKFVVVSAHNLVTIGDNV
HEPL     PLTSPSPSGQNTERKIHLSKHSRLYFGALFKAISVFASSLSNGQPPEVFITQSKLVITVGQKLV---DTL


                0176628999999999999999999999999999974088880789999999999999999999999999887389
                -HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH-----HHHHHHHHHHHHHHHHHHHHHHHHHHHHHH--
p130Cas  SRQAKAADVRSQVTHYSNLLCDLLRGIVATTKAAALQYPSPSAAQDMVDRVKELGHSTQQFRRVLGQLAAA*
mHEF1    TRQVAAQDIRNKVRNSSNQLCEQLKTIVMATKMAALHYPSTTALQEMVHQVTDLSRNAQLFKRSLLEMATF*
EFS      GRLAASAALRAQVGAAGTMLAQTLRATVLAVKGAALGYPSDTAVQEMARCVAELAGQALRFTTLLAGLLP*
DmCAS    YRNISKEALREKILRCTNALSDALKTCVLKSKKAAAHFPSGSAVQEMVDSVVHINNLARDLKSVMLQAVHL
HEPL     CSETQEKDERNEILCGSSHLCGLLKDLALATKSAVIQYPSPSALSLLQSEVERLEHHSRKFRDTLE*
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Figure 9: Cas-family C-terminal homology domains. Shown in the amino acid sequence alignment are mouse p130Cas residues 734-874 (UniProt ID Q61140), mouse "Human Enhancer of Filamentation 1" residues 693-833 (mHEF1, UniProt ID O35177), mouse "Embryonal Fyn-associated substrate" residues 421-560 (EFS, UniProt ID Q64355), *Drosophila melanogaster* p130Cas homolog residues 683-823 (DmCas, UniProt ID A4IJ58), and mouse "HEF1-EFS-p130Cas-like" residues 672-804 (HEPL, UniProt ID Q08EC4). C-termini are indicated by asterisks. Positions of amino acid identity have a black background, while other well-conserved positions have a grey background. A secondary structure prediction for the p130Cas CCH domain, made using Jpred 3 (Cole et al, 2008), is shown at the top of the alignment, with α-helices indicated by HHH and β-strands by EEE. The numbers ranging from 0 to 9 indicate prediction reliability, with larger numbers indicating greater reliability.
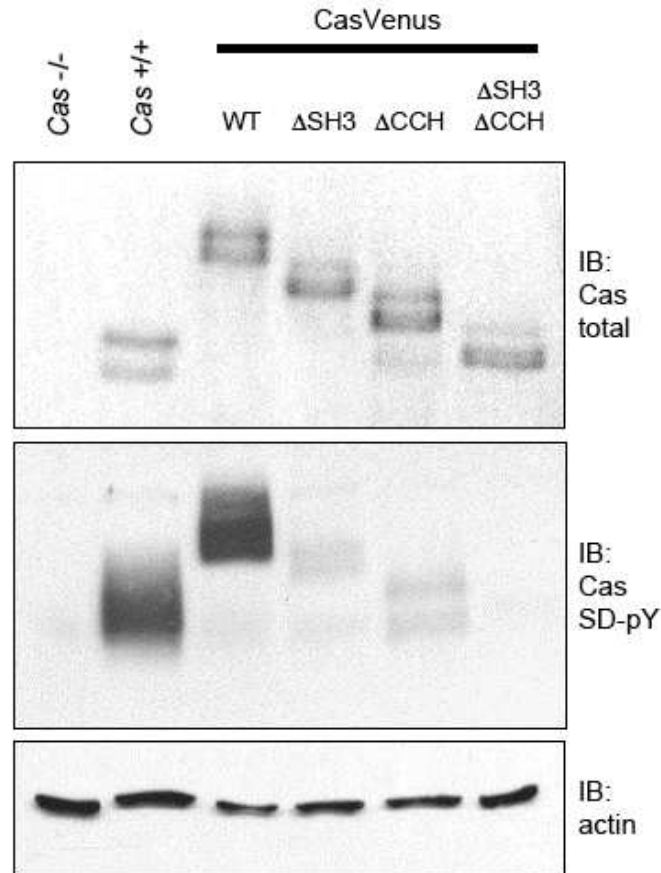
Figure 10: Venus-YFP tagged p130Cas variants lacking either the SH3 or CCH domain are deficient in SD phosphorylation. Immunoblot (IB) analysis was carried out on whole cell lysates from *Cas* -/- MEFs reconstituted with either p130CasVenus(WT), p130CasVenus(ΔSH3), p130CasVenus(ΔCCH), or p130CasVenus(ΔSH3/ΔCCH). Parental *Cas* -/- MEFs and normal *Cas* +/+ MEFs were included as controls. Blotting with an antibody for total p130Cas indicated that the variants were expressed to equivalent levels (top). SD tyrosine phosphorylation was assessed, from vanadate-treated cells, using a mixture of pCas phospho-specific antibodies that recognize SD YxxP sites (middle). Actin was detected as a loading control (bottom).

of SD phosphotyrosines, cells were pretreated with Na_3VO_4 to inhibit tyrosine phosphatases. p130CasVenus(WT) achieved a level of SD tyrosine phosphorylation close to that of endogenous p130Cas (Fig. 10, middle panel).

However, deletion of either the SH3 or CCH domain significantly impaired SD tyrosine phosphorylation such that only faint pCas immunoreactivity could be detected, and only upon prolonged exposure of the blot (Fig. 10, middle panel). Thus both the SH3 and CCH domains are required for proper p130Cas signaling. By pCas Y165 (phospho-specific antibody to p130Cas tyrosine 165) immunostaining, phosphorylation of these p130Cas variants was readily seen at FA sites (Fig. 11). As was seen by immunoblotting of whole cell lysates, p130CasVenus(WT) appeared to be robustly phosphorylated at FAs, whereas the SH3 and CCH mutants exhibited less phosphorylation, with the SH3 mutant showing the greatest reduction in phosphorylation (Fig. 11). P130CasVenus targeting to FAs appeared to be reduced in mutants experiencing losses in tyrosine phosphorylation.

Both the SH3 and CCH domains are important for targeting p130Cas to FAs

The localization of p130CasVenus mutants was analyzed in live cells using paxillin as a control FA marker. Cells were transiently transfected to express paxillin tagged with the mCherry fluorophore. Paxillin is an established marker of FAs throughout their lifetime (Zaidel-Bar et al, 2003; Webb et al, 2004) and was therefore a desirable standard to which p130Cas could be compared. By total internal reflection fluorescence (TIRF) microscopy, p130CasVenus(WT) is observed to localize quite distinctly to FAs, with little Venus-YFP signal seen in adjacent ventral areas (Fig. 12). However, neither p130CasVenus(ΔCCH) nor p130CasVenus(ΔSH3) was able to achieve the same discrete FA localization. Rather, both of these deletion mutants became more broadly distributed in the ventral region of cells, with only modest enrichment
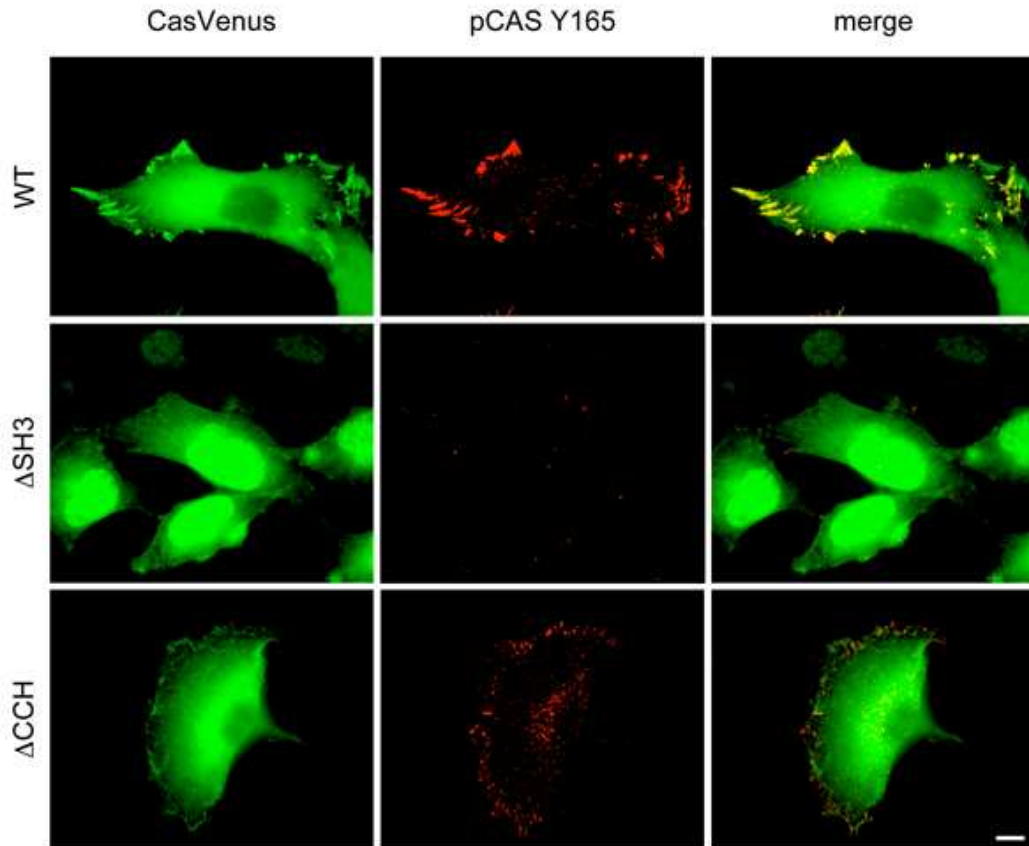
Figure 11: Tyrosine phosphorylation of p130Cas occurs at FAs and is reduced in SH3 and CCH mutants. The colocalization of p130CasVenus variants and p130Cas tyrosine phosphorylation was assessed by wide-field fluorescence microscopy. Immunostaining with a phospho-specific antibody to p130Cas Y165 served to mark tyrosine phosphorylated p130Cas. Representative images of cells expressing p130CasVenus(WT), (ΔSH3), and (ΔCCH) are shown. In the merged images, the green channel represents p130CasVenus (left panels), red identifies phospho-Cas staining with pY165 antibody (center panels), and yellow indicates areas of colocalization (right panels). Scale bar is 10 μm.

in and around the vicinity of FAs (Fig. 12 B-C). The CCH domain alone also exhibited this limited capacity for FA targeting, similarly to the ΔCCH and ΔSH3 variants (Fig. 12 D). The double mutant was excluded from these studies as cells expressing p130CasVenus(ΔSH3/ΔCCH) were difficult to distinguish by TIRF (since most of the fluorophore appeared in the cytoplasm and not in the ventral FA regions).

To further assess the mechanism of p130Cas targeting to FAs, the Venus-tagged variants, including the ΔSH3/ΔCCH mutant, were visualized at the ventral region of fixed cells using confocal microscopy. Paxillin immunostaining served to mark the FAs. By this method, the prominent FA localization of p130CasVenus(WT) was again evident, although cytoplasmic localization is more apparent than with TIRF microscopy (Fig. 13, top panels). The much weaker FA targeting of p130CasVenus(ΔSH3) and p130CasVenus(ΔCCH) was also apparent (Fig. 13, middle panels). For p130Cas-Venus(ΔSH3/ΔCCH), however, there was no FA localization (Fig. 13, bottom panels).

To quantify the FA targeting efficiencies of the variants, mean Venus fluorescence values were determined from measurements of 50 FAs, within the boundaries set by paxillin staining. Relative to p130CasVenus(WT), the FA targeting efficiency of p130CasVenus(ΔCCH) was reduced by ∼3-fold and ∼6-fold for p130CasVenus(ΔSH3) — see Fig. 14.

The difference in FA targeting between p130CasVenus(ΔCCH) and p130Cas(ΔSH3) was significant. The FA localization of p130CasVenus(ΔSH3/ΔCCH) was below the background level of fluorescence in adjacent areas (Fig. 14). Thus, it is clear that both the SH3 and CCH domains make important contributions to the proper FA targeting of p130Cas, and that no FA targeting is achieved in the absence of both domains.
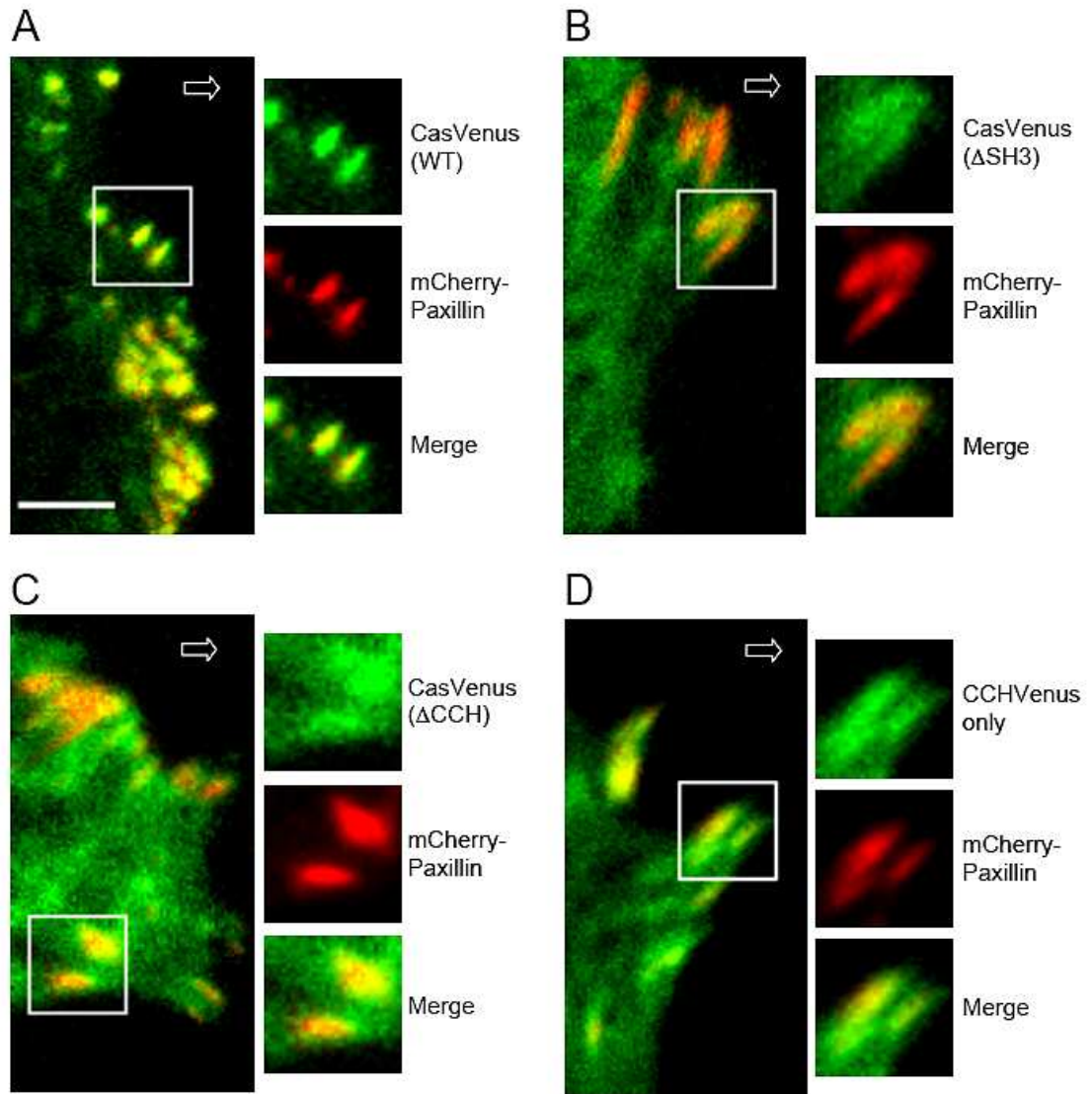
Figure 12: p130Cas mutants lack discrete localization to FAs. Live TIRF microscopy was used to assess the localization of p130CasVenus variants (WT, ΔCCH, ΔSH3, or CCH only) within the ventral plane of cells migrating from the edge of a wounded cell monolayer. To mark adhesion sites, cells were transfected to transiently express mCherry-paxillin. The larger panels show merged images of representative single cells at the wound margin, with arrows indicating direction of migration. Boxed areas enclosing well-defined FAs are shown slightly enlarged in adjacent panels that separately show the Venus, mCherry, and merged fluorescence. Note that both ΔCCH and ΔSH3 variants lack the discrete FA localization that is apparent for p130CasVenus(WT). Also note that the CCH only variant localizes poorly to FAs. Scale bar is 5 μm.
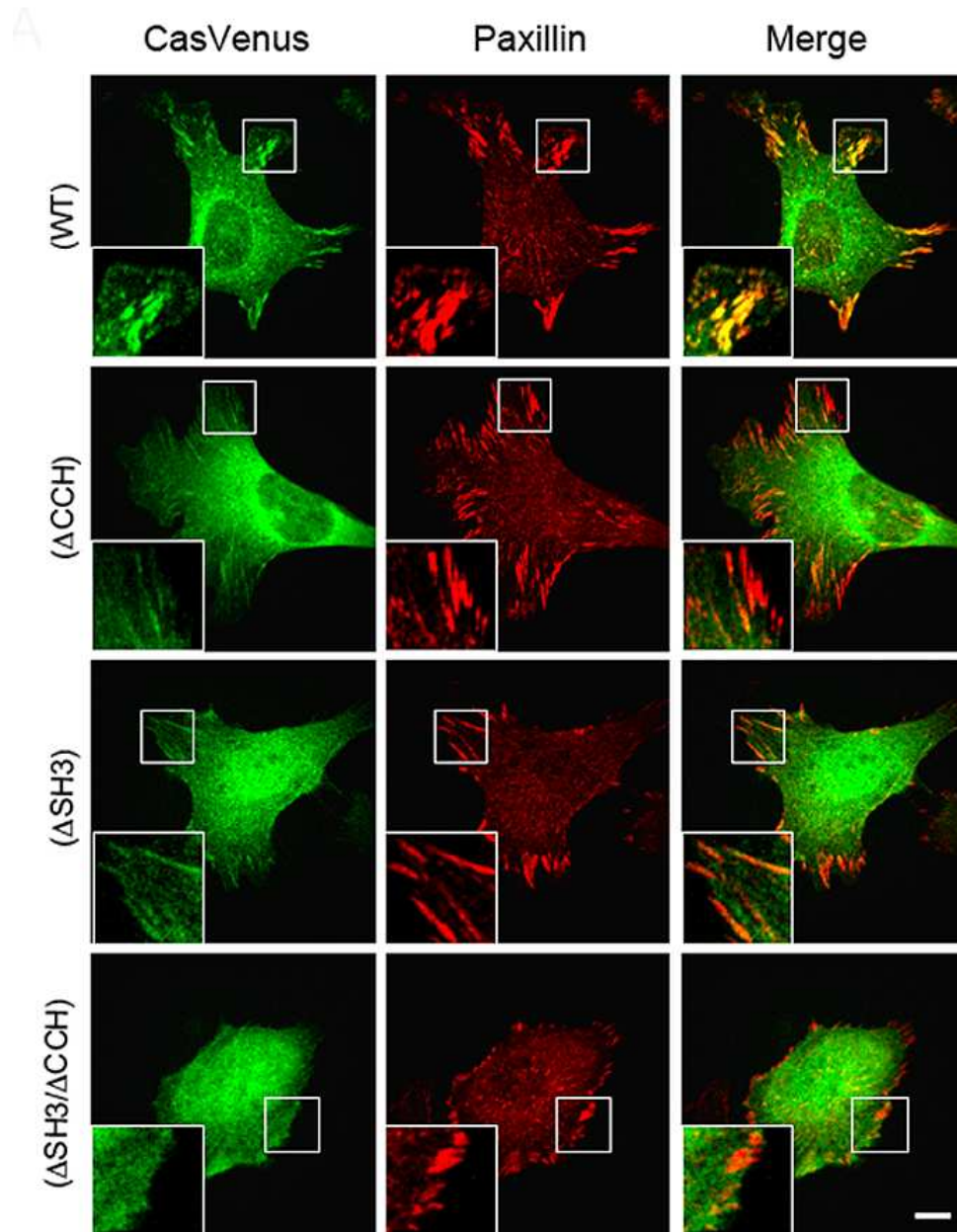
Figure 13: Both the CCH and SH3 domains play important roles in targeting p130Cas to FAs. The localization of p130CasVenus variants to FAs was assessed by fluorescence confocal microscopy at a single ventral slice. Immunostaining for endogenous paxillin served to mark FAs. Representative images of cells expressing the p130CasVenus variants: WT, ΔCCH, ΔSH3, or ΔSH3/ΔCCH. P130CasVenus fluorescence is shown as green (left panels), paxillin immunofluorescence as red (center panels), and colocalization as yellow in the merged images (right panels). Insets represent 2-fold enlargements of selected areas containing FAs. Scale bar is 10 μm.
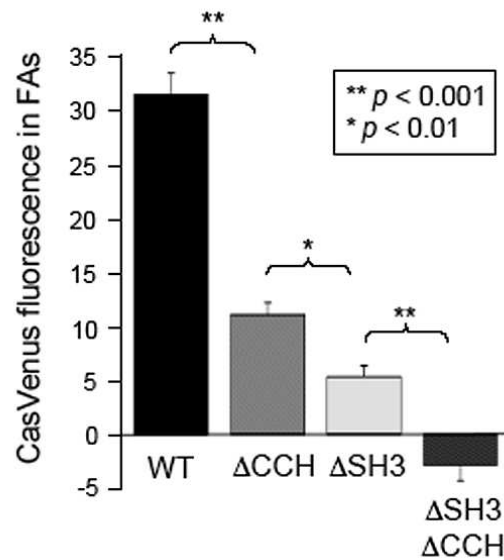
Figure 14: Deletion of either targeting domain significantly decreases p130Cas localization to FAs. Quantitative analysis of mean p130CasVenus fluorescence at FAs. Paxillin immunofluorescence was used to delineate FA borders, and mean Venus-YFP fluorescence values were measured from within the FA boundary. For each p130Cas variant, mean Venus-YFP fluorescence was determined from 50 FAs (10 each from 5 separate cells). Bars indicate SEM. Statistical significance was determined by Student's t-test.

