A Cas-BCAR3 co-regulatory circuit controls lamellipodia dynamics

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Abstract

Cell migration involves positive and negative feedback loops that coordinate integrin engagement with lamellipodial dynamics. Phosphorylation of Cas (p130Cas, BCAR1), induced by integrins, stimulates lamellipodium ruffling and migration but also triggers Cas downregulation by the ubiquitin-proteasome pathway. While the mechanism of integrin-mediated Cas phosphorylation remains unclear, a Cas-binding protein, BCAR3, is known to be involved. Here, we find that Cas phosphorylation, and consequent lamellipodium ruffling and migration, requires BCAR3 phosphorylation at a specific site. This site also targets BCAR3 inactivation by the ubiquitin-proteasome system. Thus, mutation of this site inhibits BCAR3 turnover, Cas phosphorylation, lamellipodium ruffling and migration. Additionally, BCAR3 requires Cas for localization to adhesions. This places BCAR3 in a co-regulatory positive-feedback circuit with Cas, with Cas requiring BCAR3 for activation and BCAR3 requiring Cas for localization. The use of a single phosphorylation site in BCAR3 for activation and degradation ensures reliable negative feedback by the ubiquitin-proteasome system.

Introduction

Cell migration requires accurate spatial and temporal coordination of actin polymerization, cell-cell and cell-matrix adhesions, microtubules, actomyosin contraction, vesicle traffic, and membrane composition. Breakdown of coordination leads to pathologies in morphogenesis, wound healing and cancer (Trepat et al., 2012). At the front of the cell, formation of nascent adhesion complexes activates branched actin polymerization to form a thin, dynamic lamellipodium (Bachir et al., 2017, Legate et al., 2009). Actin polymerization creates a rearward flow of actin that interacts with exposed actin-binding sites on integrin-associated talin and vinculin, forming catch bonds and clustering the integrins into focal complexes (FCs) (Case et al., 2015, Galbraith et al., 2002, Partridge et al., 2006). Most FCs are short-lived, but some mature into focal adhesions (FAs) at the base of the lamellipodium, anchoring actin stress fibers and providing resistance to actomyosin contractile forces (del Rio et al., 2009, Puklin-Faucher et al., 2009, Tadokoro et al., 2003). Integrin adhesions are also sources of biochemical signals, where multiple proteins are phosphorylated by focal adhesion kinase (FAK) and Src-family kinases (SFKs) (Burridge et al., 1992, Huang et al., 1993, Miyamoto et al., 1995, Schaller et al., 1992). Some integrin-stimulated phosphorylation events regulate protein-protein interactions within the adhesion (Pasapera et al., 2010, Stutchbury et al., 2017, Zaidel-Bar et al., 2007), while others activate signaling proteins that coordinate adhesion with lamellipodium dynamics and other aspects of cell biology, such as cell survival (Mitra et al., 2006). One important adhesion-regulated signaling protein is Cas (Crk-associated substrate, also called p130Cas or BCAR1), which plays critical roles in lamellipodium protrusion, membrane ruffling, adhesion assembly, adhesion turnover, resistance to anoikis and malignant transformation (Honda et al., 1999, Sanders et al., 2005, Sharma et al., 2008, Webb et al., 2004) (reviews (Defilippi et al., 2006, Tikhmyanova et al., 2010)).

Cas activation involves phosphorylation at multiple YxxP sites in a predicted unstructured region near the N terminus, known as the “substrate domain” (see Figure 1a for a diagram of Cas structure). Phosphorylation at these sites recruits adaptor proteins Crk and CrkL and their associated guanine nucleotide exchange factors (GEFs): C3G for Rap1 and DOCK1 (also called DOCK180) for Rac1 (Fonseca et al., 2004, Goldberg et al., 2003, Hasegawa et al., 1996, Kiyokawa et al., 1998, Klemke et al., 1998, Sakai et al., 1994, Sakai et al., 1997, Tanaka et al., 1994). Active Rap1 increases talin recruitment to integrins to promote adhesion maturation (Boettner et al., 2009), while Rac1 stimulates polymerization of branched actin structures by WAVE, leading to lamellipodial protrusion and ruffling (Cheresh et al., 1999, DeMali et al., 2002,

Ridley 2001, Ridley et al., 1992, Stradal et al., 2004). Thus, Cas is part of a self-reinforcing cycle in which actin polymerization stimulates Cas phosphorylation, which leads to more actin polymerization, lamellipodium protrusion and membrane ruffling. Cas also stimulates migration by increasing focal adhesion turnover as the cell move forwards (Ren et al., 2000, Rottner et al., 1999, Webb et al., 2004).

Cas is also subject to negative feedback. Phosphorylation of a YDYV sequence, C-terminal to the substrate domain (Figure 1a), mediates turnover of pY-Cas. This phosphosite binds to the SH2 domain of SOCS6 (suppressor of cytokine signaling 6), recruiting CRL5 (Cullin 5-RING-ligase) and targeting Cas to the ubiquitin-proteasome system (Teckchandani et al., 2014). SOCS6 co-localizes with phosphorylated Cas in adhesions at the leading edge of migrating cells where it suppresses adhesion dynamics (Teckchandani et al., 2016). Mutation of the YDYV sequence or knockdown of SOCS6 or Cullin 5 (Cul5) stabilizes Cas and increases lamellipodia protrusion and ruffling (Teckchandani et al., 2014). Thus, phosphorylation provides negative feedback onto Cas, restraining leading edge dynamics and stabilizing adhesions.

While the effects of Cas YxxP phosphorylation are well-understood, it is less clear how Cas phosphorylation is regulated. Phosphorylation is stimulated by integrin-dependent actin polymerization at the leading edge and requires an intact actin cytoskeleton (Vuori et al., 1996, Vuori et al., 1995, Zhang et al., 2014, Zhao et al., 2016). The substrate domain is more accessible for phosphorylation when it is extended (Hotta et al., 2014, Sawada et al., 2006, Tamada et al., 2004). One idea is that the substrate domain is extended by forces acting on proteins bound to the N- and C-terminal Src homology 3 (SH3) and Cas C-terminal homology (CCH)/focal adhesion targeting (FAT) domains (Figure 1a). Both domains are required to localize Cas to integrin adhesions (Branis et al., 2017, Donato et al., 2010, Harte et al., 1996, Nakamoto et al., 1997, Nojima et al., 1995, Vuori et al., 1995). The Cas SH3 binds adhesion proteins vinculin and FAK, either of which could localize Cas to adhesions and exert force (Harte et al., 1996, Janostiak et al., 2014, Polte et al., 1995). In addition, FAK bridges the Cas SH3 to N-WASP, which stimulates actin polymerization at the membrane during cell spreading (Kostic et al., 2006, Wu et al., 2004, Zhang et al., 2014). The role of the Cas CCH/FAT domain is less clear. It binds to LIM domain proteins paxillin, zyxin and ajuba, and to a CDC25 homology (CDC25H) domain found in NSP family proteins (Mace et al., 2011, Pratt et al., 2005, Wallez et al., 2012, Yi et al., 2002, Zhang et al., 2017) (Figure 1a). It is unclear which, if any, of these proteins is responsible for localizing the Cas CCH/FAT domain to adhesions.

The NSP proteins, comprised of BCAR3 (breast cancer anti-estrogen resistance 3, also called AND-34, NSP2, Sh2d3b), NSP1 (Sh2d3a) and NSP3 (Shep1, Chat, Sh2d3c), are defined by a common structure with an SH2 domain and CDC25H domain (Figure 1a). NSP proteins cooperate with Cas-family proteins in cell migration (Wallez et al., 2012). For example, over-expressed BCAR3 increases Cas phosphorylation, Cas localization to membrane ruffles, cell migration, cell proliferation, and, like Cas, protects estrogen-dependent breast cancer cells from inhibitory actions of anti-estrogens (Cai et al., 2003, Cross et al., 2016, Riggins et al., 2003, Roselli et al., 2010, Schrecengost et al., 2007, Schuh et al., 2010, van Agthoven et al., 1998). BCAR3 regulates EGF- and insulin-stimulated membrane ruffling (Oh et al., 2013, Schrecengost et al., 2007). BCAR3 is phosphorylated at unidentified tyrosine residues in response to serum and adhesion (Cai et al., 1999) and the SH2 domain can bind the epidermal growth factor (EGF) receptor (Oh et al., 2008). In mouse fibroblasts, the BCAR3 SH2-domain binds phosphorylated PTPRA (RPTPα), a protein phosphatase that activates SFKs and is required to localize BCAR3 to the cell edge and activate Cas (Sun et al., 2012, von Wichert et al., 2003, Zheng et al., 2000). These studies show that BCAR3 is intimately involved in Cas regulation, but leave open questions of how BCAR3 itself is regulated and how it regulates Cas.