FAK Has a Role in the FA Targeting of p130Cas

The finding that the SH3 domain is required for the proper targeting p130Cas to FAs suggests that the interaction of this domain with FAK may be involved in the process. To evaluate the role of FAK in targeting p130Cas to FAs, p130CasVenus(WT) and p130Cas(ΔCCH) were stably expressed in TetFAK cells, which are FAK -/- MEFs engineered for inducible FAK expression upon tetracycline withdrawal (Owen et al, 1999). The equivalent expression of the p130CasVenus variants in the TetFAK cells was confirmed by immunoblot analysis, under both non-induced and FAK-induced conditions (Fig. 15).
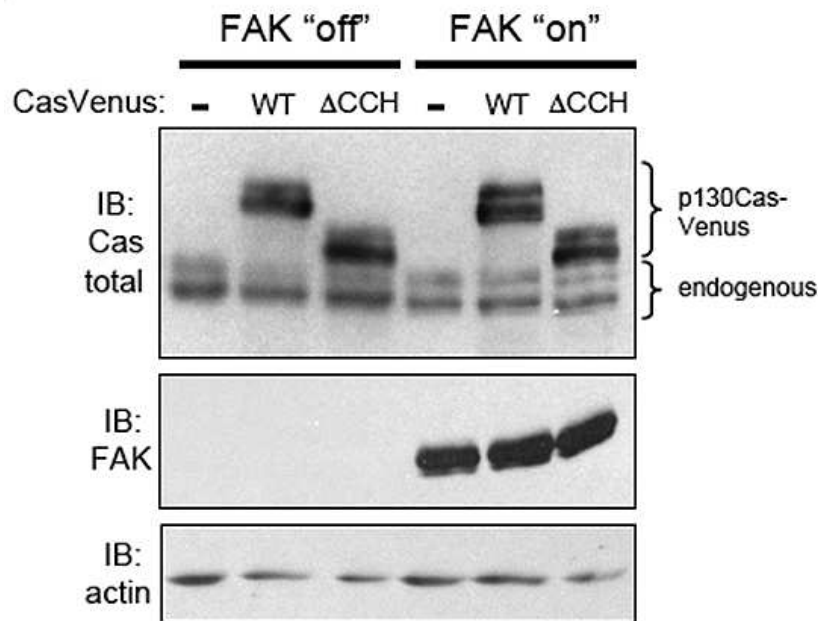
Figure 15: Expression of FAK and p130CasVenus in TetFAK cells. Immunoblot analysis of whole cell lysates from TetFAK parental cells and cells expressing either p130CasVenus(WT) or p130CasVenus(ΔCCH) that were either induced to express FAK or kept in the non-induced condition. Detection with a total p130Cas antibody (top) shows the expression of the two p130CasVenus variants in comparison to endogenous p130Cas. A total FAK antibody was used to confirm induced FAK expression (middle), while actin detection was used as a loading control (bottom)

The FA targeting efficiencies of the two p130CasVenus variants, both in the presence and absence of FAK, were quantified as described above from peripheral FAs at the leading edge of cells. FAK expression significantly increased FA targeting efficiency of both p130CasVenus(WT) and p130CasVenus(ΔCCH) (Fig. 16 and Fig. 17). For p130CasVenus(WT), the increase was $\sim$3-fold. Even in the presence of FAK, p130CasVenus(ΔCCH) localized very poorly to FAs and was $\sim$10-fold reduced in comparison to p130CasVenus(WT). In the absence of FAK, the weak FA targeting of p130CasVenus(ΔCCH) was no longer apparent. Thus, FAK has an important role in the FA targeting of p130Cas and without FAK, the FA targeting function achieved by the p130Cas SH3 domain is lost.

Both the SH3 and CCH domains are required for efficient promotion of cell motility by p130Cas.

Results presented above document the importance of the SH3 and CCH domains in p130Cas FA targeting and signaling. To extend these observations, we investigated the requirements for the SH3 and CCH domains in the ability of p130Cas to promote cell motility. For this investigation, p130Cas variants without the Venus tag were stably expressed in *Cas* -/- MEFs. By using a retroviral vector that co-expresses GFP from a bicistronic transcript, cell populations were sorted for low GFP expressers and populations were obtained that express p130Cas variants at levels comparable to the endogenous protein (Fig. 18, top panel). Vanadate treatment followed by immunoblotting with pCas antibodies revealed that the extent of SD tyrosine phosphorylation was reduced in all of the deletion mutants in comparison to WT (Fig. 18, middle panel).

SD tyrosine phosphorylation was barely detectible in the ΔSH3 and ΔSH3/ΔCCH mutants, while quantitative analysis indicated that SD tyrosine phosphorylation of the ΔCCH mutant was reduced by 50% in comparison to p130Cas(WT). The impairment
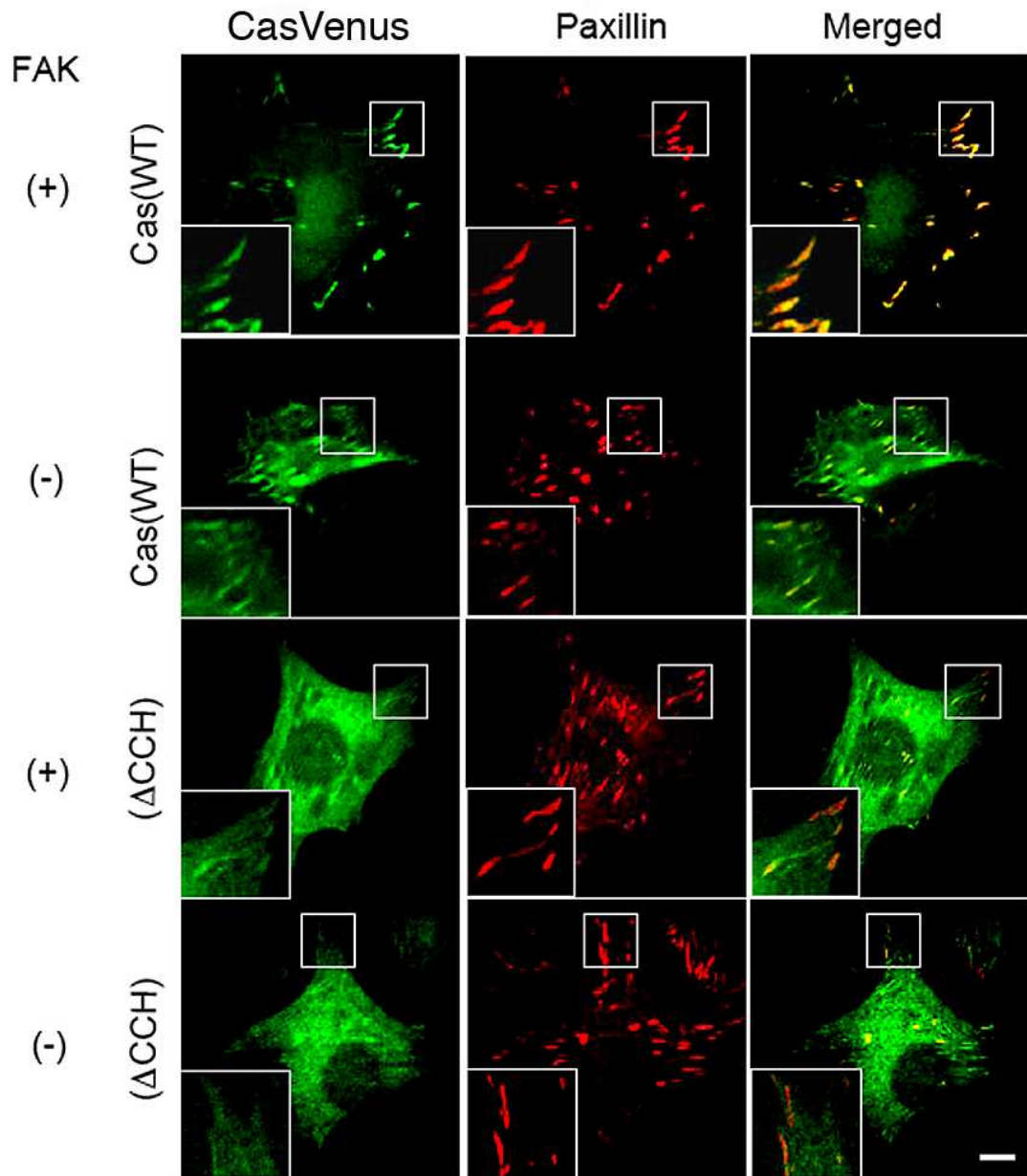
Figure 16: Confocal Images of TetFAK cells expressing p130CasVenus mutants. The localization of p130CasVenus variants to FAs was assessed by fluorescence confocal microscopy at a single ventral slice. Immunostaining for endogenous paxillin served to mark FAs. Representative images of cells expressing p130CasVenus(WT) and (ΔCCH) are shown in the presence (+) or absence(-) of FAK. p130CasVenus fluorescence is shown as green (left panels), paxillin immunofluorescence as red (center panels), and colocalization as yellow in the merged images (right panels). Insets represent 2-fold enlargements of selected areas containing FAs. Scale bar is 10 μm.
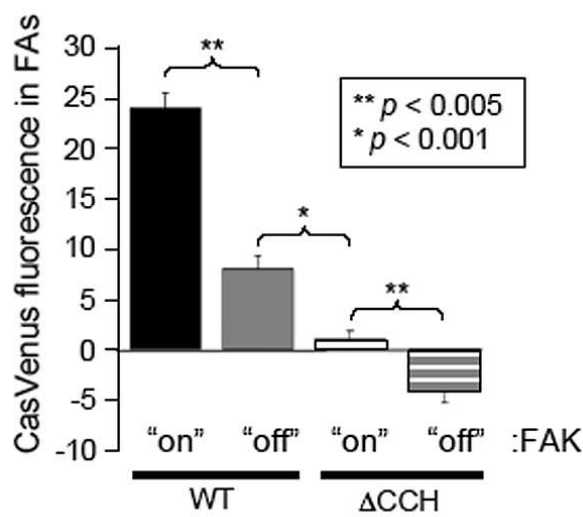
Figure 17: FAK mediates the localization of p130Cas to FAs through the SH3 domain. Quantitative analysis of mean p130CasVenus fluorescence at FAs. Paxillin immunofluorescence was used to delineate FA borders, and mean Venus-YFP fluorescence values were measured from within the FA boundary. For each p130Cas variant, mean Venus-YFP fluorescence was determined from 50 FAs (10 each from 5 separate cells). Bars indicate SEM. Statistical significance was determined by Student's t-test.
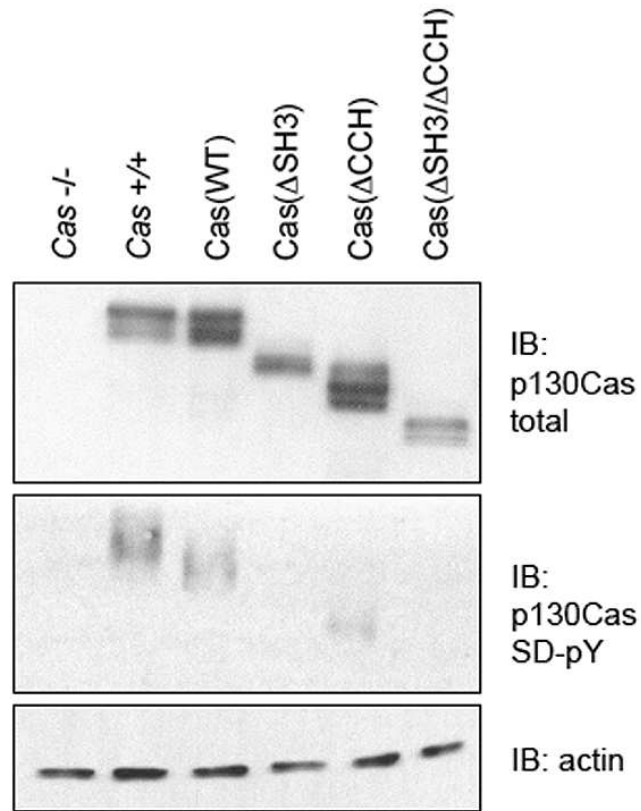
Figure 18: Both the SH3 and CCH domains are required for p130Cas signaling. Immunoblot analysis of whole cell lysates from *Cas* -/- MEFs reconstituted with untagged p130Cas(WT), p130Cas(ΔSH3), p130Cas(ΔCCH), or p130Cas(ΔSH3/ΔCCH). Parental *Cas* -/- MEFs and *Cas* +/+ MEFs were included as controls. Expression of the p130Cas variants was demonstrated using an antibody that recognizes both phosphorylated and unphosphorylated protein (top), while SD tyrosine phosphorylation was assessed using a mixture of pCas antibodies (middle). Prior to lysis, cells were treated with vanadate to enhance detection of SD phosphorylation. Actin was detected as a loading control (bottom).

of SD tyrosine phosphorylation observed for the ΔCCH mutant is not as striking as the defect observed for p130CasVenus(ΔCCH), indicating that the Venus-YFP tag has a limited negative effect on this signaling function. Nevertheless, it remains evident that the CCH domain has an important role in p130Cas SD signaling.

To study cell motility, scratch wounds were made in confluent cell monolayers and live, differential interference contrast (DIC) microscopy was used to monitor the movement of cells into wounds for determination of mean cell migration rates. Consistent with results from other studies (Huang et al, 2002; Shin et al, 2004), p130Cas(WT) expression significantly increased the cell migration rate as compared to parental *Cas* -/- cells (Fig. 19). Notably, the abilities of the deletion mutants to promote migration correlated with their extent of tyrosine phosphorylation. Thus, neither the ΔSH3 nor the ΔSH3/ΔCCH mutants promoted migration above the level observed for *Cas* -/- cells, while the ΔCCH mutant was not able to promote migration above the level of *Cas* -/- MEFs initially (at 5 hours), but eventually recovered the ability to migrate at an intermediate level to *Cas* -/- MEFs and p130Cas(WT) MEFs (Fig. 19).

As evident in the representative wounds shown in Fig. 20, many p130Cas(WT) cells are well separated from the margin at the 5 hour time point, whereas *Cas* -/- cells and cells expressing the signaling-deficient p130Cas mutants still maintain a uniform edge. Thus, in addition to promoting p130Cas FA targeting, both the SH3 and CCH domains are important for achieving proper SD tyrosine phosphorylation in order to promote cell motility.
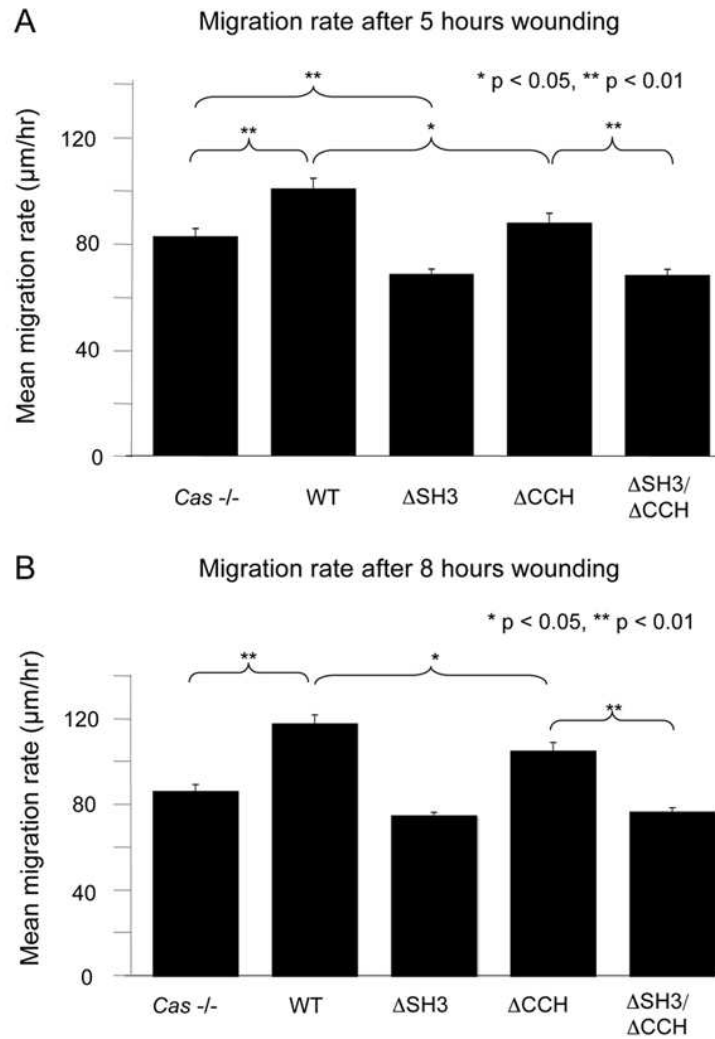
Figure 19: Deletion of either the SH3 or CCH domain significantly reduces cell motility. Scratch wounds made in confluent cell monolayers were monitored by live DIC microscopy. For quantitative analysis, 60 individual cells (12 cells per 5 separate wounds) were tracked from each cell population, and mean migration rates were determined from the total distances migrated over a 5- (A) and 8-hour period (B). Bars indicate SEM, and significance was determined by Student's t-test.
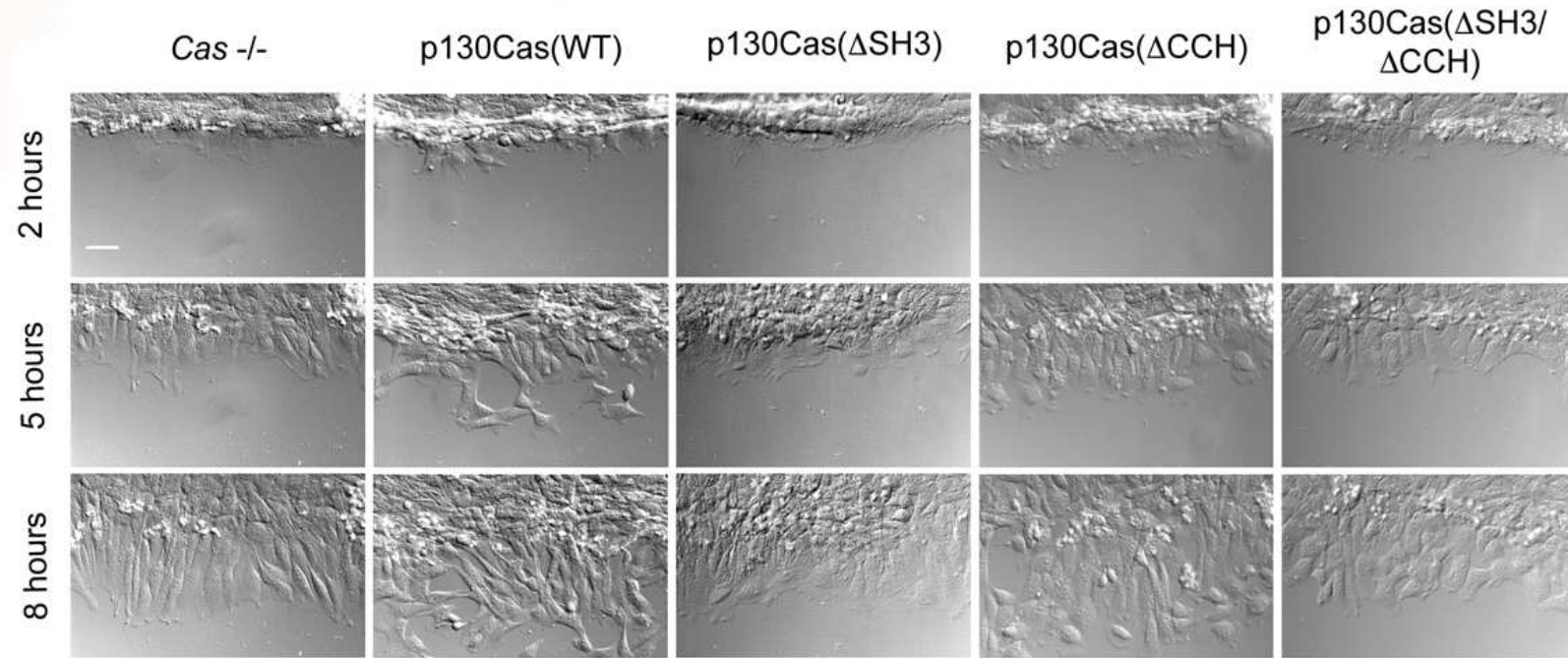
Figure 20: Both the SH3 and CCH domains are required for p130Cas to promote cell motility. Scratch wounds made in confluent cell monolayers were monitored by live DIC microscopy. Representative images of wound margins taken at 2, 5, and 8 hours post-wounding are shown. Scale bar is 50 μm.

Discussion

In this chapter, the FA localization of Venus-tagged p130Cas variants was studied in live and fixed cells in order to clarify the FA targeting mechanism of p130Cas. Two past studies addressed this question using immunostaining to detect epitope-tagged p130Cas variants (Nakamoto et al, 1997; Harte et al, 2000). Both studies found that deletion of the SD had no effect on FA targeting, indicating that SD tyrosine phosphorylation was not involved. However, the two studies reached fundamentally different conclusions as to which domains were required for the FA targeting of p130Cas. Nakamoto et al (1997) found that the SH3 domain was essential for p130Cas targeting to FAs. In contrast, Harte et al (2000) reported no inhibitory effect on FA localization when the SH3 domain was functionally impaired by mutation in the context of the full-length protein. Both studies supported the existence of an FA targeting sequence lying at or near the p130Cas C-terminus, but disagreed as to which domain is involved. Nakamoto et al (1997) concluded that the SBD is required for efficient FA targeting based on studies of a deletion mutant lacking this domain. However, Harte et al (2000) saw no effect on the FA targeting of a p130Cas variant with SBD mutations and instead reported that the C-terminal 182 amino acid residues contained a region essential for FA targeting. Neither of these previous studies included quantitative analyses to evaluate the p130Cas FA targeting mechanism, which may have contributed to the different conclusions reached.