Here, we find that BCAR3, like Cas, is subject to negative regulation by the ubiquitin-proteasome system. BCAR3 phosphorylation at Y117 recruits SOCS6 and leads to Cul5-dependent degradation. BCAR3 is needed for cell migration and invasion, and for increased lamellipodial ruffling and collective migration when Cul5 is absent. Using gene silencing and mutant analysis, we find that BCAR3 localization to adhesions requires the Cas-binding site but not Y117 or the SH2 domain. Reciprocally, Cas phosphorylation requires BCAR3's Y117, SH2 domain and Cas-binding site. BCAR3 and Cas thus form a signaling hub that localizes to active integrins and coordinates actin dynamics under negative control by the ubiquitin-proteasome system.

Results

CRL5 regulates BCAR3 protein turnover

We previously reported that CRL5 inhibits Src activity and Src-dependent transformation of MCF10A epithelial cells, in part by targeting pY proteins such as pYCas for degradation by the ubiquitin-proteasome system (Teckchandani et al., 2014). Because overexpression of Cas does not increase migration (Teckchandani et al., 2014), we infer that CRL5 may target additional phosphoproteins that, with Cas, are required for increased migration. We sought to identify such phosphoproteins by screening for pY peptides whose abundance increases when Cul5 is inhibited. To this end, control and Cul5-deficient MCF10A cells were lysed under denaturing conditions, proteins were digested with trypsin, and peptides were labeled with isobaric TMT tags for quantitative pY proteomics (Zhang et al., 2007). In one experiment, samples were prepared from control and Cul5-deficient cells that were starved for epidermal growth factor (EGF) for 0, 24 or 72 hr. Starvation time had no systematic effect on peptide abundance, so, in a second experiment, we prepared biological triplicate samples from growing control and Cul5-deficient cells. Sixteen pY peptides increased significantly in Cul5-deficient cells in both experiments, including pY128 from Cas and pY117 and pY266 from BCAR3 (Figure 1b, Figure 1 – Figure Supplement 1, Supplementary Table). We decided to focus on BCAR3 because Cul5 regulates Cas and Cas binds BCAR3 (Wallez et al., 2012).

The increased quantity of BCAR3 pY peptides in Cul5-deficient cells could result from increased phosphorylation, increased protein level, or both. We used immunoblotting to test whether BCAR3 protein level is regulated by Cul5. BCAR3 protein level increased approximately 4-fold in Cul5-deficient cells, in either growth or starvation conditions (Figure 1c). However, RNA levels were unaltered (Figure 1c), consistent with altered protein synthesis or degradation. To measure degradation, we inhibited new protein synthesis with cycloheximide and monitored BCAR3 protein level. BCAR3 half-life was approximately 4 hours in control cells but greater than 8 hours in Cul5-deficient cells (Figure 1d). BCAR3 degradation was inhibited by Cullin neddylation inhibitor MLN4924 or proteasome inhibitor MG132, but not by lysosome inhibitor Bafilomycin (Fig 1e). These results suggest that CRL5 regulates BCAR3 turnover by the ubiquitin-proteasome system and that the increase in BCAR3 pY117 and pY266 in Cul5-deficient cells is likely due to an increased availability of BCAR3 protein for phosphorylation rather than, or in addition to, an increase in kinase activity.

BCAR3 regulates epithelial cell migration

BCAR3 is required for the migration of cancer cells and fibroblasts (Cross et al., 2016, Schrecengost et al., 2007, Sun et al., 2012) but its importance in normal epithelial cells is unknown. BCAR3 loss of function induced by siRNA transfection or *BCAR3* gene disruption in MCF10A cells slowed migration and invasion toward a source of the chemoattractant EGF in modified Boyden chamber assays (Figure 2a-c, Figure 2 - Figure Supplement 1). BCAR3 was required for the migration and invasion of both normal and Cul5-deficient cells, suggesting the existence of multiple pathways acting in parallel to control single-cell migration. In contrast, in scratch wound assays without added EGF, loss of BCAR3 slowed migration in Cul5-deficient cells but had little effect in normal cells, suggesting that Cul5 inhibits collective-cell migration through BCAR3 (Figure 2d). Inspection of the wound edge revealed that BCAR3 is also needed for the increased lamellipodia length and ruffling in Cul5-depleted cells (Figure 2e-g). This epistatic relationship is consistent with CRL5 inhibiting BCAR3-dependent collective-cell migration and lamellipodia dynamics. EGF may overcome this regulation in single-cell migration assays by stimulating parallel pathways.

CRL5 separately targets BCAR3 and Cas through SOCS6

CRL5 promotes ubiquitination and degradation of substrate proteins bound to adaptor proteins (Okumura et al., 2016). CRL5 adaptors include SOCS family proteins, which contain SH2 domains for binding to pY. To test whether SOCS proteins bind BCAR3, we transiently expressed T7-tagged SOCS proteins and assayed binding to endogenous BCAR3 by immunoprecipitation and immunoblotting. BCAR3 specifically co-precipitated with SOCS6, the same adaptor that binds Cas (Figure 3a) (Teckchandani et al., 2014). Accordingly, SOCS6 loss of function in MCF10A cells increased BCAR3 steady-state protein levels and decreased the rate of BCAR3 turnover (Figure 3b,c). Together, these results suggest that $CRL5^{SOCS6}$ mediates BCAR3 turnover.

Since BCAR3 and Cas bind each other and both are bound and regulated by SOCS6, it is possible that SOCS6 binds BCAR3 indirectly, through Cas. We tested this possibility by two strategies (Figure 3d). First, we used a BCAR3 mutant, L744E/R748E, called here $BCAR3^{EE}$, that does not bind Cas (Wallez et al., 2014). This mutation inhibited binding to Cas but not SOCS6 (Figure 3e). Second, we found that BCAR3-SOCS6 binding occurred in cells from which Cas had been depleted with siRNA (Figure 3f). Collectively, these data suggest that SOCS6 binds BCAR3 and Cas independently.

CRL5 binds BCAR3 pY117

We considered that SOCS6 might bind BCAR3 through pY-dependent or -independent interactions. Pervanadate, a cell-permeable phosphotyrosine phosphatase inhibitor, increased BCAR3-SOCS6 association, suggesting pY dependence (Figure 4a). In addition, disrupting the SOCS6 SH2 domain by deletion (ΔC) or point mutation (R407K) inhibited binding to BCAR3 (Figure 4b). This suggests that the SOCS6 SH2 domain binds pY-BCAR3. Serum or adhesion stimulates tyrosine phosphorylation of BCAR3 in mouse fibroblasts (Cai et al., 1999), but the specific sites have not been identified. High-throughput pY proteomics surveys have identified phosphorylation of BCAR3 at tyrosine residues 42, 117, 212, 266 and 429 in over 25 mouse and human cell lines (Hornbeck et al., 2019). We tested whether these sites were required for binding SOCS6 using site-directed mutagenesis (Figure 4c). An F5 mutant, in which all five tyrosines were changed to phenylalanines, was unable to bind SOCS6 (Figure 4d). By mutating each pY site individually, we found that Y117 is necessary to bind SOCS6 (Figure 4d). Furthermore, mutating all sites except Y117 (42, 212, 266 and 429) had little effect on SOCS6 binding (BCAR3 4F mutant, Figure 4e). These results support a model in which phosphorylation of BCAR3 at Y117 is both necessary and sufficient to bind the SOCS6 SH2 domain.

BCAR3 Y117 and Cas, but not the SH2 domain, regulate BCAR3 turnover

To test whether Y117 or other domains of BCAR3 are required for CRL5-dependent BCAR3 protein turnover, we measured the effect of various mutations on the level of tagged BCAR3 protein in control and Cul5-deficient cells. To avoid possible artifacts due to over-expression, we used a doxycycline-inducible promoter (Baron et al., 1997). MCF10A cells were first transduced to express the reverse *tet* transactivator (rtTA), and then transduced to express tagged wildtype or mutant BCAR3 under control of the *tet* operator. BCAR3 was tagged at the N-terminus with a V5 epitope for immunoblotting and a SNAP domain (O^6-alkylguanine-DNA alkyltransferase) that allows covalent labelling with benzyl guanine compounds (Keppler et al., 2003). Selected cell lines were treated with doxycycline (dox) to induce protein expression and with various siRNAs to regulate expression of endogenous BCAR3 or Cul5.