Use of Venus-tagged p130Cas variants in these studies has allowed me to clarify the domain requirements for FA targeting of p130Cas. In contrast to the conclusions reached by either Nakamoto et al (1997) or Harte et al (2000), it is now clear that both the N-terminal SH3 and C-terminal CCH domains are necessary for p130Cas to properly localize to FAs. Quantitative analysis of fixed cells showed that deletion of either the SH3 or CCH domain leads to a significant impairment in the FA localization of p130CasVenus, while complete loss of FA targeting occurs only when both domains

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are deleted. Live cell TIRF imaging of the SH3 or CCH domain deletion mutants provided further evidence of their targeting defects. Even when these mutants are detected at FAs, it is apparent that this is an abnormally indiscrete association. Our identification of a C-terminal FA targeting sequence is consistent with the findings of Harte et al., while further defining this region to include just the CCH domain. While the main conclusion of Harte et al. was that the C-terminal region is sufficient to target p130Cas to FAs, they did recognize that the SH3 domain has the capacity for FA targeting and must be functionally impaired in the context of their C-terminal deletion mutant before FA localization was fully lost. The findings presented in this chapter, however, emphasize that the CCH domain is unable to properly localize p130Cas to FAs in the absence of the SH3 domain.

Note that the SBD deletion mutant used in the Nakamoto et al (1997) study was derived by excision of a large Hind III fragment (Nakamoto et al, 1995) that encodes not only the SBD but also a significant portion of the CCH domain. Thus the observed deficiency in the FA targeting of this mutant is likely due to the partial loss of the CCH domain, rather than loss of SBD function.

As noted in Chapter 1, FAK is a prominent FA protein that interacts with the p130Cas SH3 domain (Polte and Hanks, 1995; Harte et al, 1996; Polte and Hanks, 1997). Results obtained in this study using an inducible FAK expression system provide the first solid evidence that the FAK interaction is the main mechanism by which the p130Cas SH3 domain targets to FAs. Thus FAK serves the dual functions of recruiting p130Cas to FAs and recruiting Src to phosphorylate the p130Cas SD in order to promote downstream signaling.

As discussed in Chapter 1, it has been speculated that the CCH domain may adopt a fold similar to the FAK FAT domain, a four-helix bundle stabilized by a hydrophobic core (Arold et al, 2002; Hayashi et al, 2002; Liu et al, 2002). The results presented here lend support to the possibility that the CCH domain may have a similar tertiary

structure to the FAK FAT domain by demonstrating that the p130Cas CCH domain does, indeed, have the ability to function as an FA targeting domain. However, our results emphasize that the CCH domain by itself is a rather inefficient FA targeting domain. Unlike the FAK FAT domain, only three helices are strongly predicted to lie within the CCH domain. Until the CCH domain tertiary structure is determined, it will remain a matter of speculation as to whether or not the CCH domain actually adopts a four-helical bundle structure.

The nature of the target protein(s) for the p130Cas CCH domain in promoting FA localization is also uncertain. One possibility is paxillin, which plays a major role in targeting by the FAK FAT domain (Tachibana et al, 1995), with paxillin LD motifs binding to opposite faces of the four-helix bundle (Bertolucci et al, 2005). However, in preliminary studies using mCherry-zyxin as an FA marker, no deficiency in the FA targeting of p130CasVenus(WT) was observed in *paxillin* -/- MEFs (Fig. 21).

Two proteins known to interact with the C-terminal region of p130Cas are AND-34/BCAR3 (Riggins et al, 2003) and Ajuba (Pratt et al, 2005). However, neither protein is a strong candidate as the CCH domain target in localizing p130Cas to FAs. AND-34/BCAR3 is not a prominent FA protein, and was actually implicated in recruiting p130Cas away from FAs to the general cell periphery (Riggins et al, 2003). From studies of Ajuba -/- cells, it is also evident that Ajuba is not required for p130Cas localization to FAs and Ajuba has no effect on p130Cas tyrosine phosphorylation (Pratt et al, 2005). Thus additional studies are needed to identify the CCH domain-interacting protein(s) responsible for the recruitment of p130Cas to FAs.

Finally, the findings presented in this chapter are highly relevant to the proposed role for p130Cas signaling in cell motility and mechanotransduction. Consistent with a role for the CCH domain in FA targeting, we further showed that the CCH domain is required for maximal SD tyrosine phosphorylation and promotion of cell motility by p130Cas. Tyrosine phosphorylation of the p130Cas SD has been strongly linked
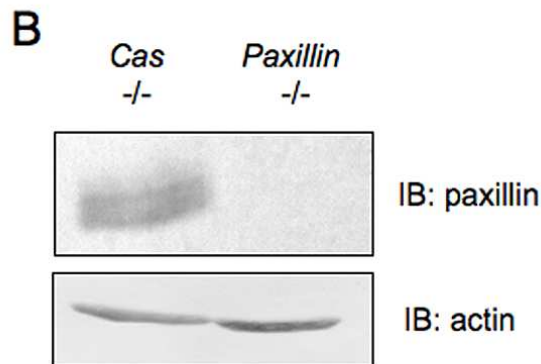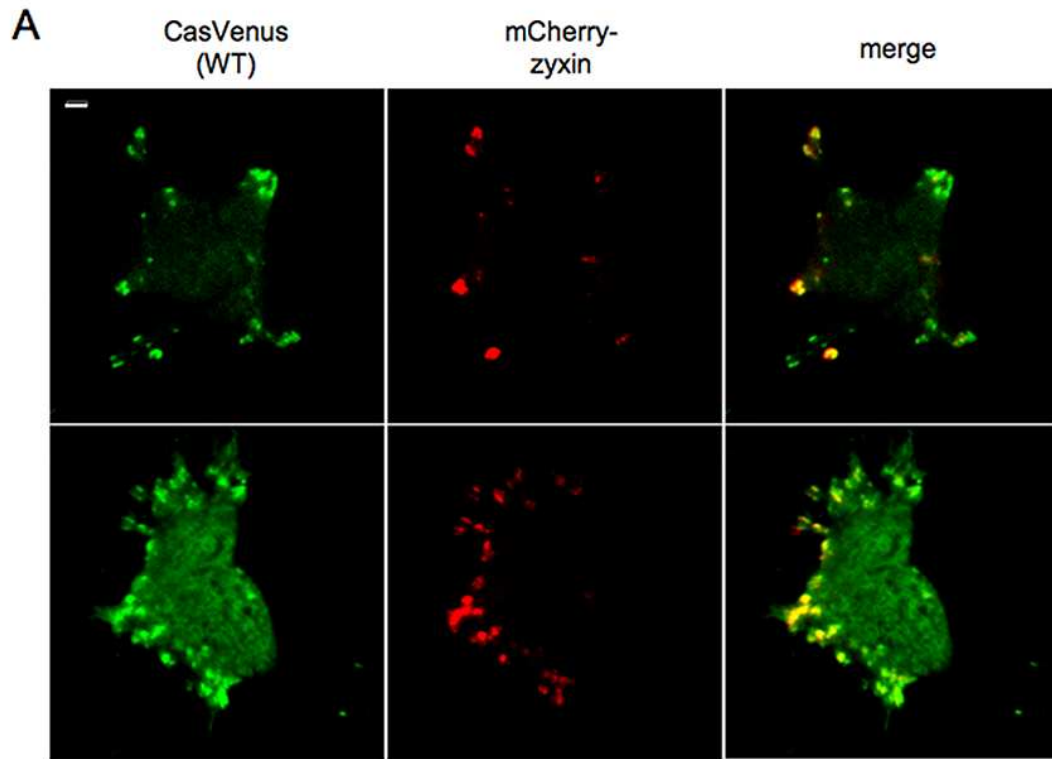
Figure 21: The localization of p130CasVenus(WT) to FAs is not lost in *paxillin* -/- MEFs. A. Two representative *paxillin* -/- MEFs stably expressing p130Cas(Venus) and transiently expressing mCherry-zyxin are shown. P130CasVenus(WT) is in green, mCherry-zyxin in red, and colocalization is indicated by yellow. Images were taken through a 100X objective lens by TIRF microscopy. Scale bar is 10 μm. B. Whole cell lysates from *Cas* -/- MEFs or *paxillin* -/- MEFs expressing p130CasVenus(WT) were subjected to immunoblotting to detect paxillin expression. Actin is shown as a loading control.

to its ability to promote cell motility, with subsequent recruitment of Crk and/or Nck adaptors acting to promote actin dynamics and plasma membrane protrusion (Klemke et al, 1998; Cho and Klemke, 2000; Huang et al, 2002; Shin et al, 2004; Brabek et al, 2005; Rivera et al, 2006). In Chapter 1, the strong correlation of p130Cas tyrosine phosphorylation and FA localization was furthermore noted. Interestingly, previous studies of cell migration in ΔSH3 mutants suggested a more modest reduction in cell migration (Huang et al, 2002). These results were difficult to interpret, however, since the protein was expressed at far lower levels than the full-length p130Cas variant and was analyzed in a manner different from that used in this study. Specifically, Huang et al (2002) calculated the area still left denuded after 24 hours of wound-healing migration, whereas migration was analyzed as the translocation of individual nuclei in the current study at time points no greater than 8 hours post-wounding.

Recently, a mechanosensing role for p130Cas in FAs has been proposed whereby the physical extension of the SD makes it more accessible for phosphorylation by Src (Sawada et al, 2006). The physical extension requires anchor points on both sides of the SD, and it was proposed that anchoring is achieved by distinct interactions made in FAs by the p130Cas SH3 domain and SBD. The proposed role for the SBD in this mechanosensing model is based on the above-mentioned studies (Nakamoto et al, 1997) that used a deletion mutant lacking the SBD but also part of the CCH domain. The studies presented here now establish the CCH domain as the C-terminal FA targeting region of p130Cas and thereby implicate the CCH domain, rather than the SBD, as the C-terminal anchor point in the mechanosensing mechanism.

CHAPTER III

THE DYNAMICS OF P130CAS LOCALIZATION IN FOCAL ADHESIONS

Introduction

It is known that tyrosine phosphorylation is important in regulating FA disassembly, as inhibition of tyrosine phosphatases leads to the formation of large, stable FAs (Inagaki et al, 2000). However, defects in FA disassembly are also seen when Src and several Src substrates, such as p130Cas, are knocked down or mutated on tyrosine residues (Webb et al, 2004).

Despite the role that p130Cas appears to have in regulating FA disassembly, nothing is known about the dynamics of p130Cas localization and turnover in FAs. Most studies of p130Cas localization and signaling have been performed by immunohistochemistry experiments. Insights from live cell microscopy studies, therefore, could enrich the understanding of how p130Cas regulates integrin-mediated signaling and FA turnover. As live microscopy techniques have improved, much has been learned about the structure of FAs and the dynamics of signaling in these structures. Such advances have led to the understanding that the number of proteins in FAs, which were previously thought to be in the range of 50, is actually likely to be more than 100 (Zaidel-Bar et al, 2003). Despite the fact that p130Cas is one of the most prominently tyrosine phosphorylated proteins in FAs, it has been largely excluded from larger adhesome studies (Zamir et al, 2000; Zaidel-Bar et al, 2003; Shroff et al, 2007; Zamir et al, 2008; Winograd-Katz et al, 2009). In this chapter, I have incorporated data about the residence of p130Cas in FAs, as well as data on the molecular exchange of p130Cas in FAs that shed light on its transient tyrosine phosphorylation state.

Materials and Methods

Cells and cell culture

Cas -/- MEFs were kindly provided by Hisamaru Hirai (University of Tokyo). MEFs were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) from Atlanta Biologicals (Lawrenceville, GA), 1% antibiotic/antimicotic (Mediatech, Manassas, VA), 5 μg/ml plasmocin (InvivoGen, San Diego, CA), and 1% non-essential amino acids (Invitrogen, Carlsbad, CA). TetFAK cells were previously described in Owen et al (1999) and Chapter 2 "Materials and Methods" and were maintained in the media mentioned above, which was furthermore supplemented with 5 μg of tetracycline (Calbiochem, La Jolla, CA) per ml of media to repress FAK expression. Phoenix Ecotropic (E) viral packaging cells (a gift from Gary Nolan, Stanford University) were maintained in DMEM, supplemented with 10% heat-inactivated FBS, 1% antibiotic/antimicotic, and 1% non-essential amino acids.

Plasmid construction and protein expression of p130Cas variants

Standard PCR-mediated mutagenesis strategies were used to create p130Cas-Venus fusion proteins with Venus-YFP fused to the C-terminus of the p130Cas coding sequence. For more details on the plasmid construction of LZRS-zeo-CasVenus(WT), ΔSH3, and ΔCCH, please see the "Materials and Methods" section in Chapter 2.

Plasmid mCherry-C1-paxilllin was generated to provide an FA marker for use in live cell studies. The coding region of paxillin-α was subcloned into plasmid mCherry-C1 (provided by Maria Nemethova, Vienna, Austria) from pGEX-2T-paxillin- α (provided by Hajime Yano, Osaka, Japan), such that paxillin is expressed with an N-terminal mCherry tag (for more details on plasmid construction, see the "Materials and Methods" section in Chapter 2). All plasmid sequences were confirmed before use.

Stable expression of p130CasVenus variants in *Cas* -/- MEFs was achieved by retroviral infection using viral supernatants of Phoenix ecotropic (E) packaging cells transfected with LZRS-zeo-Cas-Venus plasmids. Phoenix E cells were subjected by calcium phosphate transfection in 60 mm culture dishes of cells plated at ∼60% confluence. For a more complete description of the calcium phosphate transfection technique and retroviral transduction, please see "Materials and Methods" in Chapter 2.

Three rounds of infection with viral supernatants were performed. For p130Cas-Venus(ΔSH3), it was necessary to select three clones of higher expressers to produce a clonal population that expressed at levels equivalent to sorted cell populations (prior attempts at achieving equivalent expression using cell sorting failed due to a rapid loss of expression). All other p130CasVenus cell populations were further selected by FACS to obtain a population in the lower half of the fluorescence ranges.

After infection and sorting for low levels of Venus-YFP, cells were periodically maintained in media containing 10 μg/ml zeocin (Invitrogen) to assure continued expression from the bicistronic transcript that includes the bleomycin resistance gene expressed from the internal ribosome entry site. Transient expression of mCherry-paxillin was achieved by transfection with Lipofectamine LTX according to manufacturer's instructions (Invitrogen) using 12 μl of reagent to 3 μg of DNA in OptiMEM media (Invitrogen). Cells were incubated for 16 hours for transfection after which cells were placed in normal DMEM supplemented as indicated above in "Cells and Cell Culture".

Quantification of pixel intensities at focal adhesions

Cas -/- MEFs expressing p130CasVenus variants transiently transfected with plasmid mCherry-C1-paxillin were grown to confluence on coverslips coated with 1 μg/ml fibronectin. Two hours prior to analysis, scratch wounds were prepared by creating

denuded areas using a pipette tip. Media was exchanged with complete media supplemented with 10mM HEPES and cells were placed on the microscope stage in a warming chamber maintained at 37°C.

Cells migrating into the wound and expressing both fluorophores to appropriate levels were chosen for analysis of dynamic FAs using TIRF microscopy, which was performed on a Nikon ECLIPSE TE2000-E inverted microscope equipped with a Perfect Focus System and a TIRF 100X/1.49 NA oil-immersion lens. Fluorophores were excited with an 18 mW argon laser (Melles Griot, Albuquerque, NM) and 10 mW DPSS laser 85YCA010 (Melles Griot). A custom-made double-dichroic TIRF mirror and emission filters (Chroma, Rockingham, VT) in a Ludl (Hawthorne, NY) filter wheel were used to capture two-color movies.

Images were captured by a back-illuminated EM-CCD camera (Cascade 512B) driven by IPLab software. Images were imported into ImageJ where file type was converted to 8-bit grayscale. Background (non-cellular) was zeroed on each image by adjusting the lower limit of the display range using the brightness/contrast function in ImageJ.

Quantitative assessment of the dynamic localization of p130CasVenus and mCherry-paxillin was made from plot profiles (obtained using ImageJ) of pixel intensities for each fluorophore within a 3 pixel-wide line through the central longitudinal axes. For p130CasVenus(WT), this analysis included 12 FAs from 4 different cells. These images were captured every 25 seconds for 180 frames. The mean pixel intensities were plotted as a function of time to find the time point at which fluorescence peaked (this value was then used to delineate the end of assembly and the beginning of disassembly). Apparent rate constants for FA assembly and disassembly were then calculated from the slopes of semilogarithmic plots of the mean fluorescence intensities as a function of time (methodology adapted from Webb et al (2004)). To obtain these semilogarithmic plots, the following equations for assembly or disassembly were

plotted on the y axis and time was represented on the x axis:

$$Assembly = \ln(|I|/|I_0|)$$
$$Disassembly = \ln(|I_0|/|I|)$$

In these equations, 'I$_0$' represents the initial fluorescence intensity of the FA, and 'I' represents the fluorescence intensity of the FA at a particular time point. Arrays were constructed in Excel to determine the slope of a best fit line to these semilogarithmic plots using the "least squares" regression approach. Statistical significance of mean rate constants were determined using an unpaired, two-tailed, Student's t-test.

For FRAP analysis in *Cas* -/- MEFs expressing p130CasVenus(WT), 20 FAs (each from a different cell expressing p130CasVenus(WT) and mCherry-paxillin) were photobleached for 8 seconds with both 10 mW DPSS 85YCA010 and 30 mW argon lasers (Melles Griot) by focusing the beam in the TIRF focal plane with a custom-made lens (Nikon) placed in the position of the filter cube. In TetFAK cells, the number of FAs was reduced to 7. Two-color movies were recorded every 2 seconds for 4 minutes using TIRF microscopy as described above. FRAP curves and times of 50% recovery were calculated and adjusted for chromophore activated laser inactivation effect according to specifications previously described (Puthenveedu et al, 2006) using single control measurements taken from each cell. Each channel in each FA was calculated only in relationship to its own fluorescence recovery, with the value before bleaching being set as the 100% mark from which the 50% recovery point was calculated.

Results

P130CasVenus(WT) localizes to FAs throughout their lifetime

To further study dynamic aspects of p130Cas targeting to FAs, p130CasVenus(WT) was visualized at the ventral interface of living cells using total internal reflection fluorescence (TIRF) microscopy. Dynamic adhesions in motile cells at the margins of monolayer scratch wounds were analyzed. Fig. 22A shows images of a representative FA recorded over a fifty minute time period.

Similarly to mCherry-paxillin, p130CasVenus(WT) localizes to FAs at the outset of their assembly and persists throughout the process of disassembly. P130CasVenus (WT) was consistently seen in a wide range of FAs from small FXs to larger, mature FAs at the front and rear of the cell. The representative image in Fig. 22A shows an FA where p130CasVenus(WT) happens to be more readily seen at the first stages of FA assembly. However, in other instances paxillin could be more readily seen at these earlier stages. Therefore, these data are not indicative of any differences in p130Cas and paxillin arrival times in assembling FAs. The apparent extension of p130Cas residence in FAs, however, was a consistent trait, which was furthermore quantitated.

As a further measure of p130CasVenus(WT) residence in FAs, assembly and disassembly rate constants were determined by quantifying fluorescence pixel intensities over time, through the centers of the long axes of FAs. The bottom panels of Fig. 22A show the resulting fluorescence intensity plots obtained for the representative FA shown. Such measurements obtained from 12 different FAs indicated that the rate of p130CasVenus(WT) assembly into FAs was not significantly different from that of mCherry-paxillin (Fig. 22B).

However, p130CasVenus(WT) appeared to disassemble from FAs at a slower rate than mCherry-paxillin as was illustrated by calculating the apparent rate constant of assembly and disassembly (Fig. 22B). These values were obtained as the slopes of semilogarithmic plots wherein the initial fluorescence intensity of the FA was plotted
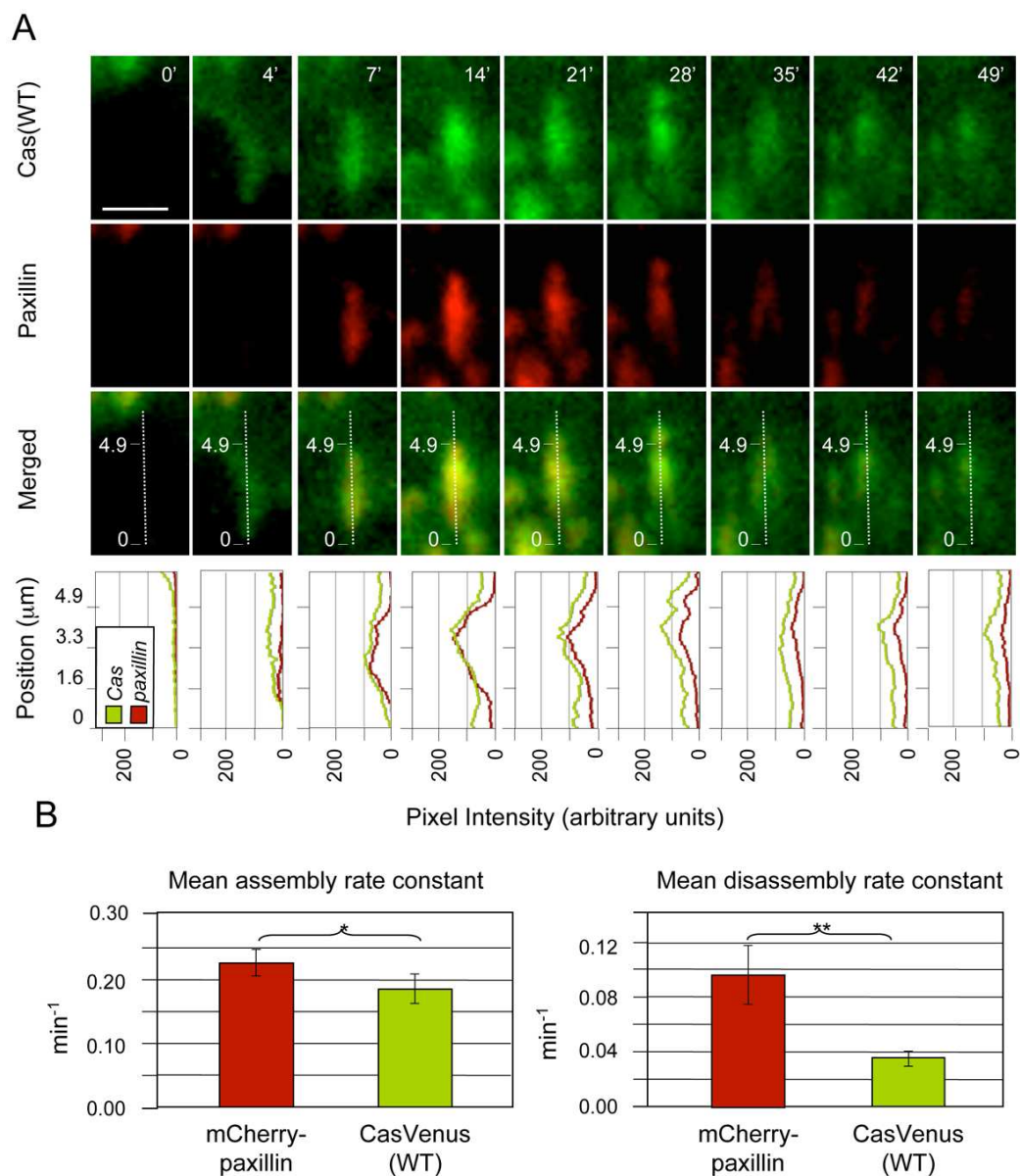
Figure 22: P130CasVenus(WT) exists in FAs with similar dynamics to paxillin, but disassembles in FAs at a slower rate. P130CasVenus(WT) cells transiently expressing mCherry-paxillin were imaged by live TIRF microscopy at the leading edge of wound margins to assess the dynamics of p130Cas localization to FAs relative to paxillin. A. Images of a representative FA taken over a 49-minute time period. The graphs show fluorescence intensity profiles obtained through the center of the long axis of the FA (dotted line) at each time point. Scale bar is 5 μm. B. Mean assembly and disassembly rates for p130CasVenus and mCherry-paxillin, calculated from the fluorescence intensity profiles of 12 FAs from 4 different cells. Bars indicate the standard error of the mean (SEM). Significance values were determined by Student's t-test where * indicates p = 0.22 and ** indicates p = 0.02.

as a function of the FA fluorescence intensity over time (see "Materials and Methods" and Webb et al (2004) for a more detailed explanation of this function). Clearly, p130Cas localizes to FAs throughout their lifetime.