We first examined the role of Y117 in BCAR3 turnover and binding to Cas. Initial characterization showed that 5-10 ng/mL dox induced expression of SNAP-V5-BCAR3^{WT} to approximately the same level as endogenous BCAR3, while SNAP-V5-BCAR3^{Y117F} was expressed at approximately two-fold higher level (Figure 5a). Both proteins co-immunoprecipitated with Cas (Figure 5b). Cul5 depletion increased the level of SNAP-V5-

BCAR3^{WT} more than 2-fold while the level of SNAP-V5-BCAR3^{Y117F} was unchanged (Figure 5c). These results suggest that phosphorylation at Y117 does not regulate Cas binding but is required for Cul5-dependent BCAR3 protein turnover in cells, consistent with SOCS6 binding to this site.

We extended this approach to test the importance of the BCAR3 SH2 domain and binding to Cas. To inhibit Cas binding, we used the aforementioned L744E/R748E mutant (BCAR3^{EE}). To inactivate the BCAR3 SH2 domain, we created an arginine to lysine at residue 177 (R177K), which lies in the consensus FLVRES motif and is required to bind the pY phosphate (Jaber Chehayeb et al., 2020, Marengere et al., 1994). This mutation did not inhibit Cas binding (Figure 5b. Cul5-depletion increased the level of SNAP-V5-BCAR3^{R177K} but not SNAP-V5-BCAR3^{EE} (Figure 5d). Taken together, the results suggest that CRL5^{SOCS6} regulates BCAR3 protein turnover dependent on Cas binding and on Y117 phosphorylation. The BCAR3 SH2 domain is dispensable for Cas binding and BCAR3 degradation, and, by implication, may not be needed for Y117 phosphorylation.

Lamellipodial ruffling and cell migration require BCAR3 Y117, SH2 domain and Cas binding

Since BCAR3^{Y117F} accumulates to higher levels in cells, we suspected it may be more active in biological assays. Over-expression of BCAR3 in fibroblasts and breast cancer cells stimulates Cas-dependent functions, such as lamellipodia ruffling (Cai et al., 2003, Wallez et al., 2014, Wilson et al., 2013). Similarly, induction of SNAP-V5-BCAR3^{WT} increased membrane ruffling in MCF10A cells (Figure 6a). In contrast, over-expressed SNAP-V5-BCAR3^{Y117F} did not increase ruffling (Figure 6a). SNAP-V5-BCAR3^{R177K} and SNAP-V5-BCAR3^{EE} were also inactive. This suggests that not only is Y117 needed for downregulation of BCAR3 by CRL5^{SOCS6}, it is also needed, together with the SH2 domain and Cas binding, for over-expressed BCAR3 to increase lamellipodial ruffling.

While BCAR3 over-expression was not sufficient to increase migration (Figure 6 - Figure Supplement 1), BCAR3 was necessary for the increased migration and ruffling of Cul5-deficient cells (Figure 2). To test which domains of BCAR3 are necessary, we knocked down Cul5 and endogenous BCAR3 and induced wildtype or mutant SNAP-V5-BCAR3 at endogenous levels (Figure 5 c, d). While wildtype SNAP-V5-BCAR3 rescued the increased migration and ruffling of Cul5-deficient cells, none of the mutants, Y117F, R177K or EE, were active (Figure 6b). Thus,

all three of these regions of BCAR3 regulate lamellipodial dynamics and cell migration when Cul5 is absent.

Cas recruits BCAR3 to integrin adhesions, where BCAR3 stimulates Cas phosphorylation, dependent on BCAR3 Y117 and SH2

These findings raise the question of how BCAR3 Y117, SH2 domain, and Cas binding cooperate to regulate membrane ruffling and migration. BCAR3 is localized to sites of integrin adhesion, which is consistent with its role in regulating integrin-stimulated lamellipodial dynamics (Cross et al., 2016, Sun et al., 2012). Over-expressed BCAR3 can relocalize Cas to membranes (Riggins et al., 2003). Cas could then activate lamellipodia dynamics through the Crk/DOCK180/Rac pathway (Klemke et al., 1998, Sakai et al., 1994, Sanders et al., 2005, Sharma et al., 2008). In that case, BCAR3's Y117, SH2 domain and/or Cas-binding site may be needed for Cas localization. Alternatively, BCAR3 may regulate Cas activation but not localization. To explore these possibilities, we monitored the effect of BCAR3 depletion on the localization of YFP-Cas and the effect of Cas depletion on the localization of SNAP-V5-BCAR3^{WT}. Localization in leading edge adhesions was quantified relative to the adhesion marker paxillin. We found that YFP-Cas localized properly to adhesions in control and BCAR3-depleted cells (Figure 7a). In contrast, SNAP-V5-BCAR3^{WT} localization to adhesions was disrupted when Cas was depleted (Figure 7b). Thus, BCAR3 requires Cas to localize to adhesions, and not vice versa. Consistent with this interpretation, SNAP-V5-BCAR3^{Y117F} and BCAR3^{R177K}, which bind Cas (Figure 5b), localized normally, while SNAP-V5-BCAR3^{EE} did not (Figure 8a, b).

Since BCAR3 does not regulate Cas localization, we tested whether BCAR3 is needed to activate Cas using antibodies to pY165, one of the repeated YxxP motifs in Cas that recruit Crk (Fonseca et al., 2004, Sakai et al., 1994, Songyang et al., 1993). We found that BCAR3 depletion inhibited Cas but not FAK phosphorylation, suggesting a specific role for BCAR3 in Cas activation (Figure 8 – figure supplement 1). As expected, SNAP-V5-BCAR3^{WT} rescued Cas phosphorylation in BCAR3-depleted cells (Figure 8a, c). However, Cas was not phosphorylated in cells expressing Y117F, R177K or EE mutant SNAP-V5-BCAR3 (Figure 8a, c). This suggests that BCAR3 not only has to be bound to Cas but also needs to be phosphorylated at Y117 and to interact with proteins through its SH2 domain for Cas phosphorylation to occur. The phosphorylation of Cas in adhesions correlates with the rescue of ruffling (Figures 6b and 8). Taken together, these results suggest that BCAR3 is brought to integrin adhesions by binding to

Cas, then uses its pY117 and SH2 domain to activate Cas phosphorylation and downstream signaling, leading to lamellipodial ruffling.

Discussion

Cell migration requires biophysical and biochemical signaling between the leading edge and adhesion complexes to coordinate adhesion assembly and disassembly with actin polymerization, anchorage of stress fibers and generation of traction forces (Trepat et al., 2012). Our results address one aspect of this complicated process: the linkage between integrin engagement and lamellipodial extension. Previous studies have found that Cas and BCAR3 synergize for lamellipodium ruffing and cell migration, but they also synergized for protein expression, making the mechanism unclear (Cai et al., 1999, Cross et al., 2016, Riggins et al., 2003, Schrecengost et al., 2007, Wallez et al., 2014). Here, using loss-of-function approaches, we find that there is positive feedback between Cas and BCAR3 phosphorylation, and dual negative feedback on both proteins by the ubiquitin-proteasome system.

Our results support a multi-step model (Figure 9). First, localization signals in Cas bring Cas to active integrins near the leading edge of migrating cells. Cas recruitment does not require BCAR3, but BCAR3 is recruitment requires binding to Cas. Since most BCAR3 in the cell is complexed with Cas (Cross et al., 2016), the two proteins are likely recruited jointly, via localization signals on Cas. We do not know where or how BCAR3 Y117 is phosphorylated, but after phosphorylation on Y117, BCAR3 stimulates Cas phosphorylation on its YxxP motifs in a mechanism that also requires the BCAR3 SH2 domain. BCAR3-dependent activation of Cas is specific in the sense that FAK activation does not require BCAR3. Cas phosphorylation then drives membrane ruffling, presumably utilizing the well-documented mechanisms involving Crk, DOCK180 and Rac (Cheresh et al., 1999, Klemke et al., 1998, Sakai et al., 1994, Sanders et al., 2005, Schaller et al., 1995, Sharma et al., 2008). Thus, the BCAR3 SH2 and Y117 provide a critical switch to activate Cas. Our results also suggest that phosphorylation at Y117 promotes SOCS6 binding and targets BCAR3 for ubiquitination and degradation by the CRL5 E3 ubiquitin ligase. pY-Cas is independently targeted by $CRL5^{SOCS6}$ binding to its pYDYV motif (Teckchandani et al., 2014). Ubiquitination likely occurs within the adhesion (Teckchandani et al., 2016). Ubiquitination and degradation of either protein is expected to terminate signaling until new Cas and BCAR3 molecules are recruited from the cytosol, providing double insurance against excess activity.