P130Cas mutants display aberrant assembly and disassembly at FAs

P130CasVenus deletion mutants, ΔSH3 and ΔCCH, and CCHVenus display aberrant localization to FAs as compared to mCherry-paxillin. Attempts at determining assembly and disassembly rates in these mutants were complicated by the indiscrete manner with which they localize to FAs. Overall assembly of these proteins in FAs does not correlate well with paxillin localization, which rises in a discernible peak. Instead, the mutant fluorescence profile intensities in FAs display a number of 'valleys and hills' during assembly, which fall outside of the FA area itself (Fig. 23). The ΔSH3 mutant, in particular, displayed very static levels of protein localization over time (Fig. 23, left panels), indicating that it was not able to appropriately assemble into these sites and/or disassemble from them. The ability of the ΔCCH mutant to assemble and disassemble in FAs varied widely, with some FA fluorescence profiles roughly mimicking paxillin profiles (Fig. 23, middle panels) and other FAs exhibiting a more static phenotype as was seen in the ΔSH3 mutant. CCHVenus fluorescence profiles appeared dynamic, peaking in areas similar to paxillin, but also localizing, assembling, and disassembling in areas adjacent to, but not within the FA (Fig. 23, right panels).
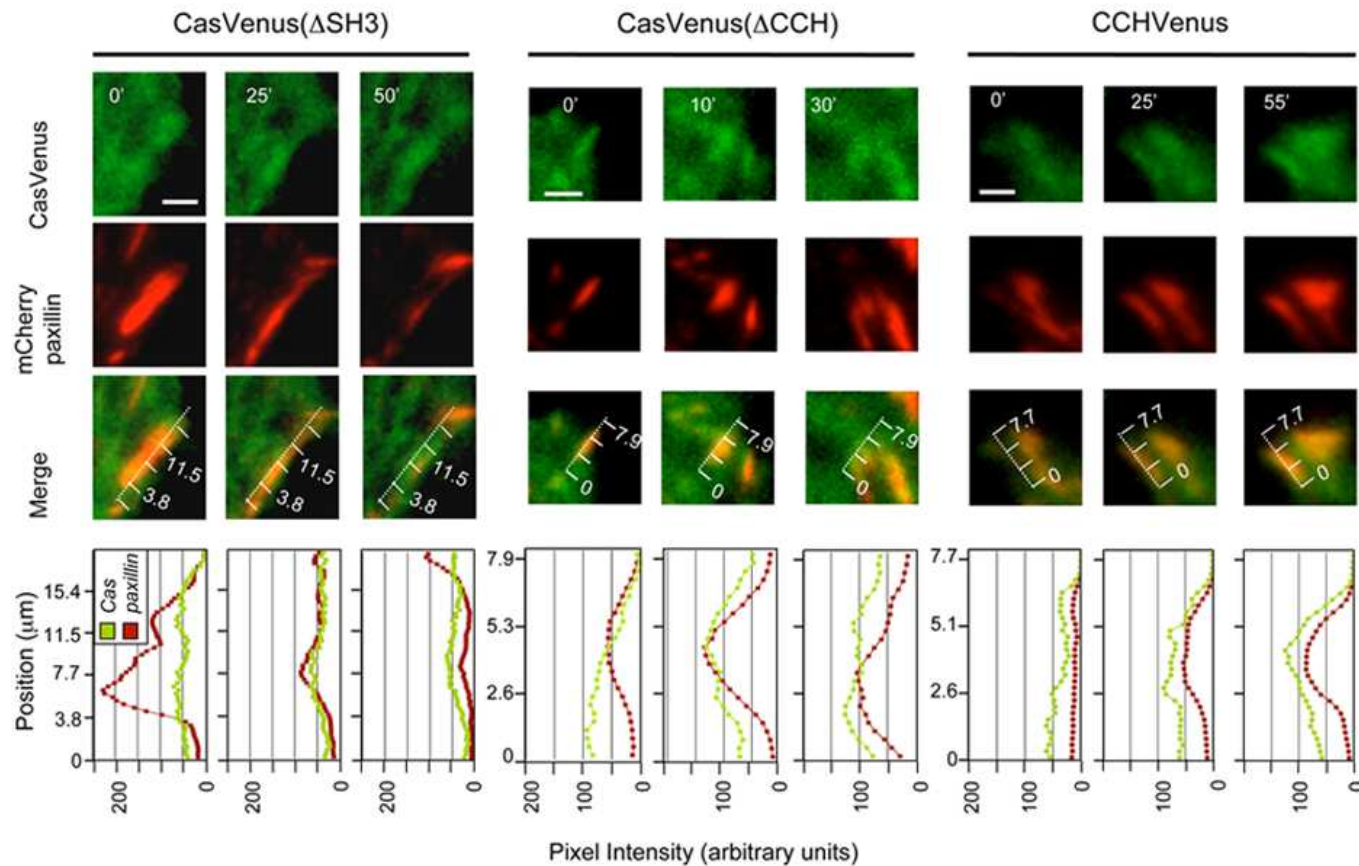
Figure 23: The dynamic localization of p130CasVenus mutants to FAs is non-discrete. P130CasVenus variants stably expressed in *Cas* -/- MEFs were transiently transfected with mCherry-paxillin and imaged by live TIRF microscopy at the leading edge of wound margins to assess the dynamics of p130Cas localization to FAs relative to paxillin. Images of representative FAs are shown. The graphs show fluorescence intensity profiles obtained through the center of the long axes of FAs (dotted line) at each time point. Scale bar is 5 μm.

P130CasVenus(WT) exists in FAs with a high mobile fraction

To investigate the dynamic mobility of p130CasVenus(WT) in existing FAs, fluorescence recovery after photobleaching (FRAP) experiments were performed using TIRF microscopy. In this manner, the recovery of fluorophores was only measured within the ventral area of the cell where FAs exist. Again, mCherry-paxillin was used as the point of reference. FAs were photobleached for 8 seconds and the subsequent recovery of unbleached fluorophores was observed at 2-second intervals. The mean time of 50% fluorescence recovery for p130CasVenus(WT) was ~6 seconds, while 50% recovery of mCherry-paxillin was significantly slower at ~43 seconds (see Fig. 24). The recovery curve for p130CasVenus(WT) plateaued at a higher level compared to that of mCherry-paxillin. Thus, p130Cas exists dynamically in FAs with a high mobile fraction.

Attempts at calculating recovery times for p130CasVenus mutants were again complicated by their indiscrete localization. While recovery of fluorophore was detected in the general area of the FA, it was clear from the recovery images that these fluorophores did not incorporate into those areas in a significant manner as was seen with p130CasVenus(WT) — data not shown.

Interestingly, by TIRF analysis the FA localization of p130CasVenus(WT) in Tet-FAK non-induced cells is less adversely affected than is seen in p130CasVenus(ΔSH3) expressed in *Cas* -/- MEFs. Therefore, FRAP analysis of p130Cas defective in FAK binding appeared more feasible by this methodology. When the rate of 50% recovery was calculated, no significant differences in recovery were seen under the FAK-induced conditions, but repressing FAK expression resulted in a more rapid fluorescence recovery of p130CasVenus(WT). The increased recovery of p130CasVenus(WT) was also concomitant with a larger mobile fraction (Fig. 25). The rate of paxillin recovery was not significantly affected when FAK expression was repressed. However, the difference in paxillin and p130Cas recovery was significant when FAK expression was repressed.
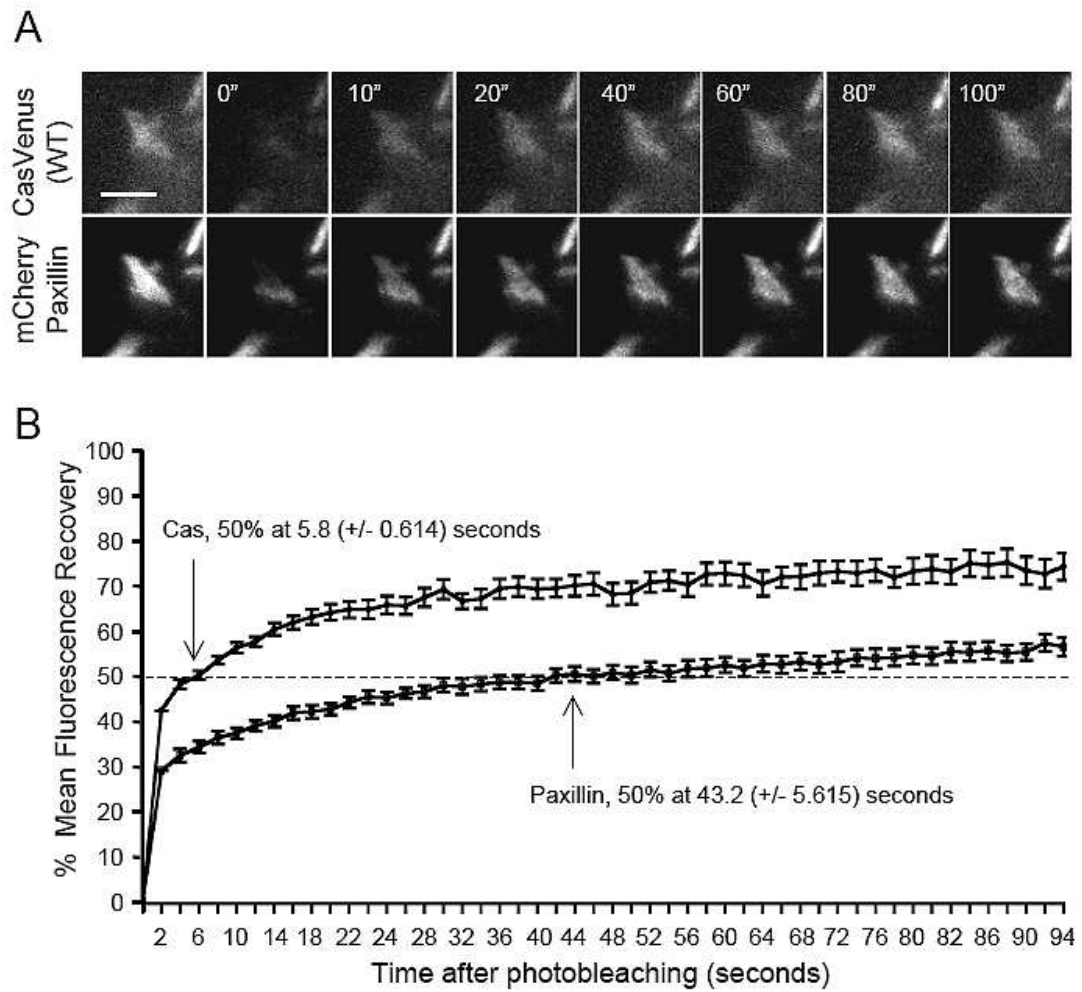
Figure 24: p130Cas exists in FAs with a high mobile fraction. TIRF-FRAP was used to study the exchange of p130CasVenus(WT) and mCherry-paxillin in individual FAs at the leading edge of a wounded cell monolayer. After photobleaching, fluorescence recovery was recorded at 2-second intervals. A. A representative FA is shown before photobleaching (left panels), immediately after photobleaching (0"), and during fluorescence recovery (10"-100"). Scale bar is 5 μm. B. Plot comparing the fluorescence recoveries after photobleaching of p130CasVenus(WT) and mCherry-paxillin. Data points represent the mean values of 20 individual FAs, with error bars indicating SEM. The 50% mean fluorescence recovery time points are marked by arrows. Student's t-test showed the difference in 50% recovery points for p130Cas and paxillin was significant at p < 0.001.
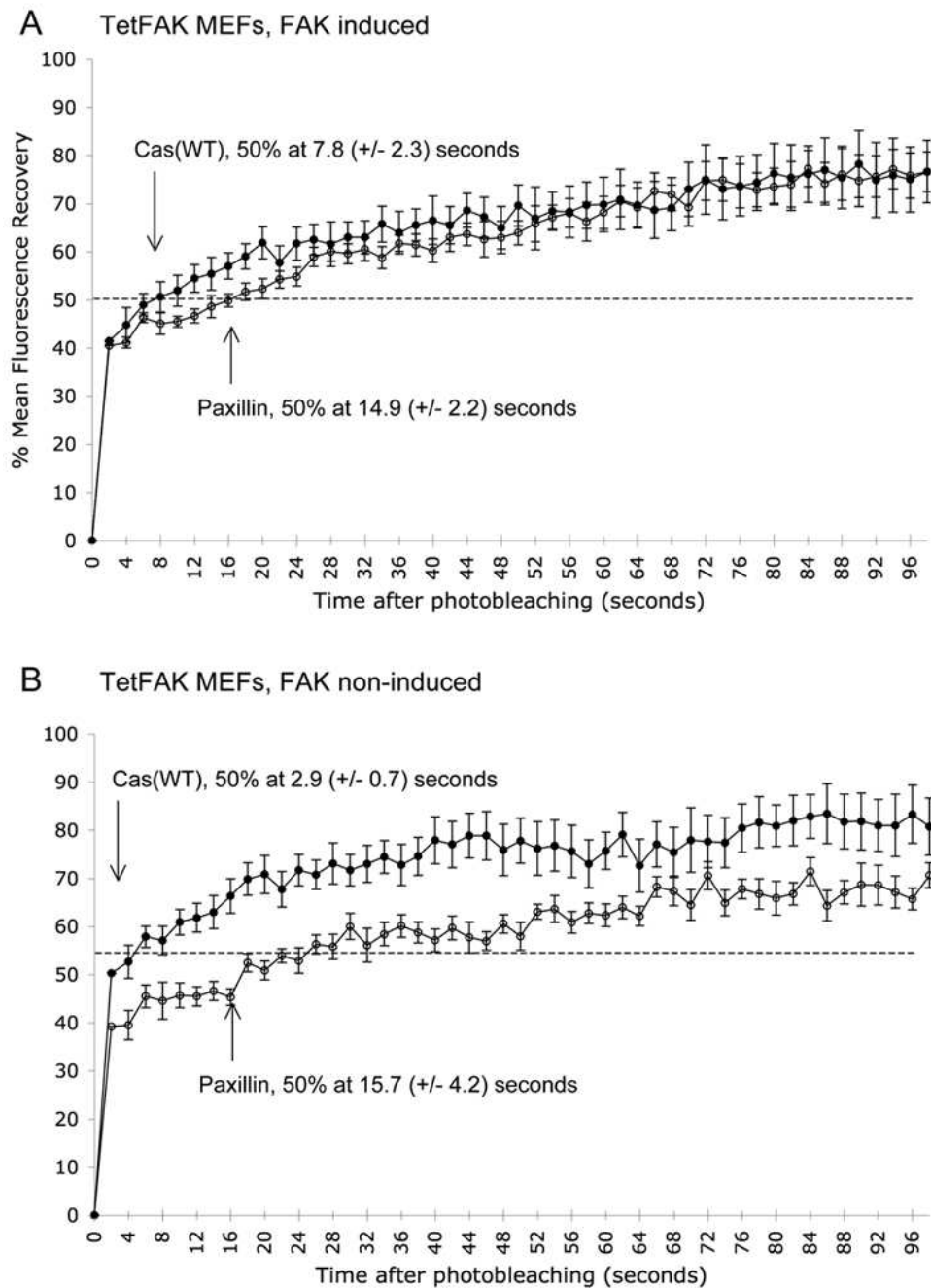
Figure 25: In the absence of FAK expression, p130CasVenus(WT) exists as a higher mobile fraction. Seven FAs from either TetFAK induced (A) or non-induced (B) cells were photobleached for 8 seconds and the recovery of p130CasVenus and mCherry-paxillin were monitored for 2 minutes in 2-second intervals. While there was no statistical difference found between p130CasVenus(WT) and mCherry-paxillin in TetFAK induced cells, in TetFAK non-induced cells this difference is significant at the level of $p < 0.001$. Error bars represent SEM.

Discussion

While it is recognized that p130Cas localizes to FAs where it engages in integrin-mediated signaling via tyrosine phosphorylation (Petch et al, 1995; Fonseca et al, 2004; Ballestrem et al, 2006), the dynamics of p130Cas targeting to these sites remain poorly understood. To investigate these issues, TIRF microscopy was used to visualize fluorophore-tagged proteins in migrating fibroblasts.

Evaluation of p130Cas localization to FAs over time showed that p130Cas arrives early during the assembly of FAs at the leading edge of motile cells, reaches peak levels as FAs reach full maturity, and departs late during FA disassembly. These observations are consistent with p130Cas having the capacity to function in FAs throughout their lifetime. The rate of p130Cas assembly in FAs is similar to that of paxillin, which is one of the first FA proteins detected in nascent FAs (Zaidel-Bar et al, 2003). The early localization of p130Cas to assembling FAs supports the notion that p130Cas engages in signaling at the leading edge of migrating cells to promote plasma membrane protrusion. During FA disassembly, p130Cas actually appears to persist longer than paxillin. This persistence is supportive of a proposed role for p130Cas in promoting FA disassembly (Webb et al, 2004).

Note that the overall morphology of FAs (as marked by paxillin) appears normal in cells expressing p130Cas mutants deficient in FA targeting, which is not surprising as this is also true for *Cas* -/- MEFs (Honda et al, 1998). However, p130Cas has been implicated in promoting FA disassembly (Webb et al, 2004) and SD tyrosine phosphorylation in FAs could be involved in this process.

Though it is not possible to discern whether or not p130CasVenus(ΔSH3) and (ΔCCH) contribute to the rate of p130Cas assembly and disassembly in FAs from the anecdotal observations presented here, it is clear that these proteins are important

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for maintaining the spatial localization of p130Cas in the FA as it assembles and disassembles. From the studies in Chapter 2, we can now deduce that the interaction of p130Cas with FAs through the SH3 domain is mediated by FAK. Previous studies have already shown that paxillin assembly and disassembly rates in FAs are reduced 14-fold in *FAK* -/- MEFs (Webb et al, 2004). However, in *Cas* -/- MEFs, this rate is reduced an extra 5-fold. Therefore, it is likely that the CCH-binding partner furthermore accounts for this reduced rate of paxillin disassembly. Finding the CCH-binding partner, therefore, may create greater insight into the function of p130Cas during disassembly.

Using FRAP, we found that p130Cas exchanges more rapidly with its binding partner(s) in FAs as compared to paxillin. Thus the interactions made by p130Cas in FAs appear to be relatively more transient and of lower affinity. Unlike paxillin, there is a large cytoplasmic reservoir of p130Cas that could be reflective of the higher mobile fraction of p130Cas in FAs. As p130Cas SD phosphorylation is linked to its localization in FAs (Fonseca et al, 2004), the high mobility of p130Cas in FAs can account for the rapid turnover of SD tyrosine phosphorylation, as evidenced by sodium vanadate treatment. Though interpretation of the FRAP data on p130CasVenus mutants was complicated by the inability of mutants to specifically relocalize to these sites, performing FRAP experiments with TetFAK cells expressing p130CasVenus(WT) demonstrated that the interaction of FAK with p130Cas may in part regulate the mobility of p130Cas in FAs. Expression of FAK shifted more p130Cas into the immobile fraction, whereas repression of FAK expression resulted in a faster recovery of p130Cas to FA sites, which may be indicative of a faster release of p130Cas from remaining binding partners. It is significant to note that the 50% recovery rate for paxillin was faster ($\sim$3-fold) in TetFAK cells in comparison to *Cas* -/- MEFs. Perhaps the expression of Venus-tagged p130Cas in excess to endogenous p130Cas can influence the rate of paxillin exchange in FAs (most likely through some

indirect mechanism). Alternately, the level of FAK expression achieved in TetFAK cells after 24 hours shifting into tetracycline-free media may differ significantly enough from levels of FAK expression in *Cas* -/- MEFs to alter these rates.

While the interaction with p130Cas SH3 and CCH binding partners may influence the molecular exchange of p130Cas in FAs, this rate may also be related to the degree of p130Cas tyrosine phosphorylation in the SD. As previously discussed, FAK plays a role in helping p130Cas achieve maximal SD tyrosine phosphorylation by acting as a scaffold from which Src can phosphorylate p130Cas. Clearly, in TetFAK cells wherein FAK expression is repressed, the tyrosine phosphorylation of p130Cas is reduced (Fonseca et al, 2004). As we know that tyrosine phosphorylation of the p130Cas SD is only detected in FAs (Fonseca et al, 2004; Ballestrem et al, 2006), it is likely that the unphosphorylated species of p130Cas in the cytoplasm represents at least part of the high mobile fraction of p130Cas.