Our results reveal that BCAR3 Y117 is required for both signaling and degradation. This means that BCAR3 activation triggers BCAR3 degradation in a negative feedback loop. The dual use of a single site for activation and degradation resembles Y198 of the neuron migration protein

Dab1, which binds downstream signaling molecules and also recruits SOCS6/7 for CRL5-dependent degradation (Simo et al., 2013). Mutating the SOCS-binding site in BCAR3 or Dab1 generates a non-functional protein that accumulates at higher level. In contrast, the SOCS6-binding site in Cas is not needed for downstream signaling, so mutating the site causes increased levels of an active protein and a dominant gain-of-function phenotype (Teckchandani et al., 2014).

BCAR3 Y117 is conserved across vertebrates, including zebrafish. How might it signal? Its phosphorylation could recruit an SH2 domain protein to stimulate Cas phosphorylation. The Y117 sequence, pYVKF, contains hydrophobic residues at +1 and +3 that characterize other SOCS6 SH2 binding sites (Krebs et al., 2002, Simo et al., 2013, Teckchandani et al., 2014, Zadjali et al., 2011) (Figure 9 – Figure Supplement 1). However, it also contains a lysine residue at position +2 and basic residues tend to inhibit SH2 domain binding (Songyang et al., 1993). Therefore, pY117 pmay instead stimulate Cas phosphorylation by an allosteric mechanism or by altering binding to other charged molecules, such as membrane phospholipids.

BCAR3 is one of three human NSP proteins. NSP1/SH2D3A is also expressed in MCF10A cells, but it may be unable to functionally substitute for BCAR3 because it lacks a Y117 homolog. The third family member, SH2D3C/SHEP1, contains a Y117 homolog but is not expressed in MCF10A cells (Vervoort et al., 2007). Cas may also be non-redundant in MCF10A cells because it is the only member of its protein family that is expressed (http://lincs.hms.harvard.edu/db/datasets/20348/results). Thus, the lack of a Y117 homolog in SH2D3A and the absence of SH2D3C expression could explain why depleting BCAR3 from MCF10A cells phenocopies depletion of Cas.

Our results add to our understanding of how Cas localizes to adhesions, which requires its CCH/FAT domain (Branis et al., 2017, Donato et al., 2010, Janostiak et al., 2014, Nakamoto et al., 1997). BCAR3 may regulate Cas localization in breast cancer cells and fibroblasts (Makkinje et al., 2009, Schrecengost et al., 2007, Sun et al., 2012), but our data suggest that BCAR3 does not regulate Cas localization to adhesions in normal epithelia. Whether these differences are due to the difference in cell type or expression level is unclear. In the absence of BCAR3, which other proteins may localize Cas to adhesions? Other reported Cas CCH/FAT-binding proteins include paxillin, zyxin and ajuba (Pratt et al., 2005, Yi et al., 2002, Zhang et al., 2017). Paxillin is a bona fide adhesion protein, but its binding to the Cas CCH/FAT in vitro is very weak (Zhang et

al., 2017). Zyxin binds the Cas homolog NEDD9 (Yi et al., 2002) but zyxin appears in adhesions after Cas (Zaidel-Bar et al., 2004), so is unlikely to be responsible for Cas localization. Ajuba contains a pre-LIM region that binds tightly to the Cas CCH/FATand a LIM region that binds to actin filaments under tension and localize to adhesions (Pratt et al., 2005, Winkelman et al., 2020). Moreover, Cas is mis-localized in ajuba-deficient cells (Pratt et al., 2005). Thus, ajuba or other LIM domain proteins may be responsible for localizing Cas to adhesions.

We found that BCAR3 regulates phosphorylation of Cas but not FAK in adhesions. This is consistent with other studies suggesting that Src, not FAK, phosphorylates Cas, with FAK serving as a scaffold (Ruest et al., 2001, Sharma et al., 2008). BCAR3 may help activate SFKs. Previous studies reported that SFK autophosphorylation is regulated by BCAR3, but the mechanism was unclear (Riggins et al., 2003, Schuh et al., 2010, Sun et al., 2012). In fibroblasts, phosphorylated PTPRA binds to the BCAR3 SH2 domain and brings Cas to FAs for phosphorylation by SFKs (Sun et al., 2012). Since PTPRA is able to activate SFKs by SH2 competition or dephosphorylating a C-terminal inhibitory tyrosine residue (Ponniah et al., 1999, Su et al., 1999, Zheng et al., 2000), this is an attractive model. However, in our studies, the BCAR3 SH2 domain is not needed to localize either BCAR3 or Cas, suggesting that Cas-BCAR3 would bring PTPRA to FAs rather than vice versa. In addition, PTPRA depletion does not inhibit Cas phosphorylation (data not shown). Therefore, how BCAR3 regulates SFK activity in this system will require further study.

Our finding that BCAR3 is subject to complex post-translational regulation may be helpful in reconciling the observation that BCAR3 over-expression in cell culture increases transformed phenotypes whereas BCAR3 RNA expression correlates with favorable outcomes in patients (Guo et al., 2014, Wallez et al., 2012, Zhang et al., 2018). In addition to the phosphorylation-dependent mechanism investigated here, a previous study showed that TGFβ stimulates proteasomal degradation of BCAR3 (Guo et al., 2014), and over-expressed BCAR3 and Cas appear to stabilize each other in breast cancer cells, dependent on their mutual binding (Wallez et al., 2014). Changes in BCAR3 protein stability or phosphorylation state might influence BCAR3 activity in patients, but that such regulation at the protein level might not be accounted for when correlating patient outcomes with gene expression. Thus, further study is required to understand mechanisms of BCAR3 regulation and how mis-regulation may contribute to disease progression.

Materials and Methods

Plasmids

pMXpuroII and pMXpuroII-shCul5 plasmids were made as previously described (Teckchandani et al., 2014). pLKO.1-nontarget small hairpin RNA (shRNA) control vector (SHC002) and pLKO.1-shSOCS6 (TRCN0000284351) were purchased (Sigma Aldrich).

pCAG-T7-mSOCS1, pCAG-T7-mSOCS2, pCAG-T7-mSOCS3, pCAG-T7-mSOCS4, pCAG-T7-mSOCS5, pCAG-T7-mSOCS6, pCAG-T7-mSOCS7, and pCAG-T7-mCisH were made as previously described (Simo et al., 2013, Teckchandani et al., 2014). All pCAG-T7-SOCS plasmids used in this work have LC-QQ mutations in the SOCS box to prevent binding to CRL5, and were made using PfuTurbo DNA polymerase to perform site-directed mutagenesis followed by DpnI digestion. pCAG-T7-mSOCS6 R407K was made as previously described (Teckchandani et al., 2016). pCAG-T7-mSOCS6 ΔC was made by PCR amplifying codons 1-381 of mSOCS6 using a 5' primer complementary to the T7 tag and a 3' primer that inserts a stop codon and a NotI site after codon 381. The PCR product was inserted into pCAG-T7-SOCS6 by BamH1/NotI restriction digest and ligation.

pCMV-SPORT6-mBCAR3 was purchased from the Harvard Plasmid Database (MmCD00318547). pcDNA5-3xFlag-mBCAR3 FRT/TO was made by Gateway cloning. mBCAR3 was PCR amplified with flanking attB sites from pCMV-SPORT6-mBCAR3 and inserted into pDONR221 with a BP reaction (Thermo Fisher Scientific). pDEST-5'-3xFlag-pcDNA FRT/TO was a kind gift of Dr. Anne-Claude Gingras. pDONR221-mBCAR3 was moved into pDEST-5'-3xFlag-pcDNA FRT/TO with a LR reaction (Thermo Fisher Scientific).

pLX304-hBCAR3-V5 was obtained from the human ORFeome version 8.1 Entry clone collection (Yang et al., 2011). pLX304-hBCAR3-F5-V5 was made by Gibson Assembly (New England Biolabs). A gBlock (Integrated DNA Technologies) containing BCAR3 Y42F, Y117F, Y212F, Y266F and Y429F mutations was assembled into pLX304-hBCAR3-V5.

rtTA-N144 was a gift from Andrew Yoo (Addgene plasmid # 66810).

pLTRE3G-SNAP-V5-hBCAR3 was made as follows. A gBlock containing sequence for SNAP-V5 tags was inserted into pLenti CMVTRE3G eGFP Blast (w818-1) (gift of Eric Campenau, Addgene #27568) at the AgeI restriction site using Gibson Assembly to make pLTRE3G-SNAP-

V5-eGFP. hBCAR3 was PCR amplified with flanking AgeI and XbaI sites and inserted into AgeI and XbaI-digested pLTRE3G-SNAP-V5-eGFP to make pLTRE3G-SNAP-V5-hBCAR3.

pLTRE3G-SNAP-V5-hBCAR3 mutants were made as follows. Single mutants Y42F, Y212F, Y266F, Y429F, R177K, and double mutant EE (L744E/R748E) were made by site-directed mutagenesis with Q5 polymerase (New England Biolabs) and Dpn1 digestion. The Y117F mutant was made by Gibson Assembly. A gBlock containing the BCAR3 Y117F mutation was assembled into pLTRE3G-SNAP-V5-hBCAR3 WT. pLTRE3G-SNAP-V5-hBCAR3 F5 was made by BamHI restriction digest of pLTRE3G-SNAP-V5-hBCAR3 Y42F, discarding the region between codon 107 and 763 and inserting the corresponding region from pLX304-hBCAR3-F5-V5. pLTRE3G-SNAP-V5-hBCAR3 F4 was made by PshAI restriction digest of pLTRE3G-SNAP-V5-hBCAR3 F5, discarding the region between codon 67 and 163 inserting the corresponding region from pLTRE3G-SNAP-V5-hBCAR3 WT.

Cell lines

MCF10A cells were cultured in DMEM/F12 (Thermo Fisher Scientific) supplemented with 5% horse serum (Thermo Fisher Scientific), 0.1 μg/ml cholera toxin (EMD Millipore), 10 μg/ml insulin (Thermo Fisher Scientific), 0.5 μg/ml hydrocortisone (Sigma-Aldrich), and 20 ng/ml EGF (Thermo Fisher Scientific). Indicated experiments used assay media (DMEM/F12, 2% horse serum, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 0 ng/ml EGF). The cells were passaged using 0.02% trypsin in PBS/EDTA. Trypsin was inactivated with an equal volume of DMEM/10% FBS and cells were harvested by centrifugation before resuspending in growth media. HeLa and 293T cells were grown in DMEM/10% FBS and passaged with trypsin as above.

MCF10A empty vector (EV) and shCul5 cells were made as previously described (Teckchandani et al., 2014). MCF10A shControl and shSOCS6 cells were made as follows. Viruses containing pLKO.1-control and pLKO.1-shSOCS6 were packaged using HEK 293T cells and MCF10A cells were infected. Stable cell lines were selected using 4 µg/mL puromycin.

BCAR3-/- MCF10A cells were made as follows. Early passage MCF10A cells were serially diluted and subclones were grown and characterized. Clone J8 was selected for its epithelial morphology. The J8 clonal cell line was infected with lentiCRISPR v2 (a gift from Feng Zhang; Addgene plasmid # 52961), lacking or containing guide RNA against BCAR3. pLCRISPRv2-

sgRNA-hBCAR3(30) and (31) were made by cloning annealed oligos (5'-CACCGTCAGAGAGCTACCTGCCGAT-3' or 5'-CACCGCCCGAAACATACCAATCGGC-3') into the BsmBI site of plentiCRISPRv2. Potential knockouts were isolated by single cell expansion. Validation of *BCAR3* knockout was done through genomic DNA isolation, PCR, and sequencing, as well as Western blotting.

MCF10A dox-inducible SNAP-V5 BCAR3 cells were made as follows. Viruses containing rtTA-N144 were packaged using 293T cells and used to infect MCF10A cells. A stable line was selected using 50 µg/mL hygromycin. The MCF10A cell line stably expressing rtTA was then infected with viruses containing one of the pLTRE3G-SNAP-V5 constructs (pLTRE3G-SNAP-V5-eGFP, pLTRE3G-SNAP-V5-BCAR3 WT, Y42F, Y117F, Y212F, Y266F, Y429F, F5, F4, R177K, or EE). Stable lines were selected using 10 µg/mL blasticidin. Each cell line was hygromycin resistant and blasticidin resistant, however not all cells within each line expressed the SNAP-V5-containing construct following induction with 50-100ng/mL dox for 48-72 hours. To remove non-inducible cells, dox-treated cells were treated with 100 nM JaneliaFluor cp646JF-SNAP-ligand (Lavis Lab, Janelia Farms) (Grimm et al., 2015) for 1 hour, washed three times with PBS, incubated in ligand-free growth media for an hour and positive cells were sorted and harvested by FACS using the APC channel to detect cp646JF.

Antibodies and Reagents

Antibody	Supplier	Catalog	Dilution
rabbit anti-BCAR3	Bethyl	A301-671A-M	Western (1:2000)
rabbit anti-Cas (C-20)	Santa Cruz	sc-960	Western (1:1000)
mouse anti-Cas	BD Biosciences	610271	Western (1:5000)
rabbit anti-pY165 Cas	Cell Signaling	4015S	IF (1:200)
rabbit anti-Cullin5	Abcam	ab184177	Western (1:1000)
rabbit anti-GAPDH (0411)	Santa Cruz	sc-47724	Western (1:1000)
mouse anti-vinculin	Sigma	V9131	Western (1:10000)
mouse anti-paxillin	BD Biosciences	610051	IF (1:200)
mouse anti-FLAG	Sigma	F1804	Western (1:1000)

mouse anti-T7	EMB Biosciences	69522-4	Western (1:5000) IP
rabbit anti-V5	Bethyl	A190-120A	Western (1:5000) IP
AlexaFluor 488 goat anti-rabbit IgG (H+L)	Invitrogen	A11008	IF (1:1000)
AlexaFluor 647 goat anti-mouse IgG (H+L)	Invitrogen	A28181	IF (1:1000)

Reagents used: cycloheximide (Sigma), epidermal growth factor (Invitrogen), MLN4924 (Active Biochem), MG132 (Fisher Scientific), bafilomycin A, doxycycline (Fisher Scientific).

Mass spectrometry

MCF10A EV and shCul5 cells were grown in 15-cm plates, two plates per condition, to approximately 80% confluence, washed in PBS, and lysed in 3 mL per plate of ice-cold 8 M urea containing 1 mM sodium orthovanadate. Proteins were reduced, alkylated and digested with trypsin as described (Zhang et al., 2005). Peptide labeling with iTRAQ reagents (TMT 6plex, Themo Fisher Scientific) was performed according to the manufacturer's instructions, using 400 μg of each sample. After reaction, the six samples were combined and concentrated under vacuum. For immunoprecipitation, protein G agrarose (60 μl, Millipore) was incubated with anti-phosphotyrosine antibodies (12 μg 4G10 (Millipore), 12 μg PT66 (Sigma) and 12 μg PY100 (Cell Signaling Technology) for 8 hours at 4 ^{o}C with rotation, then washed with 400 μl IP buffer (100 mM Tris HCl, 100 mM NaCl, and 1 % Nonidet P-40, pH 7.4). The TMT-labeled peptides were resuspended in 400 μl IP buffer and pH adjusted to 7.4, then mixed with the antibody-conjugated protein G agarose overnight at 4 ^{o}C with rotation. The antibody beads were spun down and the supernatant saved. The beads were washed with 400 μl IP buffer then four rinses with 100 mM Tris HCl pH 7.4 before elution with 70 μl of 100 mM glycine, pH 2.0 for 30 minutes at 25 ^{o}C. Offline immobilized metal affinity chromatography (IMAC) was used to further enrich for phosphotyrosine peptides (Zhang et al., 2005).

Peptides were loaded on a precolumn and separated by reverse phase HPLC using an EASY-nLC1000 (Thermo) over a 140-minute gradient from 100% solvent A (H_2O/CH_3COOH, 99:1 (v/v)) to 30% A: 70% B ($H_2O/CH_3CN/CH_3COOH$, 29:70:1 (v/v)). Eluted peptides were injected

using nanoelectrospray into a QExactive Pluss mass spectrometer (Thermo) in data-dependent mode. The parameters for full scan MS were: resolution of 70,000 across 350-2000 *m/z*, AGC $3e^6$, and maximum IT 50 ms. The full MS scan was followed by MS/MS for the top 10 precursor ions in each cycle with a NCE of 34 and dynamic exclusion of 30 s. Raw mass spectral files (.raw) were searched using Proteome Discoverer (Thermo) and Mascot version 2.4.1 (Matrix Science). Mascot search parameters were: 10 ppm mass tolerance for precursor ions; 15 mmu for fragment ion mass tolerance; 2 missed cleavages of trypsin; fixed modifications were carbamidomethylation of cysteine and TMT 6plex modification of lysines and peptide N-termini; variable modifications were methionine oxidation, tyrosine, serine and threonine phosphorylation. TMT quantification was obtained using Proteome Discoverer and isotopically corrected per manufacturer's directions, and normalized to the mean relative protein quantification ratios obtained from the total protein analysis (0.2% of the unbound peptides from the phosphotyrosine peptide immunoprecipitation). Mascot peptide identifications, phosphorylation site assignments and quantification were verified manually with the assistance of CAMV (Curran et al., 2013). Validated data were analyzed in Excel using a one-sided two-sample equal variance Student t-test.