PRELIMINARY STUDIES OF THE CCH DOMAIN STRUCTURE AND NOVEL
OBSERVATIONS OF P130CAS SUBCELLULAR LOCALIZATION

Introduction

In this chapter, I will discuss ongoing studies concerning the p130Cas CCH do-
main. As discussed in Chapter 2, a major direction of investigation that naturally
follows from the studies of the CCH domains influence on p130Cas FA targeting is to
obtain a better understanding of the protein structure. An attempt to investigate the
structure of the CCH domain indirectly by performing site-directed mutagenesis on
key amino acid residues in the putative four-helical structure will be discussed. Fig. 26
illustrates the putative tertiary structure of the CCH domain if it indeed adopts this
four-helical fold. Key residues for site-directed mutagenesis are also illustrated.
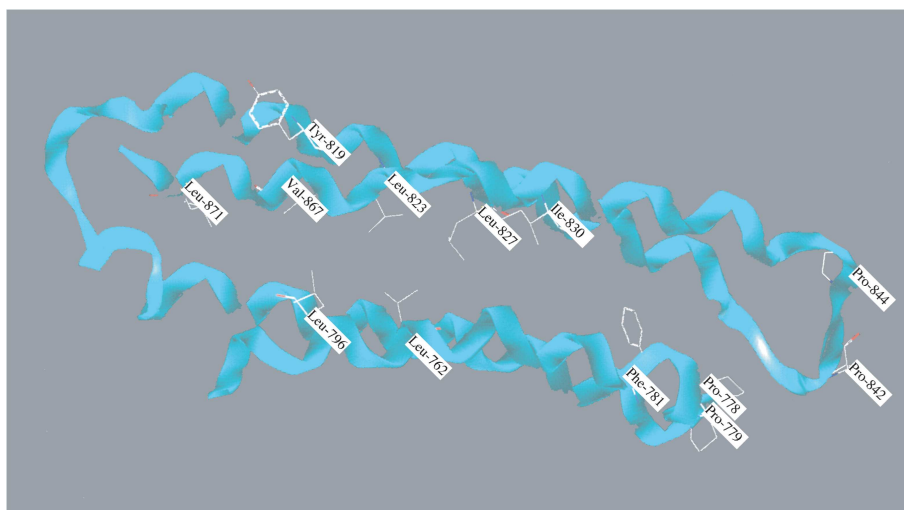

Figure 26: Putative tertiary structure of the CCH domain modeled against the FAK
FAT domain. The p130Cas CCH domain sequence was superimposed on the tertiary
structure of the FAK FAT domain to create a SWISS projection of its putative tertiary
structure with good stereochemisty. Residues postulated to be important for the
formation of the hydrophobic core (L823, L827, I830, L871)) are indicated.

Lastly, the observed localization p130CasVenus in fibrillar adhesions, filopodia, and cell-cell contacts will be discussed. These data represent preliminary and ongoing studies that have not been published, therefore few conclusions can be drawn from them. They do, however, present good opportunities for future studies on the targeting of p130Cas to its subcellular compartments. Such future studies will be discussed at greater length in Chapter 5.

Materials and Methods

Cells and cell culture

Cas -/- MEFs were kindly provided by Hisamaru Hirai (University of Tokyo). MEFs were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) from Atlanta Biologicals (Lawrenceville, GA), 1% antibiotic/antimicotic (Mediatech, Manassas, VA), 5 μg/ml plasmocin (InvivoGen, San Diego, CA), and 1% non-essential amino acids (Invitrogen, Carlsbad, CA). TetFAK cells were previously described in Owen et al (1999) and Chapter 2 "Materials and Methods" and were maintained in the media mentioned above, which was furthermore supplemented with 5 μg of tetracycline (Calbiochem, La Jolla, CA) per ml of media to repress FAK expression. Phoenix Ecotropic (E) viral packaging cells (a gift from Gary Nolan, Stanford University) were maintained in DMEM, supplemented with 10% heat-inactivated FBS, 1% antibiotic/antimicotic, and 1% non-essential amino acids.

Antibodies

Monoclonal mouse antibodies to total p130Cas and paxillin were obtained from BD Transduction Laboratories (San Jose, CA).

Plasmid construction and protein expression of p130Cas variants

Standard PCR-mediated mutagenesis strategies were used to create p130Cas-Venus fusion proteins with Venus-YFP fused to the C-terminus of the p130Cas coding sequence. For more details on the plasmid construction of LZRS-zeo-CasVenus(WT) and CCHVenus, please see the "Materials and Methods" section in Chapter 2. pCS2-Cas L823E,L827E Venus was created by site-directed mutagenesis to change leucine (Leu) residues 823 and 827 in the CCH to glutamic acid (Glu) residues. This procedure required three sequential steps of PCR amplification: 1) to amplify the mutation from the 3' end of the DNA template (pCS2-CasWT-Venus) using a pair of oligonucleotide primers wherein the reverse primer contained the mutation and the forward primer simply amplified the template sequence, 2) to amplify the mutation from the 5' end of the DNA template using another pair of oligonucleotides wherein the forward primer contained the mutation, and 3) to extend the PCR products the first two steps to obtain a complete PCR sequence. For the first step, the following oligonucleotide primers were used: 5′- GCC ACC CAC ACT TCT GAC CGC ACC -3′ (forward primer) and 5′- AC CTC GCC ACG CTC GAG GTC ACA CTC CA -3′ (reverse primer). For the second step, the oligonucleotides were: 5′-TG GAG TGT GAC CTC GAG CGT GGC GAG GT-3′ (forward primer) and 5′-CCA TGA TTA CGC CAA GCG CGC AAT TAA CCC TCA-3′ (reverse primer). The forward primer from step 1 and the reverse primer from step 2 were then used to extend the PCR sequences from steps 1 and 2 and furthermore amplify those sequences. Note that these primers were designed to induce three Glu mutations, but resulted in only two due to the difficulty of changing so many base pairs in close proximity to one another using one set of primers.

PCR products were purified and cut by restriction enzymes BglII and Xba1, where the BglII site is located in the p130Cas sequence lying at the 5' end of the CCH domain and Xba1 lies within the pCS2-Venus vector sequence. pCS2-Cas-Venus-WT was also

cut using these sites and the PCR cut product was ligated in to the larger backbone of this plasmid to replace the WT CCH sequence with the sequence containing the Glu mutations. The DNA sequence was confirmed prior to transient transfection.

Stable expression of p130CasVenus variants in *Cas* -/- MEFs was achieved by retroviral infection using viral supernatants of Phoenix ecotropic (E) packaging cells transfected with LZRS-zeo-Cas-Venus plasmids. Phoenix E cells were subjected by calcium phosphate transfection in 60 mm culture dishes of cells plated at ∼60% confluence. For a more complete description of the calcium phosphate transfection technique and retroviral transduction, please see "Materials and Methods" in Chapter 2.

Three rounds of infection with viral supernatants were performed. P130Cas-Venus(WT) and CCHVenus cell populations were further selected by fluorescence activated cell sorting (FACS) to obtain a population in the lower half of the fluorescence ranges.

After infection and sorting for low levels of Venus-YFP, cells were periodically maintained in media containing 10 μg/ml zeocin (Invitrogen) to assure continued expression from the bicistronic transcript that includes the bleomycin resistance gene expressed from the internal ribosome entry site.

TIRF imaging of p130CasVenus

Cas -/- MEFs expressing p130CasVenus variants were grown to confluence on coverslips coated with 1 μg/ml fibronectin (from human plasma, Sigma-Aldrich, St. Louis, MO). Two hours prior to analysis, scratch wounds were prepared by creating denuded areas using a pipette tip. Media was aspirated from these cells to remove cellular debris and fresh media was applied, which was furthermore supplemented with 10mM HEPES to maintain media pH when cells were placed outside of the incubator on the microscope stage. Cells were imaged by total internal reflection fluorescence

(TIRF) microscopy as previously described in Chapters 2 and 3. Fluorophores were excited with an 18 mW argon laser (Melles Griot, Albuquerque, NM). A custom-made GFP TIRF mirror and emission filters (Chroma Technology, Rockingham, VT) in a Ludl filter wheel (Ludl, Hawthorne, NY) were used to capture frames at 25 second intervals for a total of 180 frames.

Images were captured by a back-illuminated EM-CCD camera Cascade 512B (Photometrics) driven by IPLab (Scanalytics, Fairfax, VA) software. Images were imported into ImageJ (NIH – open source software), where file type was converted to 8-bit grayscale. Background (non-cellular) was zeroed on each image by adjusting the lower limit of the display range using the brightness/contrast function in ImageJ.

Imaging of filopodia by DIC and wide-field fluorescence microscopy

Scratch wounds were created, as described above, in confluent cell monolayers on coverslips coated with 1 μg/ml fibronectin and analyzed by DIC and wide-field fluorescence microscopy. The wounded monolayers were placed in complete DMEM further supplemented with 10 mM HEPES, mounted into a 37°C heating chamber on an inverted Nikon ECLIPSE TE2000-E2 microscope, and visualized through a 100X/1.4 oil Plan Apo objective lens (Nikon). Wide-field fluorescence imaging was achieved by placing a YFP filter in the excitation pathway. Frames were captured every 25 seconds for 80 frames on a CoolSnapHQ camera (Photometrics) using IPLab software.

Immunostaining CCHVenus cells for paxillin

Cas -/- MEFs expressing CCHVenus were fixed, immunostained for paxillin as an FA marker, and visualized by wide-field fluorescence microscopy. Cells were grown on coverslips coated with 10 μg/ml fibronectin (from human plasma, Sigma-Aldrich, St. Louis, MO) and fixed for 5 minutes in 4% paraformaldehyde (in a buffer containing

10mM MES, (pH 6.1), 138 mM KCl, 3mM MgCl$_2$, 2mM EGTA, and 0.32M sucrose). Paraformaldehyde was removed from slips by washing in a 50 μg/ml NaBH$_4$ dissolved in TBS and alternating with a TBS wash (washes were performed on ice). Cells were then permeabilized for 10 minutes in 0.5% Triton-X in TBS. Slips were washed in TBS. After blocking for 1 hour in a solution of 2% BSA and 0.1% Triton-X dissolved in TBS, the coverslips were immunostained for paxillin with detection by Cy3-conjugated secondary antibody. Coverslips were kept in the dark once secondary antibody was added to prevent fluorophore degradation. The coverslips were mounted in Prolong Gold Mounting Media (Invitrogen).

Images of immunostained cells were captured by QED Capture software (Media Cybernetics, Silver Spring, MD) through a 100X/1.40 oil immersion lens on an upright Nikon Eclipse 80i microscope (Nikon, Melville, NY) equipped with Nikon Epifluorescence D-FL attachments and a Photometrics Coolsnap ES camera (Tucson, AZ). Fluorophores were excited by an EXFO X-Cite 120 fluorescence illumination system (EXFO, Quebec, Canada) using Nikon C-FL G-23/C TRITC and C-FL B-2E/C FITC filter cubes. After capture, TIFF images were imported into Adobe Photoshop CS and the autocontrast function was used to adjust the images.

Results

Preliminary mutagenesis results

In Chapter 2, we showed that the CCH domain of p130Cas has a significant role in localizing p130Cas to FAs. As previously noted, the structure of the CCH domain has not been solved, yet there are at least two theories on what the secondary structure of the CCH may be: 1) the CCH may contain four α-helices, similarly to the FAK FAT domain (Arold et al, 2002; Hayashi et al, 2002; Liu et al, 2002; Garron et al, 2009), or 2) it may contain three α-helices and an area of β-strand content in the area that

would be analogous to the second helix in the FAK FAT domain (Singh et al, 2008).

Therefore, the possibility that the CCH domain may contain alpha helices that bundle similarly to the FAK FAT domain in the third and fourth areas predicted to be helices seemed plausible. Residues that may play an important role in maintaining the hydrophobic interactions between the third and fourth helix were therefore identified for site-directed mutagenesis. In the FAK FAT structure, two leucine (Leu) and a valine (Val) residue are indicated in the third helical area, as well as a Val and Leu residue in the fourth helical area. All of these sites are also well conserved in p130Cas and its family members, though the Leu residue in the fourth helix is an isoleucine (Ile) in DmCas (which is nevertheless a hydrophobic residue). Therefore, mutation of some of these analogous residues in p130Cas was used to investigate the importance of hydrophobic residues in the ability of the CCH domain to target to FAs. It stands to reason that if the CCH targeting mechanism is interrupted by one or several of these mutations, that the CCH domain may contain similar structural features to the FAK FAT domain (i.e. loss of targeting by the CCH would be correlated with a disruption of CCH domain tertiary structure).

Mutants were generated based on the existing DNA plasmid, pCS2-Cas(WT)-Venus. Mutagenesis of two Leu residues (823 and 827) implicated in helix bundling to Glu residues (effectively making these hydrophobic residues acidic) was evaluated by transiently transfecting *Cas* -/- MEFs with the DNA plasmid pCS2-Cas-L823E,L827E-Venus and imaging cells by TIRF microscopy (Fig. 27).

Localization of this p130CasVenus(L823E,L827E) was variable, though most cells appeared to lack normal FA localization. As seen in Fig. 27, some cells did present some areas of dense localization at the periphery (right image), though it is not clear from this experiment whether or not these sites are FAs. Further complicating the determination of whether or not these cells display localization of CasVenus to FAs is the fact that the cells did not appear well-spread. Further study, is therefore necessary.
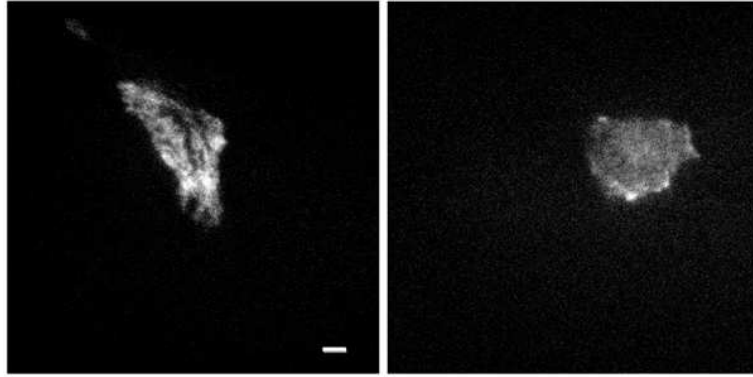
Figure 27: The FA localization of p130CasVenus(L823E,L827E) appears defective. TIRF imaging of *Cas* -/- MEFs transiently expressing Cas-L823E,L8237E-Venus through a 100X objective lens. Two representative images of transfected cells are shown. Scale bar is 10 μm.

The localization of p130Cas in fibrillar FAs is influenced by the CCH domain

In Chapters 2 and 3, the localization of p130Cas to leading edge FAs and FXs was discussed. However, there are other classes of ECM/cell contacts in the cell, including the trailing edge FAs and the fibrillar adhesions. Using TetFAK cells, it became apparent that the localization of p130CasVenus variants in leading edge FAs differed from the localization in fibrillar adhesions. When FAK was expressed, p130CasVenus(WT) could be detected at fibrillar adhesions. Surprisingly, the amount of p130CasVenus(WT) fluorescence increased in fibrillar adhesions when FAK was not expressed. The ΔCCH mutants did experience some significant loss of targeting at fibrillar adhesions, which was furthermore reduced in the absence of FAK expression. However, complete loss of targeting was never seen (Fig. 28).

Localization of p130CasVenus to filopodial tips

By TIRF, small speckles of p130CasVenus(WT) were occasionally seen beyond the edge of where the lamellipodium appeared to be, in a manner that did not appear
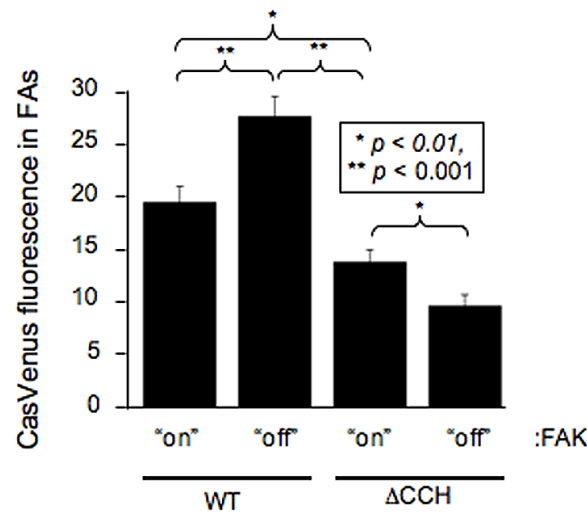
Figure 28: P130CasVenus localizes to fibrillar adhesions in a CCH-dependent, but FAK-independent manner. Quantitative analysis of meanp130CasVenus fluorescence at fibrillar adhesions. Paxillin immunofluorescence was used to delineate FA borders in confocal images of TetFAK cells expression p130CasVenus (see Chapter 2 for images), and mean Venus-YFP fluorescence values were measured from within the FA boundary. For each p130Cas variant, mean Venus-YFP fluorescence was determined from 50 FAs (10 each from 5 separate cells). Bars indicate SEM. Statistical significance was determined by Student's t-test.

Figure 29: Small speckles of p130CasVenus are seen beyond the general leading edge of cells by TIRF, which do not appear to be FA structures. *Cas* -/- MEFs expressing p130CasVenus(WT) were imaged at the edge of a wounded cell monolayer by TIRF microscopy. Arrows indicate the position of speckles. The time stamp is indicated on each image corresponding to the time in minutes:seconds. Scale bar is 5 μm.
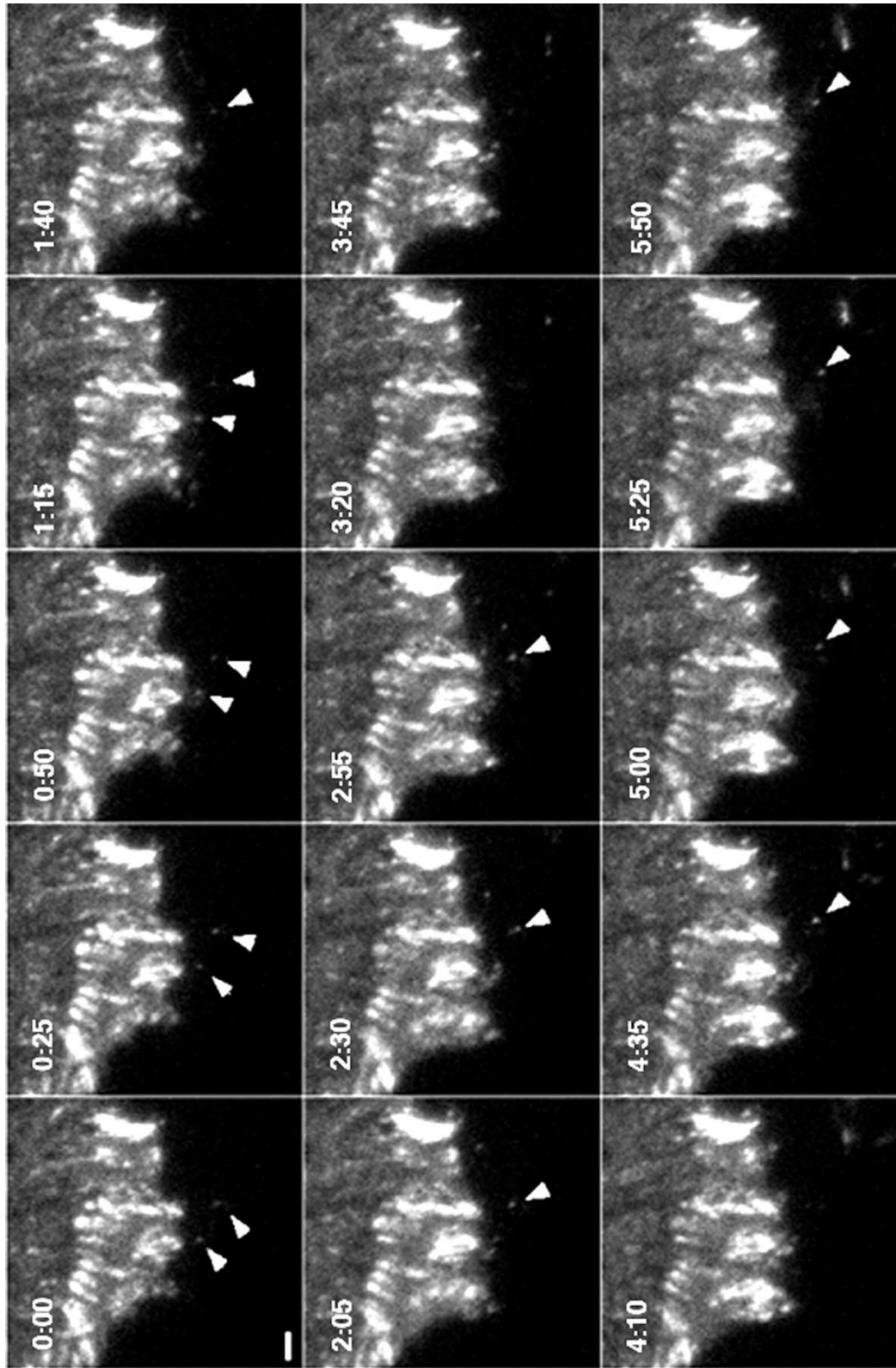
Figure 30: P130CasVenus localizes to the tips of filopodia. *Cas* -/- MEFs expressing p130CasVenus(WT) were imaged at the edge of a wounded cell monolayer by DIC and wide-field fluorescence microscopy. Arrows indicate the tip of the filopodium. The time stamp is indicated on each image corresponding to the time in minutes:seconds. Scale bar 5 μm.