siRNA Transfection

MCF10A cells were trypsinized and seeded in growth medium into a 12-well plate so as to be 50% confluent after attaching. A mixture of 50 pmol siRNA, 1.25 μl Lipofectamine 2000 (Invitrogen) in Optimem medium (Invitrogen) was added to the newly plated MCF10A cells and left for the cells to attach overnight. The next day the media was changed to fresh growth media. A second siRNA transfection was done 48 hours after the first transfection using the same protocol scaled up to use a 6-well plate (125 pmol siRNA) or scaled down to use a 96-well plate (for migration assays). One day later, cells from the 6-well dish were transferred to 4-well plates containing 12-mm coverslips for microscopy, or 12-well plates for protein analysis. Doxycline was added as needed at 10 ng/mL for 2-3 days before analysis. Cells were either lysed 48 hours after the second transfection or assays were performed as described.

siRNA	Target Sequence	Source	Notes	Catalog
Control siRNA	5'-AATTCTCCCGAACGTGTCACGT-3'	Qiagen		1027310
SOCS6 siRNA Pool	5'-TAGAATCGTGAATTGACATAA-3' 5'-CAGCTGCGATATCAACGGTGA-3'	Qiagen		SI00061383 SI03068359

	5’-TTGATCTAATTGAGCATTCAA-3’ 5’-CGGGTACAAATTGGCATAACA-3’			SI00061369 SI00061376
Cul5 siRNA Pool	5’-GACACGACGTCTTATATTA-3’ 5’-CGTCTAATCTGTTAAAGAA-3’ 5’-GATGATACGGCTTTGCTAA-3’ 5’-GTTCAACTACGAATACTAA-3’	GE Dharmacon		M-019553-01-0005
BCAR1 siRNA Pool	5’-AAGCAGTTTGAACGACTGGAA-3’ 5’-CTGGATGGAGGACTATGACTA-3’ 5’-CAGCATCACGCGGCAGGGCAA-3’ 5’-CAACCTGACCACACTGACCAA-3’	Qiagen		SI02757741 SI02757734 SI04438280 SI04438273
BCAR3 siRNA Pool	5’-CCGGAACTCTGGCGTCAACTA-3’ 5’-CCGAGCGGCCACTCTGAGTAA-3’ 5’-GCCCAACGAGTTTGAGTCAAA-3’ 5’-AAGGTATCAGTTATATGATAT-3’	Qiagen		SI03081603 SI03080196 SI00053102 SI00053095
BCAR3 siRNA	5’-GGUAACUACUGCUAAUGUUTT-3’	Life Technologies	Targets 3’UTR	AM16708

qPCR

An RNeasy Plus Mini kit (Qiagen) was used to extract RNA and an iScript reverse transcription supermix (BioRad) was used to make cDNA. Control reactions lacked reverse transcriptase. cDNA abundance was measured using an iTaq Universal SYBR Green Supermix kit (BioRad). Samples were run on the QuantStudio 5 Real-Time PCR System.

Primer	Sequence
BCAR3 forward	5’ – AATCGCTTCTCCAAACAGAGC – 3’
BCAR3 reverse	5’ – ATTCACCGGCATGTTTCTGG – 3’
Cul5 forward	5’ – TTTTATGCGCCCGATTGTTTTG – 3’
Cul5 reverse	5’ – TTGCTGGGCCTTTATCATCCC – 3’
GAPDH forward	5’ – CAGCCTCAAGATCATCAGCA – 3’
GAPDH reverse	5’ – TGTGGTCATGAGTCCTTCCA – 3’

Cell lysis and Immunoblotting

Cells were washed three times in phosphate-buffered saline (PBS) before lysis. Cells were lysed in X-100 buffer (1% Triton X-100, 150 mM NaCl, 10 mM HEPES pH 7.4, 2 mM EDTA, 50 mM NaF) or RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA) with fresh protease and phosphatase inhibitors (10 μg/mL Aprotinin, 1 mM PMSF, 1 mM sodium vanadate) added before use.

Lysates were adjusted to SDS sample buffer, heated to 95 ^{o}C, resolved by SDS-PAGE using 10% polyacrylamide/0.133% bis-acrylamide gels, and transferred onto nitrocellulose membrane. The membrane was blocked in Odyssey blocking buffer (TBS) (LI-COR Biosciences) with 5% BSA for phosphotyrosine antibodies or 5% non-fat dry milk for all other antibodies. Following blocking, the membrane was probed with a primary antibody followed by IRDye 800CW goat anti-rabbit or 680RD goat anti-mouse conjugated secondary antibodies. Membranes were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences).

DNA transfections and Immunoprecipitation

HeLa cells were plated in 6-well plates the day before transfection such that cells were 50% confluent on the day of transfection. A mixture of DNA, Lipofectamine 2000 (Thermo Fisher Scientific) and Optimem (Invitrogen) was made according to manufacturer's protocol, added to the cells and removed after 5 hours.

Immunoprecipitation experiments were conducted 24-48 hours after transfection. For indicated experiments, cells were incubated with 1 mM sodium pervanadate for 30 minutes. Cells were lysed on ice in X-100 buffer with fresh protease and phosphatase inhibitors (see above). Lysates were cleared by centrifugation for 10 minutes at 14,000 g. Lysates were rotated with 1 μg antibody at 4°C for 3 hours, after which Protein A/G plus agarose beads (Santa Cruz Biotechnology) were added for 1 hour at 4°C. Beads were collected by centrifugation and washed three times with X-100 buffer. The beads were resuspended in SDS sample buffer, boiled, lightly mixed to release bound protein and centrifuged. Immunoprecipitation samples were resolved by SDS-PAGE using 10% polyacrylamide gels and bound proteins detected by Western blotting as above. Samples of total cell lysate typically contained 5% of the protein used for immunoprecipitation.

Cycloheximide chase

Cells were grown to 80% confluency and treated with 25μg/mL cycloheximide by adding it directly to the conditioned media on the cells. Cells were treated with cycloheximide for 0, 2, 4, or 8 hours and lysed. Quantification of Western blots was done in ImageJ and BCAR3 protein levels were normalized to the loading control (GAPDH or vinculin).

Scratch wound assay

The desired proteins were knocked down using two siRNA transfections, as previously described. Cells were plated in ImageLock 96-well dishes (Essen BioScience) for the second siRNA transfection and transfection materials were scaled down proportional to dish sizes. Cells were plated at 30% confluence at the time of the second transfection. For migration assays where doxycycline (dox)-induction was required, the media was replaced with growth media containing dox (10 ng/mL except where noted) 6 hours after transfection and after two washes with PBS. For all migration assays, the confluent monolayers were placed in EGF-free assay media (with dox when required) 48 hours after the second transfection transfection. Monolayers were scratched using an Incucyte WoundMaker (Essen BioScience) 8 hours after being placed in assay media, the wells were washed with PBS three times to remove debris and cells were placed back in assay media (with dox when required). Scratch wounds were imaged once every 2 hours on an IncuCyte S3 and images were analyzed using the scratch wound function on the IncuCyte image analysis software. Overall migration was measured using the relative wound density calculated by the analysis software. Lamellipodium length was measured using the ruler in the IncuCyte image analysis software. Membrane ruffles were visualized as dark contrast at the front of the protrusion. Ruffling was scored by counting the number of leading edge cells that did and did not have membrane ruffles to calculate a percentage of ruffling cells.

Transwell migration and invasion assays

Cells were grown in EGF-free assay media for 24 hours before the assay. Migration assays were performed in 24-well chemotaxis chamber with an 8 μm pore size polyethylene terephthalate filter that separated the upper and lower wells (Thermo Fisher Scientific). Invasion assays were performed in 24-well Matrigel invasion chambers with an 8 μm pore size polyethylene terephthalate filter coated in Matrigel matrix that separated the upper and lower wells (Thermo Fisher Scientific). In both assays, the lower wells were filled with MCF10A growth media. 80,000 cells were resuspended in EGF-free assay media and added to the top well. After 24 hours, cells on the top of the membrane were removed and the cells on the bottom of

the membrane were fixed with methanol and stained with SybrSafe at 1:10,000 for 15 minutes. Membranes were rinsed, imaged and nuclei were counted.

Immunofluorescence

For experiments that required protein knockdown, cells were transfected with siRNA as previously described. On the day following the second siRNA transfection, cells were plated on 12-mm #1.5 coverslips at 30% confluency in 4-well or 24-well (1.9 cm^2) plates for scratch wound assays (with dox when required). Cells were placed in assay media overnight the day before the assay and confluent monolayers were scratched with a P200 pipette tip the following morning. Cells were fixed and permeabilized 6 hours after the start of migration.

To detect the SNAP tag, JaneliaFluor cpJF549 SNAP-ligand (Lavis Lab, Janelia Farms) (Grimm et al., 2015) was added to the cells at 100 nM 4 hours after forming the wounds. Cells were incubated for 1 hour with the SNAP-ligand, washed three times with PBS and placed in fresh, ligand-free assay media for 1 hour to remove unreacted SNAP-ligand.

Fixation and immunostaining was done at room temperature. Cells were washed with PBS, fixed and permeabilized with 0.1% Triton X-100, 4% paraformaldehyde (PFA) in PBS for 2 minutes, and further fixed with 4% PFA/PBS for 15 minutes. Cells were blocked with 2% BSA, 5% normal goat serum in PBS for 30 minutes or overnight at 4ºC. Primary antibodies were diluted in block solution (typically 1:200 dilution) and added for 3 hours. Coverslips were washed three times with PBS. AlexaFluor-labeled secondary antibodies, diluted 1:1000 in PBS, were added for 1 hour. Coverslips were washed three times with PBS, mounted in Prolong Glass Antifade Mountant and left to cure overnight in the dark before imaging or storing in the cold.

Imaging and image quantification

Coverslips were imaged using 63x, 1.40 NA or 100x, 1.40 NA oil objectives on a Leica SP8 confocal microscope and deconvolved using Leica LAS acquisition software. Scale and imaging settings were consistent within each experiment.

Image quantification was done in ImageJ. A line was drawn ~6 µm from the front of the migrating cells, as described (Teckchandani et al., 2016), to isolate the leading edge. Using the threshold tool on ImageJ, paxillin-containing adhesions were identified and mean intensity of SNAP, pY-Cas, EYFP-Cas and paxillin in these leading edge adhesions and in the cytoplasm at

the rear of the cell were calculated. The SNAP/paxillin ratio was calculated by subtracting a ligand background mean intensity value (taken from a non-dox-induced sample) from the SNAP mean intensity, normalizing to the cytoplasmic SNAP mean intensity and then dividing by the paxillin mean intensity that was normalized to cytoplasmic paxillin mean intensity. The EYFP-Cas/paxillin ratio was calculated by normalizing the mean intensities of each in adhesions to the mean intensities in the cytoplasm and dividing normalized EYFP-Cas intensity by the normalized paxillin intensity. The pY-Cas/paxillin ratio was calculated by dividing the mean intensity of pY-Cas in adhesions by the mean intensity of paxillin.

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Figure 1

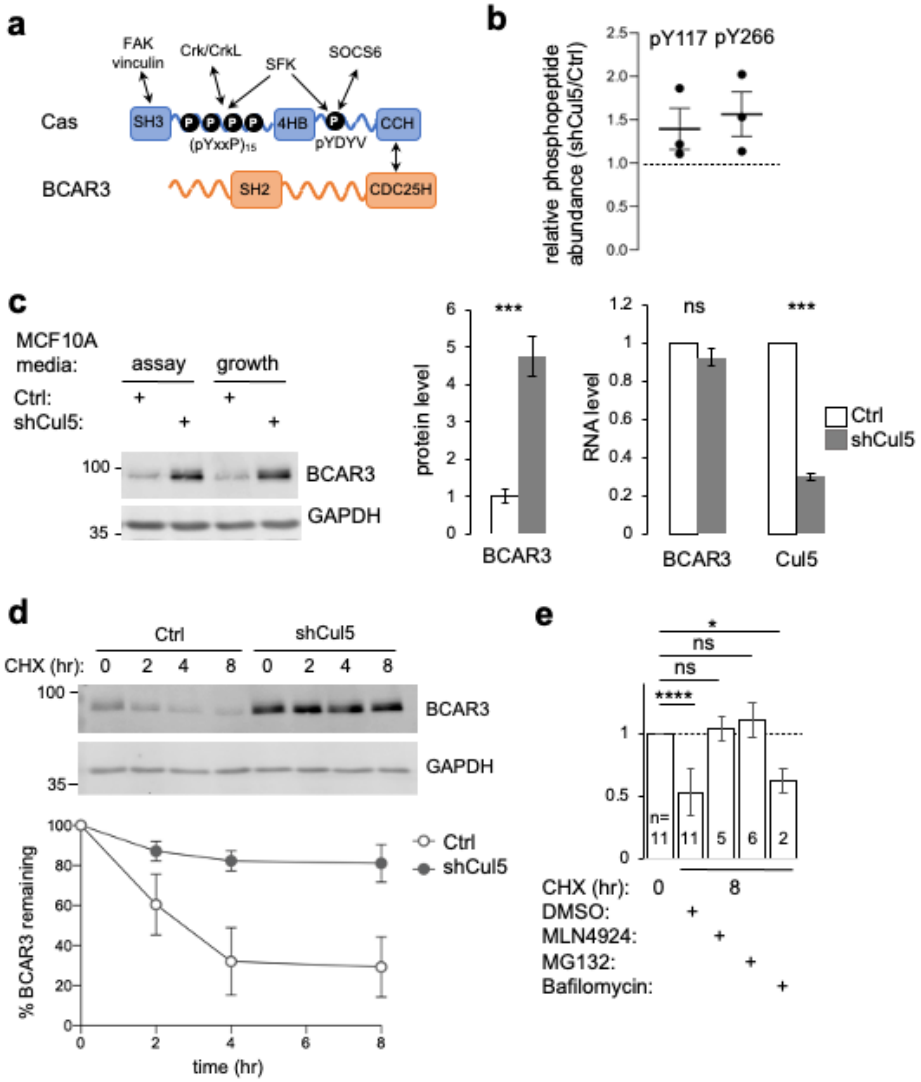

Figure 1. CRL5 regulates BCAR3 protein turnover. (a) Cas and BCAR3 structures. (b) Ratio of BCAR3 pY117 and pY266 phosphopeptide abundances in Cul5-deficient (shCul5) relative to control (Ctrl) MCF10A cells. n=3 biological replicates. (c) BCAR3 protein and RNA levels in Ctrl and shCul5 cells. Representative immunoblot of cells grown in assay media (no added EGF) or growth media (containing EGF). Graphs show immunoblot quantifcation of BCAR3 protein and qPCR quantification of BCAR3 RNA relative to GAPDH control. Mean±SD ; n=3 biological replicates. ***p < 0.001 (t-test). (d) BCAR3 degradation. Control and Cul5-deficient cells were treated with cycloheximide (CHX) for various times. Representative immunoblot and quantification. Mean±SEM ; n=3 biological replicates. (e) Proteasome-dependent BCAR3 degradation. MCF10A cells were treated for 8 hours with CHX and either MLN4924 (cullin neddylation inhibitor), MG132 (proteasome inhibitor) or bafilomycin A1 (lysosome inhibitor). Mean±SD; the number of biological replicates is noted on the graph. *p < 0.05; ****p < 0.0001 (One-way ANOVA).

Figure 1 – figure supplement 1

Protein	Position	Uniprot	Sequence	Experiment 1		Experiment 2	
				Fold[a]	p[b]	Fold[a]	p[b]
KRT6A	Y62	Q92625	sLyGLGGSk	3.21	0.012	1.85	0.011
ABL1	Y393	P00519	lMTGDTyTAHAGAk	2.90	0.010	1.77	0.001
ANKS1A	Y455	Q92625	eEDEHPyELLLTAETk	2.58	0.004	1.41	0.009
ARHGAP35	Y1105	Q9NRY4	nEEENIYsVPHDSTQGk	2.49	0.008	1.67	0.005
RIN1	Y36	Q13671	ekPAQDPLyDVPNASGGQAGGPQRPGR	2.02	0.018	1.45	0.018
BCAR3	Y117	O75815	dPHLLDPTVEyVk	1.96	0.011	1.34	0.049
MPZL1	Y263	O95297	sESVVyADIR	1.90	0.026	1.54	0.027
BCAR3	Y266	O75815	cLEEHyGTSPGQAR	1.81	0.000	1.50	0.012
BCAR1	Y128	P56945	aQQGLyQVPGPSPQFQSPPAk	1.80	0.003	1.28	0.036
SGK223	Y413	Q86YV5	eATQPEPIyAESTk	1.74	0.024	1.26	0.032
ENO1	Y44	P06733	aAVPSGASTGIyEALELR	1.64	0.004	1.18	0.032
ITGB4	Y1207	P16144	vcAYGAQGEGPySSLVScR	1.59	0.010	1.19	0.043
ANXA2	Y30	A6NMY6	ayTNFDAER	1.58	0.018	1.31	0.014
IGF1R	Y1165	P08069	dIYETDyYR	1.53	0.030	1.69	0.001
PTPRA	Y798	P18433	vVQEYIDAFSDyANFk	1.50	0.006	1.23	0.019
TLN1	Y26	Q9Y480	tMQFEPSTMVyDAcR	1.22	0.022	1.25	0.014

Figure 1 – figure supplement 1. Phosphotyrosine peptides increased in Cul5-deficient cells. Protein names, tyrosine positions, Uniprot accession numbers, peptide sequences, and quantification of phosphotyrosine peptides that were significantly increased in two independent experiments, each performed in triplicate, comparing Cul5-deficient and control MCF10A cells. In Experiment 1, the triplicate samples were obtained from cells starved for EGF for 0, 24 or 72 hours. In Experiment 2, the triplicate samples were all from unstarved cells. [a] Ratio shCul5/Ctrl. [b] p value, t-test (2-tailed, paired); n=3.