A) Widefield fluorescence images of p130CasVenus(WT)

B) DIC images of p130CasVenus(WT)
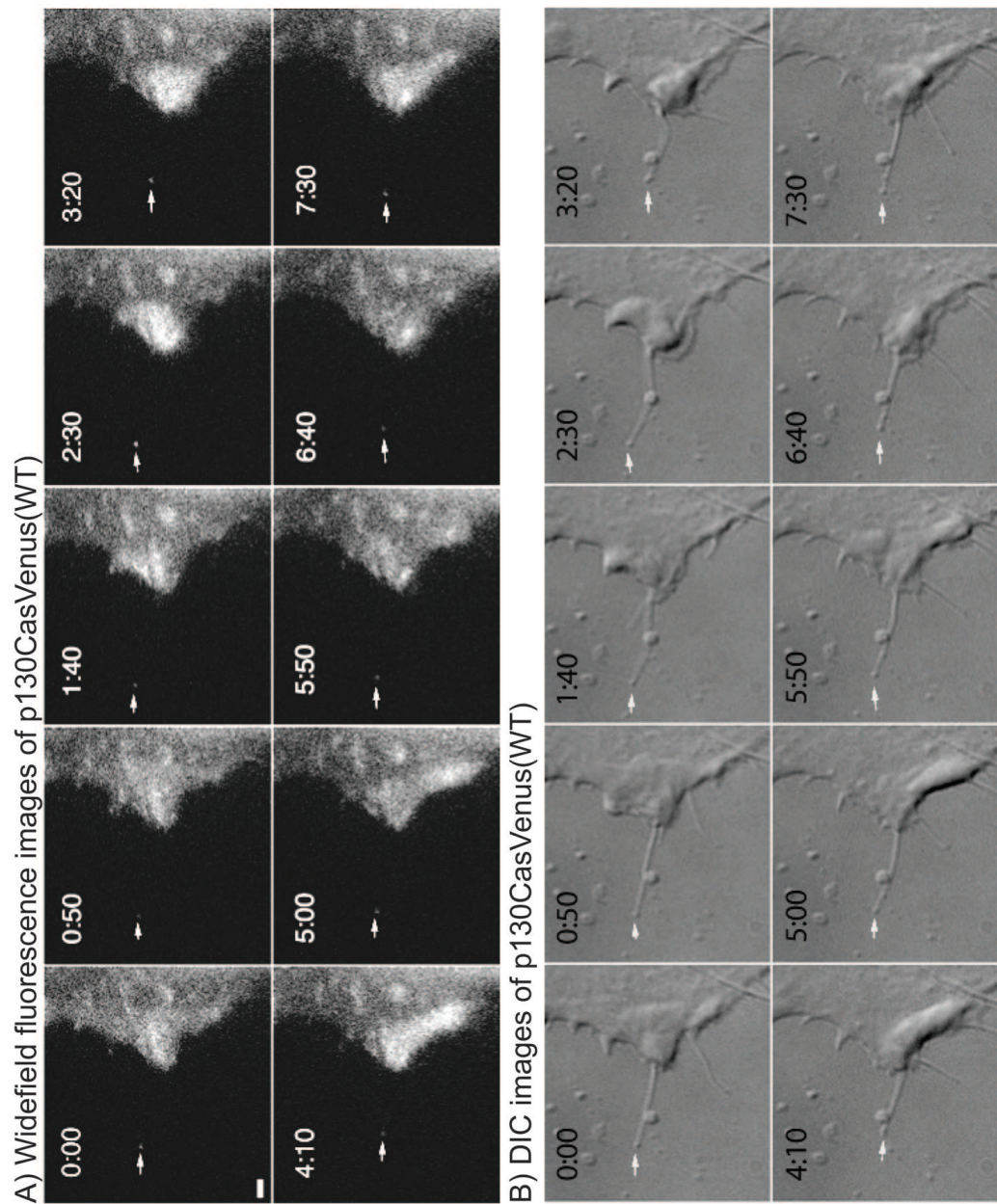
consistent with FA or FX localization (Fig. 29). These speckles moved rapidly in various directions without showing obvious turnover. Since it was not possible to discern the outline of the cell by TIRF imaging (hence making it impossible to discern what structures these speckles occurred in), DIC imaging and wide-field fluorescence microscopy were used to discern where these speckles were occurring. Using this technique, the small speckles were found to localize at the distal tips of filopodia

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(Fig. 30). Imaging of these structures over time showed that the speckle remained localized to the tip and did not track along the length of the filopodium. These data constitute the first observation of p130Cas at filopodial tips in live cells. p130Cas has been observed in areas that appear to be filopodia by immunostaining (Gustavsson et al, 2004).

CCHVenus can localize to areas of cell-cell contact

Previously, p130CasVenus(WT) was found to localize to cell-cell contacts of Caco-2 cells that were well-polarized (Lund, 2008), whereas HCF-7 cells, which are not as well polarized did not display such a localization. The mechanism by which p130Cas localized to these sites was not further investigated. By immunostaining, p130Cas had also been found to localize to cell-cell junctions in kidney epithelial cells.

It is therefore interesting to note that in fibroblasts that exhibited close contact with one another, CCHVenus could sometimes be seen in sites that appear to be nascent cell-cell contacts (Fig. 31). When cells were fixed and stained for paxillin, CCHVenus could be seen aligning along the periphery of cells where they made contact with one another, yet paxillin, which remained localized to FAs, was not seen in those sites. The localization of p130Cas at these sites appeared in a perpendicular angle to the cell-cell contacts themselves, in a pattern similar to stitches.

Discussion

Though the data presented for the p130CasVenus(L823E,L827E) mutant are by no means conclusive, this technique shows promise for the elucidation of specific residues important for the localization of p130Cas to FAs through the CCH domain. Future experiments will require that the cells be allowed to spread for longer amounts of time and that cells be cotransfected with an FA marker, such as mCherry-paxillin.
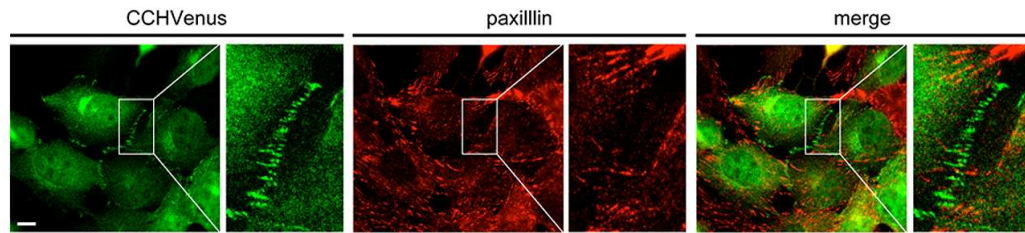
Figure 31: CCHVenus can localize to cell-cell contact areas. *Cas* -/- MEFs stably expressing CCHVenus were fixed in 4% paraformaldehyde and labeled for FAs with paxillin immunostaining. A representative cell that was imaged by wide-field fluorescence microscopy is shown along with enlarged insets of each channel. CCHVenus is shown in green and paxillin immunostaining in red. Cells were imaged with a 100X lens. Scale bar is 10 μm.

Additionally, studying this mutant in the context of the CCHVenus instead of the full-length protein should present a more definite idea of what these residues contributions to localization are. If this mutant is found to be defective in FA localization after further study, it would also serve as a useful tool for the identification of possible CCH binding partners (where this mutant could serve as a negative control in CoIP experiments.

As fibrillar adhesions are known to develop and mature for leading edge FAs (Zamir et al, 1999; Volberg et al, 2001), a loss of CasVenus fluorescence under FAK repressed conditions seems logical (since this might prevent the accumulation of other FA components in fibrillar adhesions). However, this does not appear to be the case, since CasVenus accumulates at a higher level in fibrillar adhesions when FAK is absent. Data obtained from TetFAK cells are suggestive of a particular role for the CCH domain in fibrillar adhesions and furthermore suggest that in the absence of the CCH domain, p130Cas may interact with other binding proteins either through its SH3 domain or other domains to localize p130Cas to these sites. To further explore the possibility of p130Cas switching SH3 domain partners at fibrillar adhesions, it would have been interesting to investigate the ΔSH3/ΔCCH domain mutant in this

context.

Since FAK does not appear to be a key fibrillar adhesion component (Zamir and Geiger, 2001) and its expression negatively affected CasVenus localization to fibrillar adhesions, a role for FAK in localizing p130Cas to fibrillar adhesions seems unlikely. As previously discussed, loss of the association between p130Cas and FAK leads to reduced SD tyrosine phosphorylation due to a loss of Src-binding to the FAK autophosphorylation site (Ruest et al, 2001). Though fibrillar adhesions have a relatively lower pTyr content than FAs (Katz et al, 2000), it is possible that p130Cas SD tyrosine phosphorylation is a key process in its incorporation to these sites. As discussed in Chapter 1, a distinctive feature of fibrillar adhesions is their tensin content. Interestingly, when CHO cells are stimulated by GH, p130Cas/CrkII complexes form that also contain tensin (Zhu et al, 1998). As GH stimulation leads to increased tyrosine phosphorylation p130Cas, it would be interesting to determine whether or not the loss of p130CasVenus(ΔCCH) in fibrillar adhesions was due to a reduction in tyrosine phosphorylation at these sites. Staining cells that exhibit fibrillar adhesions for Crk, may create some insight as to whether or not p130Cas SD phosphorylation and specifically, p130Cas/Crk coupling is involved in this localization. Mechanotransduction through p130Cas may also play a critical role, which might account for the reduction in targeting to fibrillar adhesions when the CCH domain is absent. In order for fibrillar adhesions to form, tensile forces on mature FAs must pull on these sites, resulting in the translocation of tensin and integrins to fibrils (Pankov et al, 2000). If mechanotransduction by p130Cas is aberrant in leading edge FAs, it may furthermore disrupt fibrillar adhesion formation. Considering the importance of p130Cas to mechanotransduction in FAs, an overall analysis of fibrillar adhesion number and size may also be warranted.

While it is difficult to come to any conclusions about the role of the p130Cas CCH in the formation of cell-cell contacts from these studies, the observation that such a

localization can occur sets a foundation for further investigation. Since fibroblasts do not form true cell-cell junctions, it would be useful to study the localization of CCHVenus is an epithelial cell line which is able to efficiently polarize in an apical-basolateral fashion. In kidney epithelial cells, p130Cas has been seen at cell-cell junctions with ZO-1 and Nephrocystin (Donaldson et al, 2000). The interaction between p130Cas and Nephrocystin was abrogated when the Nephrocystin SH3 domain was deleted. Therefore, it appears that the p130Cas SBD may recruit Nephrocystin to these sites. Since expression of Nephrocystin appears primarily in adult kidney tissues, it seems unlikely that the localization of p130CasVenus(WT) to cell-cell contacts of Caco-2 cells (intestinal epithelial cell line) would be mediated by the same interaction.

The expansion of cell-cell contacts and their maturation into cell-cell junctions is a carefully coordinated process that requires both lamellipodial protrusion driven by Rac1 and Arp2/3 as well as contractile forces exerted by RhoA and myosins (Yamada and Nelson, 2007). It is therefore possible that p130Cas is initially recruited to sites of cell-cell contact to promote actin polymerization. Alternately, p130Cas may be involved at these sites to promote an epithelial to mesynchemal transition (EMT), whereby well-polarized cells adopt a more promotile phenotype. Indeed, siRNA targeting of p130Cas in Caco-2 cells results in greater polarization than scamble siRNA as measured by a transwell resistance assay (Lund, 2008). While this localization to cell-cell contacts was observed with CCHVenus, the same observation was not made with p130CasVenus(WT) cells, suggesting that the use of this single domain may have stabilized what may otherwise be a more transient interaction. Identification of CCH binding partners, therefore, could be useful to perform overexpression experiments to attempt to stabilize the localization of the full-length p130Cas to these sites.

The observation of p130CasVenus(WT) to the distal tips of filopodia may also be related to cell-cell contact localization. Filopodia form in a response to migratory

stimuli, cell spreading, and formation of cell-cell contacts (Partridge and Marcantonio, 2006; Mattila and Lappalainen, 2008). In epithelial cells, filopodia are implicated in "zippering" epithelial cells together (Mattila and Lappalainen, 2008), a pattern that looks somewhat reminiscent of the "stitch" appearance seen in CCHVenus cell-cell contacts. Formation of these zippering sites is correlated with actomyosin contraction, which is important in establishing apical-basolateral polarization. Alternatively, p130Cas may be localizing to these filopodial tips to promote new FA formation and/or actin polymerization. During cell spreading, filopodia extend and generate *de novo* FAs. These filopodia are integrin-rich and recruit other FA proteins in a sequential manner as FAs form at their tips (Partridge and Marcantonio, 2006). One might therefore imagine a scenario, where p130Cas, which is closely linked to integrin signaling, can promote actin polymerization at tips of filopodia and may function to recruit and/or assemble FA proteins at these sites. In response to invasin (β-1 integrin ligand from *Yersinia tuberculosis*), p130Cas has been observed in areas of high pTyr staining that appear to be filopodia tips (Gustavsson et al, 2004). The authors also showed the CrkII localizes to these tips and that filopodial extension is defective when the SD is deleted (Gustavsson et al, 2004). Interestingly, Rac appears to be necessary for filopodial extension in response to invasin ligation, but not Cdc42 (Gustavsson et al, 2004). p130CasVenus variants could therefore serve as a useful tool for dissecting the manner of p130Cas targeting to filopodial tips.

CHAPTER V

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Concluding Remarks

The observation that p130Cas is elevated in tamoxifen-resistant breast cancer patients (van der Flier et al, 2000; Brinkman et al, 2000) and that it can also cause drug resistance to other drugs such as adriamycin (Ta et al, 2008), makes p130Cas a protein of interest for the development of cancer treatments. It is clear that p130Cas SD signaling is involved the ability of p130Cas to promote tumorigenesis (Soni et al, 2009; Cunningham-Edmondson and Hanks, 2009). Therefore, understanding the mechanisms by which p130Cas promotes such signaling is central to developing better therapies. The importance of p130Cas in integrin signaling and actin reorganization has long been noted (Petch et al, 1995; Nojima et al, 1995; Honda et al, 1999). Previous studies have shown that p130Cas SD signaling occurs at FAs (Fonseca et al, 2004; Ballestrem et al, 2006), yet the mechanisms by which this localization occurs was left unclear due to conflicting reports by Nakamoto et al (1997) and Harte et al (2000). The data presented in this thesis clarify the mechanism of p130Cas targeting to FAs by illustrating that the SH3 interaction with FAK plays an important role in localizing p130Cas to FAs, in addition to identifying the CCH domain as a separate site of FA targeting.

The importance of p130Cas FA localization was furthermore emphasized by showing that disruption of the p130Cas FA targeting mechanism leads to defective SD signaling and p130Cas-enhanced cell motility. It is clear from previous studies that SD signaling and p130Cas coupling of SH2 adapter proteins are key events during p130Cas signaling to promote cell motility (Klemke et al, 1998; Schlaepfer et al, 1997; Huang et al, 2002; Shin et al, 2004). Actin polymerization as a result of this

SD signaling does not appear to be the only mechanism by which p130Cas promotes cell motility, however, as evidenced by the observation that *Cas* -/- MEFs exhibit FA disassembly defects (Webb et al, 2004). Aberrant FA formation and reduced disassembly of FAs has been linked to cell motility defects in *FAK* -/- cells as well (Ilic et al, 1995; Webb et al, 2004).

Therefore, the data presented in Chapter 3 demonstrating that p130Cas resides in FAs throughout their lifetimes and disassembles at a slower rate in FAs than paxillin creates insights into the mechanisms of p130Cas regulation of overall FA disassembly. Clearly, the persistence of p130Cas in FAs at later time points of disassembly are consistent with a role for this docking protein in regulating the disassembly of other FA proteins from these sites (which was demonstrated previously by Webb et al (2004) by analyzing the turnover of paxillin in *Cas* -/- MEFs). Additionally, the observation that p130Cas has a high mobile fraction suggests that p130Cas exchanges in FAs in a dynamic manner, which may be critical to its role in FA signaling. These data provide an explanation for the transient tyrosine phosphorylation of p130Cas that was previously noted by Fonseca et al (2004). In light of observations made by Brown et al (2006) and Hu et al (2007) that FA components that bind F-actin exhibit faster dynamics than those located closer to the integrin interface, one may speculate that p130Cas might associate with an FA protein linked to F-actin. On the other hand, p130Cas clearly associates with FAK in FAs, and the retrograde flow of FAK in FAs, while it is coherent with actin retrograde flow, is still slower than the actin flow (Hu et al, 2007). If p130Cas were linked into FAs by one domain at a closer proximity to integrins (perhaps the SH3 domain) and to an actin-bound FA protein by the other domain (maybe the CCH domain), then p130Cas would be poised to act a sort of lever in FAs, whereby actomyosin contractility might be modulated. Such an association would be consistent with the mechanotransduction model of p130Cas SD signaling proposed by Sawada et al (2006). As FAs mature and greater force is applied to these

sites from stress fibers linked to FAs, p130Cas would become increasingly stretched, exposing its SD residues.

Observations of p130Cas localization to other subcellular compartments such as fibrillar adhesions, filopodial tips, and areas of cell-cell contact further emphasize that this is a protein that is closely linked to a variety of integrin- and actin-associated structures. It is likely that p130Cas can be recycled into a variety of these structures through its association with integrins and actin. For example, it is not difficult to conceive that integrins that were initially bound in FAs could be redistributed into filopodia and that p130Cas may be in complex with these integrins (directly or indirectly).

Future Directions

Identifying CCH binding partners

Though the studies presented here show that the CCH domain is an important FA targeting domain, the molecular mechanism by which this domain localizes p130Cas to FAs remains unknown. Identifying proteins which bind the p130Cas CCH domain would therefore be a logical step forward. Using CCHVenus cells to perform CoIP experiments, for instance, might be a good way to identify interacting partners. GFP antibodies that have the ability to IP YFP are commercially available. Alternatively, CoIP experiments with full-length p130Cas and the CCH deletion mutant could be used to identify binding partners that are lost in the latter case. Use of the p130Cas(WT) and p130Cas(ΔCCH) may offer the advantage of capturing interactions that may first require the assembly of p130Cas complexes through its other interaction domains. As construction of p130Cas mutants with individual residues mutated in the CCH domain are currently in development, these mutants could also be used in CoIP experiments.

One consideration that may complicate CoIP experiments is the fact that the interaction of p130Cas with FAs appears to be so transient. Therefore, it may be necessary to perform reversible cross-linking of proteins in cell lysates in order to detect these interactions. Once IP experiments are performed, the products can be run separated by SDS-PAGE, visualized by Coomassie staining, and bands of interest can be isolated for identification by LC/MS/MS (liquid chromatography and two runs of mass spectrometry).

Though IP of proteins that were processed intracellularly is preferable, binding proteins may also be identified by using a His-tagged construct of the CCH domain to perform a pull-down assay. In this scenario, the recombinant protein bound to Nickel beads would be incubated with whole cell lysates from fibroblasts in hopes of capturing interacting proteins. This assay may be complicated, however, by the fact that this recombinant protein is difficult to elute off the bead (data not shown). Use of thrombin cleavage to remove the CCH domain from the His-tag and therefore the bead, has also been unsuccessful. The CCH domain remains bound to the bead, presumably because of some other interaction that may be a result the domains tertiary folding.

Probing the CCH domain structure

An ongoing collaboration with the lab of Jie Zheng, Ph.D. at St. Jude Children's Hospital in Memphis, TN has led to a new prediction for the tertiary structure of the CCH domain. The Zheng lab used a longer His-tagged construct that also includes the SBD (including the YDYV motif thought to bind the Src SH2 domain) and suggest that the area including the SBD could form a fourth α-helix (by secondary structure prediction). Should NMR structure data corroborate these predictions, the extended domain would be a somewhat novel FA targeting structure with some similarities to the FAK FAT domain. Interestingly, the FAK FAT domain contains a YENV motif at

Tyr925 in the first α-helix whose accessibility to phosphorylation has been postulated to be unlikely without structural remodeling (Arold et al, 2002). Simulations of FAK FAT domain "unraveling" have been performed, with an estimation that mechanotransduction forces could unfold the four helical bundle of the FAK FAT domain and lead to a dissociation of FAK and paxillin (Mofrad et al, 2004). However, the effect on Tyr925 exposure was not specifically included in this analysis.

Until an NMR structure of the CCH domain is obtained, continued studies by site-directed mutagenesis of this domain may be useful to identify features important for its localization to FAs. In addition to the amino acids mutated in Chapter 4 (L823 and L827), other residues may play a role in the helical bundling of the CCH domain (if these helical bundles in fact exist): I830 and L871. A single CCH mutant plasmid (in the context of the full-length protein) has been generated for L827, but has not yet been evaluated. It will most likely be preferable to evaluate the localization of this mutant in the context of CCHVenus by itself. Additionally, the surface patch that has been implicated in the binding of paxillin LD motifs by the FAK FAT is formed between helices 2 and 3 of the FAK FAT domain. By mutating the following residues on the CCH domain, a better idea of whether or not a surface patch is involved in recruiting the CCH domain to FAs could be evaluated: Lys787, Asp801, and Lys835. Development of the Lys787 mutant (mutated to Ala) in the context of the CCH domain is currently under way. Since these mutants are being created on the basis of similarity to the FAK FAT domain, it may be interesting to compare their localization of a comparable WT FAK FAT fusion protein. Subcloning of such a DNA construct is currently under way. However, it may be difficult to evaluate differences of localization between the FAK FAT and CCH domains in the context of the same cell, as the FAK FAT domain essentially functions as a dominant negative (which may cause the mislocalization of the CCH binding partner if it is reliant on the localization of FAK to FAs). Since p130CasVenus still localized to FAs in the

absence of FAK expression, it may however, be reasonable to expect that the CCH domain could still localize to FAs.

Elucidating features of the p130Cas mobility in FAs

Using FRAP to analyze the recovery of p130CasVenus in comparison to actin and integrins might offer greater insight into the transience of p130Cas at FAs. In particular, it would be interesting to perform FRAP in nascent FXs in comparison to FAs and observe whether or not the stage of FA maturation can affect the transience of p130Cas localization. Though the SH3 and CCH mutants do show reduced phosphorylation, it may be necessary for p130Cas to be completely dephosphorylated in order to dissociate from FAs. Therefore, use of phosphatase inhibitors during FRAP may be an interesting tool. If the mobile fraction reflects a pool of p130Cas that is not phosphorylated on tyrosine, application of a phosphatase inhibitor should lead to a smaller mobile fraction. Alternately, if tyrosine phosphorylation serves as cue for the dissociation of p130Cas from FAs, the expression of an SD mutant might increase the immobile fraction and retard the recovery of fluorophores to FAs after photobleaching.

Investigating the localization of p130Cas in other subcellular compartments

Though the current studies have created a clearer understanding of what domains in p130Cas are required for its localization to leading edge FAs (and partially to fibrillar adhesions), a more complete study of domains required for the localization of p130Cas to other classes of ECM/cell contacts is still lacking. For instance, it is still not clear which domains are required for the localization of p130Cas to FXs and podosomes (actin-rich invasive structures at the ECM/cell interface). Cells developed for the studies in this thesis, which express p130CasVenus variants, would therefore be useful tools in such future studies. To understand what domains are required for focal complex localization, for instance, cells can be stimulated to form FXs by the

application of a ROCK inhibitor or by driving Rac activity by transfecting cells with a constitutively active Rac construct (Zaidel-Bar et al, 2003). Paxilllin and/or talin could be used as markers of FXs in such a setting and the localization of p130CasVenus variants can be similarly studied as was done in Chapter 2 in leading edge FAs.

To study the localization of p130CasVenus variants in podosomes, the cells used in the current study could be stimulated with phorbol esters or transfected with a constitutively active Src DNA construct to promote podosome formation. Immunostaining cells for tyrosine phosphorylated cortactin would serve as a good marker for podosomes. Colocalization of p130CasVenus constructs with cortactin in such cells could then be used to evaluate the contribution of these domains to p130Cas localization in podosomes. Previous studies in *Cas* -/- MEFs have shown that the formation of podosomes is greatly reduced in comparison to *Cas* +/+ MEFs when they are transformed with a constitutively active Src (Brabek et al, 2004). Formation of large podosome structures was also inhibited when p130Cas variants with SD and SBD mutations were expressed in *Cas* -/- MEFs (Brabek et al, 2005). However, expression of an SH3 mutant did not significantly reduce the numbers of large podosomes formed in response to Src-transformation. Yet, the activation of matrix metalloproteinase-2 was reduced in cells expressing the SH3 mutant. Using phospho-specific antibodies to the p130Cas SD, Alexander et al (2008), demonstrated that p130Cas is localized and phosphorylated in invadopodia. Since invadopodia/podosomes are ECM/cell contacts composed of many of the same components present in FAs, it is likely that some of the same mechanisms that regulate p130Cas localization to FAs may be necessary for the localization of p130Cas to podosomes.

The observation of p130CasVenus at the tips of filopodia is intriguing. dDia2 has been found at filopodial tips and is thought to act as a an inhibitor of actin filament capping, thereby promoting continued filopodial protrusion. Src is known to interact with dDia2. It would be interesting to know if p130Cas functions in

this compartment to promote 1) actin polymerization or 2) a Src/dDia2 interaction. Interestingly, myosin X has also been found at the tips of filopodia, where it has been implicated in the trafficking of integrins (Zhang et al, 2004), presumably to test out the ECM for sites of ligation. It would be interesting to further elucidate the function of p130Cas in filopodia. Using transient expression of a constitutively active form of Rho in filopodia (Rif) to produce filopodial extension leads to the formation of so many filopodia that the cells appears "hairy" (Ellis and Mellor, 2000). This approach would be a great tool do determine whether or not p130CasVenus variants, such as the ΔCCH are capable of localizing to filopodia or not. Additionally, this approach would allow useful to perform colocalization experiments in which the colocalization of p130Cas with formins or myosins in filopodial tips (either by immunostaining or cotransfection) could be monitored.

The localization of p130Cas in filopodia could also be related to its localization in cell-cell junctions (as observed in epithelial cells by Lund (2008)). One of the functions of filopodia is to sense where other cells are and make contact with them. Indeed, in endothelial cells, myosin-X and vascular endothelium-cadherin (VE-cadherin) colocalize at filopodial tips (Almagro et al, 2010), where the FERM domain binds VE-cadherin and transports VE-cadherin to the filopodial tips. Interestingly, in a yeast two-hybrid screen for proteins that interacted with the C-terminus of p130Cas (including the SBD), myosin-X was implicated as an interacting partner (unpublished data from the screen conducted by Donaldson et al (2000).) This interaction was not demonstrated in mammalian cells, however.

Clearly, the studies performed for this dissertation have elucidated many aspects of p130Cas FA localization. With the new insights created by these studies, new questions and observations have arisen that may be promising avenues of future study. Continued efforts to understand domain contributions to p130Cas subcellular localization are well-warranted, as this protein's localization is so heavily implicated in cell

signaling pathways affecting various cell functions such as cell migration, invasion, survival, and pathogenesis.

REFERENCES

Achiwa H, Lazo J (2007) PRL-1 tyrosine phosphatse regulates c-Src levels, adherence, and invasion in human lung cancer cells. Cancer Res 67:643–650

Alexander N, Branch K, Parekh A, ES C, Iwueke I, Guelcher S, Weaver A (2008) Extracellular matrix rigidity promotes invadopodia activity. Curr Biol 18:1295–9

Alexandropoulos K, Baltimore D (1996) Coordinate activation of c-Src by SH3- and SH2-binding sites on a novel, p130Cas-related protein, Sin. Genes Dev 10:1341–1355

Alexandropoulos K, Regelmann A (2009) Regulation of T-lymphocyte physiology by the Chat-H/CasL adapter complex. Immunol Rev 232:160–174

Almagro S, Durmort C, Chervin-Petinot A, Heyraud S, Dubois M, Lambert O, Maillefaud C, Hewat E, Schaal J, Huber P, Gulino-Debrac D (2010) The motor protein myosin-X transports ve-cadherin along filopodia to allow the formation of early endothelial cell-cell contacts. Mol Cell Biol 30:1703–1717

Almeida E, Ilic D, Han Q, Hauck C, Jin F, Kawakatsu H, Schlaepfer D, Damsky C (2000) Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase. J Cell Biol 149:741–754

Armulik A, Velling T, Johansson S (2004) The integrin beta1 subunit transmembrane domain regulates phosphatidylinositol 3-kinase-dependent tyrosine phosphorylation of Crk-associated substrate. Mol Biol Cell 15:2558–67

Arold S, Hoellerer M, Noble M (2002) The structural basis of localization and signaling by the focal adhesion targeting domain. Structure 10:319–327

Astier A, Manie S, Law S, Canty T, Hahayghi N, Druker B, Salgia R, Golemis E, Freedman A (1997) Association of the Cas-like molecule HEF1 with Crk following integrin and antigen receptor signaling in human B-cells: potential relevance to neoplastic lymphohematopoietic cells. Leuk Lymphoma 28:65–72

Ballestrem C, Erez N, Kirchneer J, Kam Z, Bershadsky A, Geiger B (2006) Molecular mapping of tyrosine-phosphorylated proteins in focal adhesions using fluorescence resonance energy transfer. J Cell Sci 119:866–875

Bannerman D, Sathyamoorthy M, Goldblum S (1998) Bacterial lipopolysaccharide disrupts endothelial monolayer integrity and survival signaling events through caspase cleavage of adherens junction proteins. J Biol Chem 273:35,371–35,380

Beckerle M (1997) Zyxin: zinc fingers at sites of cell adhesion. Bioessays 19:949–957

Beinke C, Van Beuningen D, Cordes N (2003) Ionizing radiation modules of the expression and tyrosine phosphorylation of the focal adhesion-associated proteins focal adhesion kinase (FAK) and its substrates p130cas and paxillin in A549 human lung carcinoma cells in vitro. Int J Radiat Biol 79:721–31

Bertolucci C, Guibao C, Zheng J (2005) Structural features of the focal adhesion kinase-paxillin complex give insight into the dynamics of focal adhesion assembly. Prot Sci 14:644–652

Black D, Bliska J (1997) Identification of p130Cas as a substrate of Yersinia YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. EMBO J 16:2730–2744

Bouton A, Riggins R, Bruce-Staskal P (2001) Functions of the adapter protein Cas: signal convergence and the determination of cellular responses. Oncogene 20:6448–6458

Brabek J, Constancio S, Shin N, Pozzi A, Weaver A, Hanks S (2004) Cas promotes invasiveness of src-transformed cells. Oncogene 23:7406–7415

Brabek J, Constancio S, Siesser P, Shin N, Pozzi A, Hanks S (2005) Crk-associated substrate tyrosine phosphorylation sites are critical for invasion and metastasis of SRC-transformed cells. Mol Cancer Res 3:307–315

Briknarova K, Nasertorabi F, Havert M, Eggleston E, Hoyt D, Li C, Olson A, Vuroi K, Ely K (2005) The serine-rich domain from Crk-associated substrate (p130cas) is a four-helix bundle. J Biol Chem 280:21,908–21,914

Brinkman A, van der Flier S, Kok E, Dorssers L (2000) BCAR1, a human homologue of the adapter protein p130Cas, and antiestrogen resistance in breast cancer cells. J Natl Cancer Inst 92:112–120

Broussard J, Webb D, Kaverina I (2008) Assymetric focal adhesion disassembly in motile cells. Curr Opin Cell Biol 20:85–90

Brown C, Hebert B, Kolin D, Zareno J, Whitmore L, Horwitz A, Wiseman P (2006) Probing the integrin-actin linkage using high-resolution protein velocity mapping. J Cell Sci 119:5204–5214

Buday L, Wunderlich L, Tamas P (2002) The Nck family of adapter proteins: regulators of actin cytoskeleton. Cell Signal 14:723–731

Burnham M, Harte M, Bouton A (1999) The role of SRC-CAS interactions in cellular transformation: ectopic expression of the carboxy terminus of CAS inhibits SRC-CAS interaction but has no effect on cellular transformation. Molecular Carcinogenesis 26:20–31

Burnham M, Bruce-Staskal P, Harte M, Weidow C, Ma A, Weed S, Bouton A (2000) Regulation of c-SRC activity and function by the adapter protein CAS. Mol Cell Biol 20:5865–5878

Cabodi S, Moro L, Baj G, Smeriglio M, Di Stefano P, Gippone S, Surico N, Silengo L, Turco E, Tarone G, Defilippi P (2004) p130Cas interacts with estrogen receptor alpha and modulates non-genomic estrogen signaling in breast cancer cells. J Cell Sci 117:1603–1611

Cabodi S, Tinnirello A, Di Stefano P, Bisaro B, Ambrosino E, Castellano I, Sapino A, Arisio R, Cavallo F, Forni G, Glukhova M, Silengo L, Altruda F, Turco E, Tarone G, Defilippi P (2006) p130Cas as a new regulator of mammary epithelial cell proliferation, survival, and HER2-neu oncogene-dependent breast tumorigenesis. Cancer Res 66:4672–4680

Cantley L, Songyang Z (1994) Specificity in recognition of phosphopeptides by src-homology 2 domains. J Cell Sci Suppl 18:121–126

Cartwright C, Simantov R, Cowan W, Hunter T, Eckhart W (1988) pp60-src expression in the developing rat brain. Proc Natl Acad Sci USA 85:3348–3352

Cary L, Han D, Polte T, Hanks S, Guan J (1998) Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. J Cell Biol 140:211–21

Casamassima A, Rozengurt E (1997) Tyrosine phosphorylation of p130(cas) by bombesin, lysophosphatidic acid, phorbol esters, and platelet-derived growth factor. signaling pathways and formation of a p130(cas)-crk complex. J Biol Chem 272:9363–70

Casamassima A, Rozengurt E (1998) Insulin-like growth factor i stimulates tyrosine phosphorylation of p130(Cas), focal adhesion kinase, and paxillin. role of phosphatidylinositol 3'-kinase and formation of a p130(Cas).Crk complex. J Biol Chem 273:26,149–56

Casanova I, Parreno M, Farre L, Gurerrero S, Cespedes M, Pavon M, Sancho F, Marcuello E, Trias M, Mangues R (2006) Celecoxib induces anoikis in human colon carcinoma cells associated with the deregulation of focal adhesions and nuclear translocation of p130cas. Int J Cancer 118:2381–2389

Chan Y, Gallo R (1998) PR-39, a syndecan-inducing antimicrobial peptide, binds and affects p130(Cas). J Biol Chem 273:28,978–28,985

Chen H, Appeddu P, Parsons J, Hildebrand J, Schaller M, Guan J (1995) Interaction of focal adhesion kinase with cytoskeletal protein talin. J Biol Chem 270:16,995–16,999

Cho S, Klemke R (2000) Extracellular-regulated kinase activation and CAS/Crk coupling regulate cell migration and suppress apoptosis during invasion of the extracellular matrix. J Cell Biol 149:223–236

Chodniewicz D, Klemke R (2004) Regulation of integrin-mediated cellular responses through assembly of a CAS/Crk scaffold. Biochim Biophys Acta 1692:63–76

Chrzanowska-Wodnicka M, Burridge K (1996) Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J Cell Biol 133:1403–1415

Cole C, Barber J, Barton G (2008) The jpred 3 secondary structure prediction server. Nucleic Acids Research 36:W197–201

Cotteret S, Chernoff J (2002) The evolutionary history of effectors downstream of cdc42 and rac. Genome Biol 3:reviews0002.1–0002.8

Cowell L, Graham J, Bouton A, Clarke C, O'neill sG (2006) Tamoxifen treatment promotes phosphorylation of the adhesion molecules, p130cas/bcar1, fak and src, via an adhesion-dependent pathway. Oncogene 25:7597–7607

Cunningham-Edmondson A, Hanks S (2009) P130Cas substrate domain signaling promotes growth, migration, and invasion of estrogen-receptor-negative breast cancer cells. Breast Cancer: Targets and Therapy 1:39–52

Czuchra A, Wu X, Meyer H, van Hengel J, Schroeder T, Geffers R, Rottner K, Brakebusch C (2005) Cdc42 is not essential for filopodium formation, directed migration, cell polarization, and mitosis in fibroblastoid cells. Mol Biol Cell 16:4473–4484

Defilippi P, Di Stefano P, Cabodi S (2006) p130Cas: a versatile scaffold in signaling networks. Trends Cell Biol 16:257–263

Dolfi F, Garcia-Guzman M, Ojaniemi M, Nakamura H, Matsuda M, Vuori K (1998) The adaptor protein Crk connects multiple cellular stimuli to the JNK signaling pathway. Proc Natl Acad Sci USA 95:15,394–15,399

Donaldson J, Dempsey P, Reddy S, Bouton A, Coffey R, Hanks S (2000) Crk-associated Substrate p130 CAS interacts with Nephrocystin and Both Proteins Localize to Cell-Cell Contacts of Polarized Epithelial Cells. Exp Cell Res 256:168–178

Dorssers L, van der Flier S, Brinkman A, van Agthoven T, Veldscholte J, Berns E, Klijn J, Beex L, Foekens J (2001) Tamoxifen resistance in breast cancer: elucidating mechanisms. Drugs 61:1721–1733

Ellis H, Mellor H (2000) The novel Rho-family GTPase rif regulates coordinated actin-based membrane rearrangements. Curr Biol 10:1387–1390

Etienne-Manneville S, Hall A (2001) Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. Cell 106:489–498

Faix J, Rottner K (2006) The making of filopodia. Curr Opin Cell Biol 18:18–25

Feller S (2001) Crk family adaptors-signalling complex formation and biological roles. Oncogene 20:6348–6371

Flevaris P, Stojanovic A, Gong H, Chishti A, Welch E, Du X (2007) A molecular switch that controls cell spreading and retraction. J Cell Biol 179:553–563

van der Flier S, Brinkman A, Look M, Kok E, Meijer-van Gelder M, Klijn J, Dorssers L, Foekens J (2000) Bcar1/p130Cas protein and primary breast cancer: prognosis and response to tamoxifen treatment. J Natl Cancer Inst 92:120–127

Fonseca P, Shin N, Brabek J, Ryzhova L, Wu J, Hanks S (2004) Regulation and localization of CAS substrate domain tyrosine phosphorylation. Cell Signal 16:621–629

Frankel P, Pellet-Many C, Lehtolainen P, D'Abaco G, Tickner M, Cheng L, Zachary I (2008) Chondroitin sulphate-modified neuropilin 1 is expressed in human tumour cells and modulates 3D invasion in the U87MG human glioblastoma cell line through a p130Cas-mediated pathway. EMBO Rep 9:983–9

Frisch S, Ruoslahti E (1997) Integrins and anoikis. Curr Opin Cell Biol 9:701–706

Garcia-Guzman M, Dolfi M, Vuori K (1999) Cell Adhesion regulates the interaction between the docking protein p130Cas and the 14-3-3 proteins. J Biol Chem 274:5762–5768

Garron M, Arsenieva D, Zhong J, Bloom A, Lerner A, GM O, Arold S (2009) Structural insights into the association between BCAR3 and Cas family members, an atypical complex implicated in anti-oestrogen resistance. J Mol Biol 386:190–203

Garton A, Flint A, Tonks N (1996) Identification of p130(cas) as a substrate for the cytosolic protein tyrosine phosphatase PTP-PEST. Mol Cell Biol 16:6408–6418

Garton A, Burnham M, Bouton A, Tonks N (1997) Association of PTP-PEST with the SH3 domain of p130cas; a novel mechanism of protein tyrosine phosphatase substrate recognition. Oncogene 15:877–885

Gerrow K, El-Husseini A (2006) Cell adhesion molecules at the synapse. Front Biosci 11:2400–2419

Goldmann W, Ezzell R, Adamson E, Niggli V, Isenberg G (1996) Vinculin, talin and focal adhesions. Journal of Muscle Research and Cell Motility 17:1–5

Gomes E, Jani S, Gundersen G (2005) Nuclear movement regulated by Cdc42, MRCK, myosin, and actin flow establishes MTOC polarization in migrating cells. Cell 121:451–463

Gotoh T, Hattori S, Nakamura S, Kitayama H, Noda M, Takai Y, Kaibuchi K, Matsui H, Hatase O, Takahashi H (1995) Identfication of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G. Mol Cell Biol 15:6746–6753

Gotoh T, Niino Y, Tokuda M, Hatasse O, Nakamura S, Matsuda M, Hattori S (1997) Activation of R-Ras by Ras-guanine nucleotide-releasing factor. J Biol Chem 272:18,602–180,607

Gotoh T, Cai D, Tian X, Feig L, Lerner A (2000) p130Cas regulates the activity of AND-34, a novel Ral, Rap1, and R-Ras guanine nucleotide exchange factor. J Biol Chem 275:30,118–30,123

Guo C, Liu Q, Yang W, Zhang Z, Yao Y (2008) Relation among p130Cas, E-cadherin and beta-catenin expression, clinicopathologic significance and prognosis in human hepatocellular carcinoma. Hebatobiliary Pancreat Dis Int 7:490–6

Gustavsson A, Yuan M, Fallman M (2004) Temporal dissection of beta1-integrin signaling indicates a role for p130Cas-Crk in filopodia formation. J Biol Chem 279:22,893–901

Hamamura K, Furukawa K, Hayashi T, Hattori T, Nakano J, Nakashima H, Okuda T, Mizutani H, Hattori H, Ueda M, Urano T, Lloyd K, Furukawa K (2005) Ganglioside gd3 promotes cell growth and invasion through p130cas and paxillin in malignant melanoma cells. Proc Natl Acad Sci USA 102:11,041–6

Hamamura K, Tsuji M, Ohkawa Y, Nakashima H, Miyazaki S, Urano T, Yamamoto N, Ueda M, Furukawa K, Furukawa K (2008) Focal adhesion kinase as well as p130cas and paxillin is crucially involved in the enhanced malignant properties under expression of ganglioside gd3 in melanoma cells. Biochim Biophys Acta 1780:513–509

Hamasaki K, Mimura T, Morino N, Furuya H, Nakamoto T, Aizawa S, Morimoto C, Yazaki Y, Hirai H, Nojima Y (1996) Src kinase plays an essential role in integrin-mediated tyrosine phosphorylation of Crk-associated substrate p130Cas. Biochem Biophys Res Comm 222:338–343

Hanks S, Polte T (1997) Signaling through focal adhesion kinase. Bioessays 19:137–145

Harte M, Hildebrand J, Burnham M, Bouton A, Parsons J (1996) p130cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. J Biol Chem 271:13,649–13,655

Harte M, Macklem M, Weidow C, Parsons J, Bouton A (2000) Identification of two focal adhesion targeting sequences in the adapter molecule p130(Cas). Biochim Biophys Acta 1499:34–48

Hayashi I, Vuori K, Liddington R (2002) The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. Nat Struct Biol 9:101–106

Honda H, Oda H, Nakamoto T, Honda Z, Sakai R, Suzuki T, Saito T, Nakamura K, Nakao K, Ishikawa T, Katsuki M, Yazaki Y, Hirai H (1998) Cardiovascular

anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. Nat Gen 19:361–365

Honda H, Nakamoto T, Sakai R, Hirai H (1999) p130Cas, an Assembling Molecule of Actin Filaments, Promotes Cell Movement, Cell Migration, and Cell Spreading in Fibroblasts. Biochem Biophys Res Comm 262:25–30

Hu K, Ji L, Applegate K, Danuser G, Waterman-Storer C (2007) Differential transmission of actin motion within focal adhesions. Science 315:111–115

Huang J, Hamasaki H, Nakamoto T, Honda H, Hirai H, Saito M, Takato T, Sakai R (2002) Differential regulation of cell migration, actin stress fiber organization, and cell transformation by functional domains of Crk-associated substrate. J Biol Chem 277:27,265–27,272

Huang J, Sakai R, Teiichi F (2006) The docking protein Cas links tyrosine phosphorylation signaling to elongation of cerebellar granule cell axons. Mol Biol Cell 17:3187–3196

Huang Z, Yazdani U, Thompson-Peer K, Kolodkin A, Terman J (2007) Crks-associated substrate (cas) signaling protein functions with integrins to specify axons guidance during development. Development 134:2337–2347

Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature 377:539–544

Inagaki K, Noguchi T, Matozaki T, Horikawa T, Fukunuga K, Tsuda M, Ichihashi M, Kasuga M (2000) Roles for the protein tyrosine phosphatase shp-2 in cytoskeletal organization, cell adhesion and cell migration revealed by overexpression of a dominant negative mutant. Oncogene 19:75–84

Ireton R, Davis M, van Hengel J, Mariner D, Barnes K, Thoreson M, Anastasiadis P, Matrisian L, Bundy LM, Sealy L, Gilbert B, van Roy F, Reynolds A (2002) A novel role for p120 catenin in E-cadherin function. J Cell Biol 159:465–476

Ishino M, Ohba T, Sasaki H, Sasaki T (1995) Molecular cloning of a cDNA encoding a phosphoprotein, Efs, which contains a Src homology 3 domain and associates with Fyn. Oncogene 11:2331–2338

de Jong R, van Wijk A, Haataja L, Heisterkamp N, Groffen J (1997) BCR/ABL-induced leukemogenesis causes phosphorylation of Hef1 and its association with Crk1. J Biol Chem 272:32,649–32,655

Kain K, Gooch S, Klemke R (2003) Cytoplasmic c-Abl provides a molecular 'Rheostat' controlling carcinoma cell survival and invasion. Oncogene 22:6071–6080

Katz B, Zamir E, Bershadsky A, Kam Z, Yamada K, Geiger B (2000) Physical state of the extracellular matrix regulates the structure and molecular composition of cell-matrix adhesions. Mol Biol Cell 11:1047–1060

Kaverina I, Krylyshkina O, Small J (2002) Regulation of substrate adhesion dynamics during cell motility. Internatl J Biochem Cell Biol 34:746–761

Kim M, Gans J, Nogueira A C an Wang, Paik J, Feng B, Brennan C, Hahn W, Cordon-Cardo C, Wagner S, Flotte T, Duncan L, Granter S, Chin L (2006) Comparative oncogenomics identifies nedd9 as a melanoma metastasis gene. Cell 125:1269–1281

Kim W, Kook S, Kim D, Teodorof C, Song W (2003) The 31-kDa Caspase-generated Cleavage Product of p130 CAS Functions as a Transcriptional Repressor of E2A in Apoptotic Cells. J Biol Chem 279:833–8342

Kirsch K, Georgescu M, Hanafusa H (1998) Direct binding of p130(Cas) to the guanine nucleotide exchange factor C3G. J Biol Chem 273:25,673–25,679

Kirsch K, Georgescu M, Ishimaru S, Hanafusa H (1999) CMS: an adapter molecule involved in cytoskeletal rearrangements. Proc Natl Acad Sci USA 96:6211–6216

Kiyokawa E, Hashimoto Y, Kobayashi S, Sugimura H, Kurata T, Matsuda M (1998a) Activation of Rac1 by a Crk SH3-binding protein, DOCK180. Genes Dev 12:3331–3336

Kiyokawa E, Hashimoto Y, Kurata T, Sugimara H, Matsuda M (1998b) Evidence that DOCK180 up-regulates signals from the Crkii-p130(Cas) complex. J Biol Chem 273:24,479–24,484

Klemke R, Leng J, Molander R, Brooks P, Vuori K, Cheresh D (1998) CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. J Cell Biol 140:961–972

Kovacic-Milivojevic B, Roediger F, Almeida E, Damsky C, Gardner D, Ilic D (2001) Focal adhesion kinase and p130Cas mediate both sarcomeric organization and activation of genes associated with cardiac myocyte hypertrophy. Mol Biol Cell 12:2290–307

Kovacic-Milivojevic B, Damsky C, Gardner D, Ilic D (2002) Requirements for the localization of p130 Cas to Z-lines in cardiac myocytes. Cell Mol Biol Lett 7:323–9

Kupfer A, Louvard D, Singer S (1982) Polarization of the Golgi apparatus and the microtubule-organizing center in cultured fibroblasts at the edge of an experimental wound. Proc Natl Acad Sci USA 79:2603–2607

Law S, Estojak J, Wang B, Mysliwiec T, Kruh G, Golemis E (1996) Human enhancer of filamentation 1, a novel p130cas-like docking protein, associates with focal adhesion kinase and induces pseudohyphal growth in Saccharomyces cerevisiae. Mol Cell Biol 16:3327–3337

Law S, Zhang Y, Klein-Szanto A, Golemis E (1998) Cell cycle-regulated processing of HEF1 to multiple protein forms differentially targeted to multiple subcellular compartments. Mol Cell Biol 18:3540–3551

Law S, Zhang Y, Fashena S, Toby G, Estojak J, Golemis E (1999) Dimerization of the docking/adaptor protein HEF1 via a carboxy-terminal helix-loop-helix domain. Exp Cell Res 252:224–235

Law S, O'neill G, Fashena S, Einarson M, Golemis E (2000) The docking protein HEF1 is an apoptotic mediator at focal adhesion sites. Mol Cell Biol 20:5184–5195

Lee T, Gold G, Workman R, Cook C, Konrad R (2004) Glucose stimulates the association of crk with p130cas in pancreatic beta cells. Pancreas 29:e100–e105

Li E, Stupack D, Brown S, Klemke R, Schlaepfer D, Nemerow G (2000) Association of p130CAS with phosphatidylinositol-3-OH kinase mediates adenovirus cell entry. J Biol Chem 275:14,729–17,435

Liu F, Hill D, Chernoff J (1996) Direct binding of the proline-rich region of protein tyrosine phosphatase 1B to the Src homology 3 domain of p130(Cas). J Biol Chem 271:31,290–31,295

Liu G, Guibao C, Zheng J (2002) Structural insight into the mechanisms of targeting and signaling of focal adhesion kinase. Mol Cell Biol 22:2751–2760

Lu Y, Brush J, Stewart T (1999) NSP1 defines a novel family of adaptor proteins linking integrin and tyrosine kinase receptors to the c-Jun N-terminal kinase/ stress-activated protein kinase signaling pathway. J Biol Chem 274:10,047–10,052

Lund S (2008) Studies on Src Tyrosine Kinase in Tumorigenic Cell Growth and Invasion. PhD thesis, Vanderbilt University School of Medicine

Luo W, Slebos R, Hill S, Li M, Brabek J, Amanchy R, Chaerkady R, Pandey A, Ham AJ, Hanks S (2008) Global impact of oncogenic Src on a phosphotyrosine proteome. J Prot Res 7:3447–3460

Makkinje A, Near R, Infusini G, Vanden Borre P, Bloom A, Cai D, Costello C, Lerner A (2009) AND-34/BCAR3 regulates adhesion-dependent p130Cas serine phosphorylation and breast cancer cell growth pattern. Cellular Signaling 21:1423–1435

Manie S, Beck A, Astier A, Law S, Canty T, Hirai H, Druker B, Avraham H, Haghayeghi N, Sattler M, Salgia R, Griffin J, Golemis E, Freedman A (1997) Involvement of p130(Cas) and p130(HEF1), a novel Cas-like docking protein, in a cytoskeleton-dependent signaling pathway initiated by ligation of integrin or antigen receptor on human b cells. J Biol Chem 272:4230–4236

Matozaki T, Nakanishi H, Takai Y (2000) Small g-protein networks: their crosstalk and signal cascades. Cell Signal 12:515–524

Matsuda M, Mayer B, Fukui Y, Hanafusa H (1990) Binding of transforming protein, P47gag-crk, to a broad range of phosphotyrosine-containing proteins. Science 248:1537–1539

Matsuda M, Mayer B, Hanafusa H (1991) Identification of domains of the v-crk oncogene product sufficient for association with phosphotyrosine-containing proteins. Mol Cell Biol 11:1607–1613

Mattila P, Lappalainen P (2008) Filopodia: molecular architecture and cellular functions. Nat Rev Mol Cell Biol 9:446–454

Mayer BJ, Hamaguchi M, Hanafusa H (1988) A novel viral oncogene with structural similarity to phospholipase C. Nature 332:272–275

Miller P, Folkmann A, Maia A, Efimova N, Efimov A, Kaverina I (2009) Golgi-derived CLASP-dependent microtubules control Golgi organization and polarized trafficking in motile cells. Nat Cell Biol 11:1069–1080

Minegishi M, Tachibana K, Sato T, Iwata S, Nojima Y, Morimoto C (1996) Structure and function of Cas-L, a 105-kD Crk-associated substrate-related protein that is involved in beta 1 integrin-mediated signaling in lymphocytes. J Exp Med 184:1365–1375

Mitra S, Hanson D, Schlaepfer D (2005) Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol 6:56–68

Miyamoto S, Akiyama S, Yamada K (1995) Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science 267:883–885

Mofrad M, Golji J, Abdul Rahim N, Kamm R (2004) Force-induced unfolding of the focal adhesion targeting domain and the influence of paxillin binding. Mech Chem Biosyst 1:253–265

Moser M, Legate K, Zent R, Faessler R (2009) The tail of integrins, talin, and kindlins. Science 324:895–899

Nagai T, Ibata K, Park E, Kubota M, Mikoshiba K, Miyawaki A (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotech 20:87–90

Nakamoto T, Sakai R, Ozawa K, Yazaki Y, Hirai H (1995) Direct binding of C-terminal region of p130Cas to SH2 and SH3 domains of Src kinase. J Biol Chem 271:8959–8965

Nakamoto T, Sakai R, Honda H, Ogawa S, Ueno H, Suzuki T, Aizawa S, Yazaki Y, Hirai H (1997) Requirements for localization of p130cas to focal adhesions. Mol Cell Biol 17:3884–3897

Nakashima H, Hamamura K, Houjou T, Taguchi R, Yamamoto N, Mitsudo K, Tohnai I, Ueda M, Urano T, Furukawa K, Furukawa K (2007) Overexpression of caveolin-1 in a human melanoma cell line results in dispersion of ganglioside gd3 from lipid rafts and alteration of leading edges, leading to attenuation of malignant properties. Cancer Sci 98:512–520

Nobes C, Hall A (1995) Rho, rac, and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. Biochem Soc Trans 23:456–459

Noguchi T, Tsuda M, Takeda H, Takada T, Inagaki K, Yamao T, Fukunaga K, Matozaki T, Kasuga M (2001) Inhibition of cell growth and spreading by stomach cancer-associated protein-tyrosine phosphatase-1 (SAP-1) through dephosphorylation of p130cas. J Biol Chem 276:15,216–24

Nojima Y, Tachibana K, Sato T, Schlossman S, Morimoto C (1995) Focal adhesion kinase (pp125FAK) is tyrosine phosphorylated after engagement of alpha 4 beta 1 and alpha 5 beta 1 integrins on human T-lymphoblastic cells. Cell Immunol 161:8–13

Ogden K, Thompson J, Hickner Z, Huang T, Tang D, Watts S (2006) A new signaling paradigm for serotonin: use of Crk-associated substrate in arterial contraction. AJP: Heart and Circulatory Physiology 291:H2857–H2863

O'Neill G, Fashena S, Golemis E (2000) Integrin signalling: a new Cas(t) of characters enters the stage. Trends Cell Biol 10:111–119

Owen J, Ruest P, Fry D, Hanks S (1999) Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. Mol Cell Biol 19:4806–4810

Pankov R, Cukierman E, Katz E, Matsumoto K, Lin D, Lin S, Hahn C, Yamada K (2000) Integrin dynamics and matrix assembly: tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. J Cell Biol 148:1075–1090

Partridge M, Marcantonio E (2006) Initiation of attachment and generation of mature focal adhesions by integrin-containing filopodia in cell spreading. Mol Biol Cell 17:4237–4248

Pellicena P, Miller W (2001) Processive phosphorylation of p130Cas by Src depends on SH3-polyproline interactions. J Biol Chem 276:28,190–28,196

Petch L, Bockholt S, Bouton A, Parsons J, Burridge K (1995) Adhesion-induced tyrosine phosphorylation of the p130 src substrate. J Cell Sci 108:1371–9

Petruzzelli L, Takami M, Herrera R (1996) Adhesion through the interaction of lymphocyte function-associated antigen-1 with intracellular adhesion molecule-1 induces tyrosine phosphorylation of p130Cas and its association with c-CrkII. J Biol Chem 271:7796–7801

Podar K, Shringarpure R, Tai Y, Simoncini M, Sattler M, Ishitsuka K, Richardson P, Hideshima T, Chauhan D, Anderson K (2004) Caveolin-1 is required for vascular endothelial growth factor-triggered multiple myeloma cell migration and is targeted by bortezomib. Cancer Res 64:7500–6

Pollard T, Borisy G (2003) Cellular motility driven by assembly and disassembly of actin filaments. Cell 112:453–465

Polte T, Hanks S (1995) Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. Proc Natl Acad Sci USA 92:10,678–10,682

Polte T, Hanks S (1997) Complexes of focal adhesion kinase (FAK) and Crk-associated substrate (p130(Cas)) are elevated in cytoskeleton-associated fractions following adhesion and Src transformation. Requirements for Src kinase activity and FAK proline-rich motifs. J Biol Chem 272:5501–5509

Pozzi A, Zent R (2003) Integrins: sensors of extracellular matrix and modulators of cell function. Nephron Exp Nephrol 94:e77–84

Prasad N, Topping R, Decker S (2001) SH2-containing inositol 5'-phosphatase SHIP2 associates with the p130(Cas) adapter protein and regulates cellular adhesion and spreading. Mol Cell Biol 21:1416–1428

Pratt S, Epple H, Ward M, Feng Y, Braga V, Longmore G (2005) The LIM protein Ajuba influences p130Cas localization and Rac1 activity during cell migration. J Cell Biol 168:813–824

Puthenveedu M, Bachert C, Puri S, Lanni F, Linstedt A (2006) GM130 and GRASP65-dependent lateral cisternal fusion allows uniform Golgi-enzyme distribution. Nat Cell Biol 8:238–248

Reddien P, Horvitz H (2000) CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in Caenorhabditis elegans. Nat Cell Biol 2:131–136

Reynolds A, Kanner S, Wang H, Parsons J (1989) Stable association of activated pp60src with two tyrosine-phosphorylated cellular proteins. Mol Cell Biol 9:3951–3958

Ridley A, Schwartz M, Burridge K, Firtel R, Ginsberg M, Borisy G, Parsons J, Horwitz A (2003) Cell migration: integrating signals form front to back. Science 302:1704–1709

Riggins R, Quilliam L, Bouton A (2003) Synergistic promotion of c-Src activation and cell migration by Cas and AND-34/BCAR3. J Biol Chem 278:28,267–28,273

Riggins R, Thomas K, Ta H, Wen J, Davis R, Schuh N, Donelan S, Owen K, Gibson M, Shupnik M, Silva C, Parsons S, Clarke R, Bouton A (2006) Physical and functional interactions between Cas and c-Src induce tamoxifen resistance of breast cancer cells through pathways involving epidermal growth factor receptor and signal transducer and activator of transcription 5b. Cancer Res 66:7007–7015

Riveline D, Zamir E, Balaban N, Schwarz U, Ishazaki T, Narumiya S, Kam Z, Geiger B, Bershadsky A (2001) Focal contacts as mechnosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. J Cell Biol pp 1175–1186

Rivera G, Antoku S, Gelkop S, Shin N, Hanks S, Pawson T, Mayer B (2006) Requirement of nck adaptors for actin dynamics and cell migration stimulated by platelet-derived growth factor B. Proc Natl Acad Sci USA 103:9536–9541

Rozengurt E (1998) Signal transduction pathways in the mitogenic response to G protein-coupled neuropeptide receptor agonists. J Cell Phys 177:507–517

Ruest P, Shin N, Polte T, Zhang X, Hanks S (2001) Mechanisms of CAS substrate domain tyrosine phosphorylation by FAK and Src. Mol Cell Biol 21:7641–7652

Rush J, Moritz A, Lee K, Guo A, Goss V, Spek E, Zhang H, Zha X, Polakiewicz R, Comb M (2005) Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotech 23:94–101

Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Mano H, Yazaki Y, Hirai H (1994) A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J 13:3748–3756

Sakai R, Nakamoto T, Ozawa K, Aizawa S, Hirai H (1997) Characterization of the kinase activity essential for tyrosine phosphorylation of p130Cas in fibroblasts. Oncogene 14:1419–1426

Sakakibara A, Hattori S (2000) Chat, a Cas/HEF-1 associated adaptor protein that integrates multiple signaling pathways. J Biol Chem 275:6404–6410

Sawada Y, Tamada M, Dubin-Thaler B, Cherniavskaya O, Sakai R, Tanaka S, Sheetz M (2006) Force sensing by mechanical extension of the Src family kinase substrate p130Cas. Cell 127:1015–1026

Schaller M (2001) Paxillin: a focal adhesion-associated adaptor protein. Oncogene 20:6459–6472

Schaller M, Otey C, Hildebrand J, Parsons J (1995) Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. J Cell Biol 130:1181–1187

Schlaepfer D, Broome M, Hunter T (1997) Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. Mol Cell Biol 17:1702–1713

Shin N, Dise R, Schneider-Mergener J, Ritchie M, Kilkenny D, Hanks S (2004) Subsets of the major tyrosine phosphorylation sites in Crk-associated substrate (CAS) are sufficient to promote cell migration. J Biol Chem 279:38,331–38,337

Shroff H, Galbraith C, Galbraith J, White H, Gillette J, Olenych S, Davidson M, Betzig E (2007) Dual-color superresolution imaging of genetically expressed probes within individual adhesion complexes. Proc Natl Acad Sci USA 104:20,308–20,313

Siesser P, Meenderink L, Ryzhova L, Michael K, Dumbauld D, Garcia A, Kaverina I, Hanks S (2008) A FAK/Src chimera with gain-of-function properties promotes formation of large peripheral adhesions associated with dynamic actin assembly. Cell Motil Cytoskeleton 65:25–39

Singh M, Dadke D, Nicolas E, Serebriiskii I, Apostolou S, Canutescu A, Egleston B, Golemis E (2008) A novel Cas family member, HEPL, regulates FAK and cell spreading. Mol Biol Cell 19:1627–1636

Small J, Stradal T, Vignal E, Rottner K (2002) The lamellipodium: where motility begins. Trends Cell Biol 12:112–120

Smith h, Marra P, Marshall C (2008) uPAR promotes formation of the p130cas-crk complex to activate rac through dock180. J Cell Biol 182:777–790

Soni S, Bor-Tyh L, August A, Nicholson R, Kirsch K (2009) Expression of a phosphorylated p130Cas substrate domain attentuates the phosphatidylinositol 3-kinase/ Akt survival pathway in tamoxifen resistant breast cancer cells. J Cell Biochem 107:364–375

Ta H, Thomas K, Schrecengost R, Bouton A (2008) A novel association between p130cas and resistance to the chemotherapeutic drug adriamycin in human breast cancer cells. Cancer Res 68:8796–8804

Tachibana K, Sato T, D'Avirro N, Morimoto C (1995) Direct association of pp125FAK with paxillin, the focal adhesion-targeting mechanism of pp125FAK. J Exp Med 182:1089–1099

Tikhmyanova N, Little J, Golemis E (2010) CAS proteins in normal and pathological cell growth control. Cell Mol Life Sci 67:1025–48

Vicente-Manzanares M, Webb D, AR H (2005) Cell migration at a glance. J Cell Sci 118:4917–4919

Volberg T, Romer L, Zamir E, Geiger B (2001) pp60(c-src) and related tyrosine kinases: a role in the assembly and reorganization of matrix adhesions. J Cell Sci 114:2279–2289

Vuori K, Ruoslahti E (1995) Tyrosine phosphorylation of p130Cas and cortactin accompanies integrin-mediated cell adhesion to extracellular matrix. J Biol Chem 270:22,259–22,262

Vuori K, Hirai H, Aizawa S, Ruoslahti E (1996) Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. Mol Cell Biol 16:2606–13

Wang X, Weng L, Yu Q (2000) Specific inhibition of FGF-induced MAPK activation by the receptor-like protein tyrosine phosphatase LAR. Oncogene 19:2346–2353

Wang Y (2003) Zyxin and paxillin proteins: focal adhesion plaque LIM domain proteins go nuclear. Biochim Biophys Acta - Mol Cell Res 1593:115–120

Webb D, Donais K, Whitmore L, Thomas S, Turner C, Parsons J, Horwitz A (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 6:154–161

Weidow C, Black D, Bliska J, Bouton A (2000) CAS/Crk signalling mediates uptake of Yersinia into human epithelial cells. Cell Microbiol 2:549–560

Wendt M, Smith J, Schiemann W (2009) p130cas is required for mammary tumor growth and transforming growth factor-beta-mediated metastasis through regulation of Smad2/3 activity. J Biol Chem 284:34,145–56

Winograd-Katz S, Itkovitz S, Kam Z, Geiger B (2009) Multiparametric analysis of focal adhesion formation by RNAi-meidated gene knockdown. J Cell Biol 186:423–436

Xing L, Donlin L, Miller R, Alexandropoulos K (2004) The Adapter Molecule Sin Regulates T-Cell-Receptor-Mediated Signal Transduction by Modulating Signaling Substrate Availability. Mol Cell Biol 24:4581–4592

Yamada S, Nelson W (2007) Localized zones of Rho and Rac activities drive initiation and expansion of epithelial cell cell adhesion. J Cell Biol 173:517–527

Yamakita Y, Totsukawa G, Yamashiro S, Fry D, Zhang X, Hanks S, Matsumura F (1999) Dissociation of FAK/p130CAS/c-Src Complex during Mitosis: Role of Mitosis-specific Serine Phosphorylation of FAK. J Cell Biol 144:315–324

Yang L, Alexandropoulos K, Sap J (2002) c-SRC Mediates Neurite Outgrowth through Recruitment of Ccrk to the Scaffolding Protein Sin/Efs without Altering the Kinetics of ERK Activation. J Biol Chem 277:17,406–17,414

Yoshizumi M, Abe J, Haeneler J, Huang Q, Berk B (2000) Src and Cas mediate JNK activation but not ERK1/2/ and p38 kinases by reactive oxygen species. J Biol Chem 275:11,706–11,712

Yu H, Schrader H, Otte J, Schmidt W, Schmitz F (2004) Rapid tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130cas by gastrin in human colon cancer cells. Biochem Pharmacol 67:135–46

Zaidel-Bar R, Ballestrem C, Kam Z, Geiger B (2003) Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. J Cell Sci 116:4605–4613

Zamir E, Geiger B (2001) Molecular complexity and dynamics of cell-matrix adhesions. J Cell Sci 114:3583–3590

Zamir E, Katz B, Aota S, Yamada K, Geiger B, Kam Z (1999) Molecular diversity of cell-matrix adhesions. J Cell Sci 112:1655–1669

Zamir E, Katz M, Posen Y, Erez N, Yamada K, Katz B, Lin S, Lin D, Bershadsky A, Kam Z, Geiger B (2000) Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. Nat Cell Biol 2:191–196

Zamir E, Geiger B, Kam Z (2008) Quantitative multicolor compositional imaging resolves molecular domains in cell-matrix adhesions. PLoS ONE 3:e1901

Zhang H, Berg J, Li Z, Wang Y, Lang P, Sousa A, Bhaskar A, Cheney R, Stromblad S (2004) Myosin-X provides a motor-based link between integrins and the cytoskeleton. Nat Cell Biol 6:523–531

Zhu T, Goh E, LeRoith D, Lobie P (1998) Growth hormone stimulates the formation of a multiprotein signaling complex involving p130(Cas) and Crkii. Resultant activation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). J Biol Chem 273:10,682–10,689

Zouq N, Keeble J, Lindsay J, Valentijn A, Zhang L, Mills D, Turner C, Streuli C, Gilmore A (2009) FAK engages multiple pathways to maintain survival of fibroblasts and epithelia - differential roles for paxillin and p130Cas. J Cell Sci 122:357–367