Figure 2

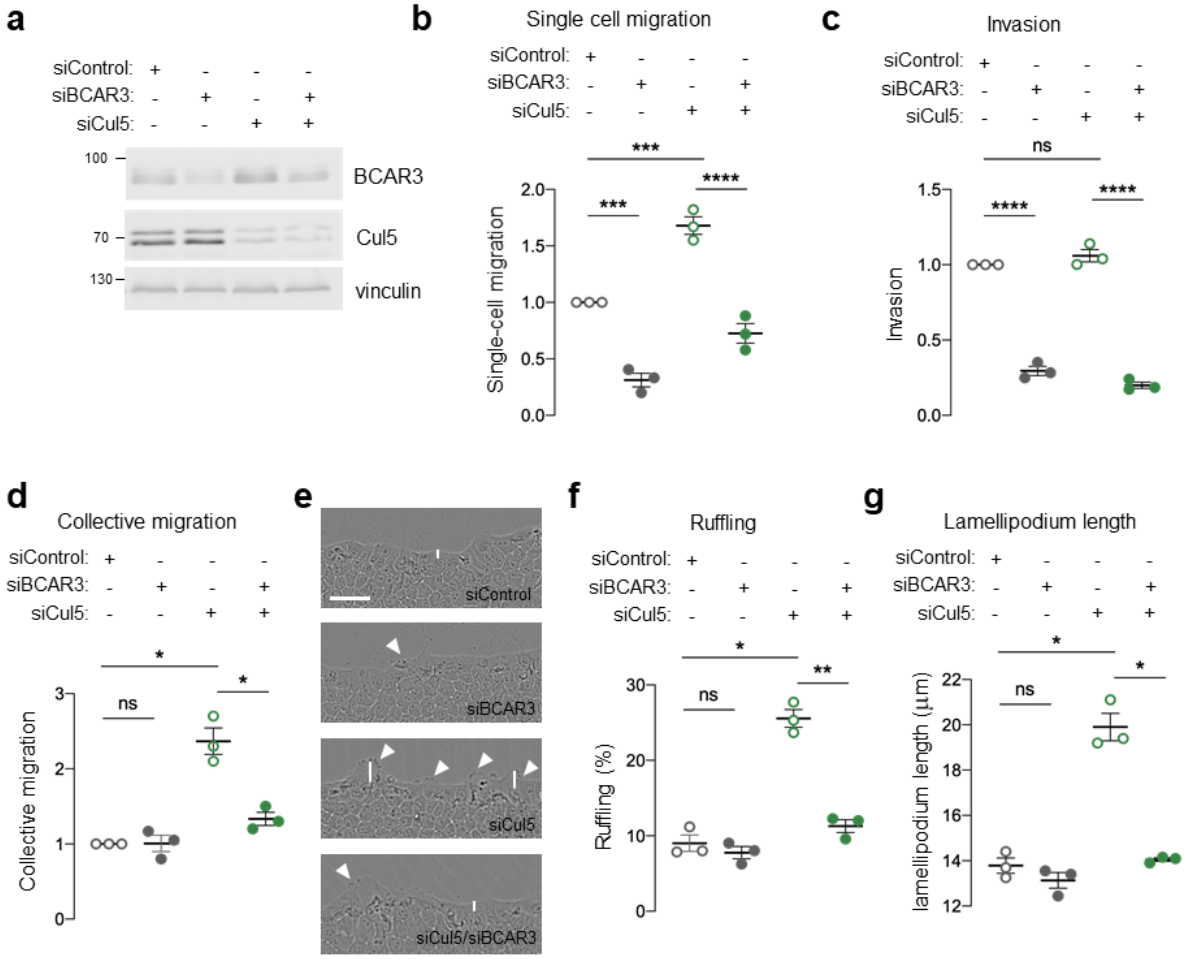

Figure 2. BCAR3 regulates epithelial cell migration. MCF10A cells were transfected with control, BCAR3 or Cul5 siRNA. (a) Representative immunoblot showing BCAR3, Cul5 and vinculin protein levels. (b) Single cell migration using Boyden chamber assay. Migration from EGF-free assay media towards EGF-containing growth media was measured. Mean±SEM; n=3 biological replicates, each with 5 technical replicates. ***p<0.0005 and ****p<0.0001 (One-way ANOVA). (c) Invasion using Boyden chamber containing Matrigel. Mean±SEM; n=3 biological replicates, each with 5 technical replicates. ****p<0.0001 (One-way ANOVA). (d-g) Collective migration. Confluent monolayers were placed in EGF-free media and wounded. (d) Relative migration after 12 hours. Mean±SEM; n=3 biological replicates each with 8-12 technical replicates. *p<0.05 (One-way ANOVA). (e) Representative images of scratch wounds after 6 hours of migration. Arrows indicate cells with membrane ruffles and lines indicate lamellipodia length measurements. Scale bar: 100 µm. (f) Percentage of ruffling cells. Mean±SEM of >250 cells per condition from n=3 biological replicates. *p<0.05 and **p<0.005 (One-way ANOVA). (g) Lamellipodia length. Mean±SEM of 50 cells per condition from n=3 biological replicates. *p<0.05 (One-way ANOVA).

Figure 2 – figure supplement 1

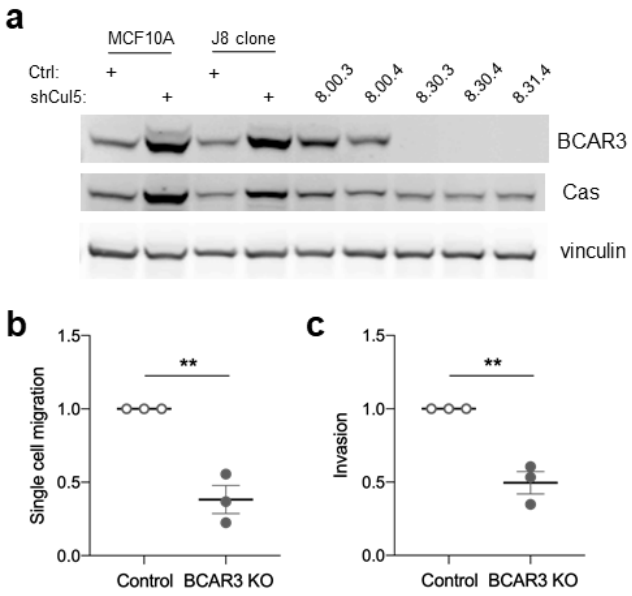

Figure 2 - figure supplement 1. ***BCAR3*** **gene deletion inhibits single-cell migration and invasion**. (a) MCF10A subclone J8, selected for its epithelial morphology, was infected with an all-in-one CRISPR plasmid lacking or containing guide RNA against BCAR3 (guide 30 or 31). Potential knockouts were isolated through single cell expansion. Levels of BCAR3 and Cas in control clonal cell lines, 8.00.3 and 8.00.4, were similar to those in J8 or uncloned MCF10A cells. *BCAR3-/-* (KO) cell lines, 8.30.3, 8.30.4, and 8.31.4, express Cas but not BCAR3. These clones were confirmed by PCR and sequencing (Materials and Methods). (b) Single-cell migration toward EGF using a Boyden chamber assay. Mean±SEM; n=3. **p=0.003 (unpaired t-test). (c) Invasion through Matrigel toward EGF. Mean±SEM; n=3. **p=0.0028 (unpaired t-test).

Figure 3

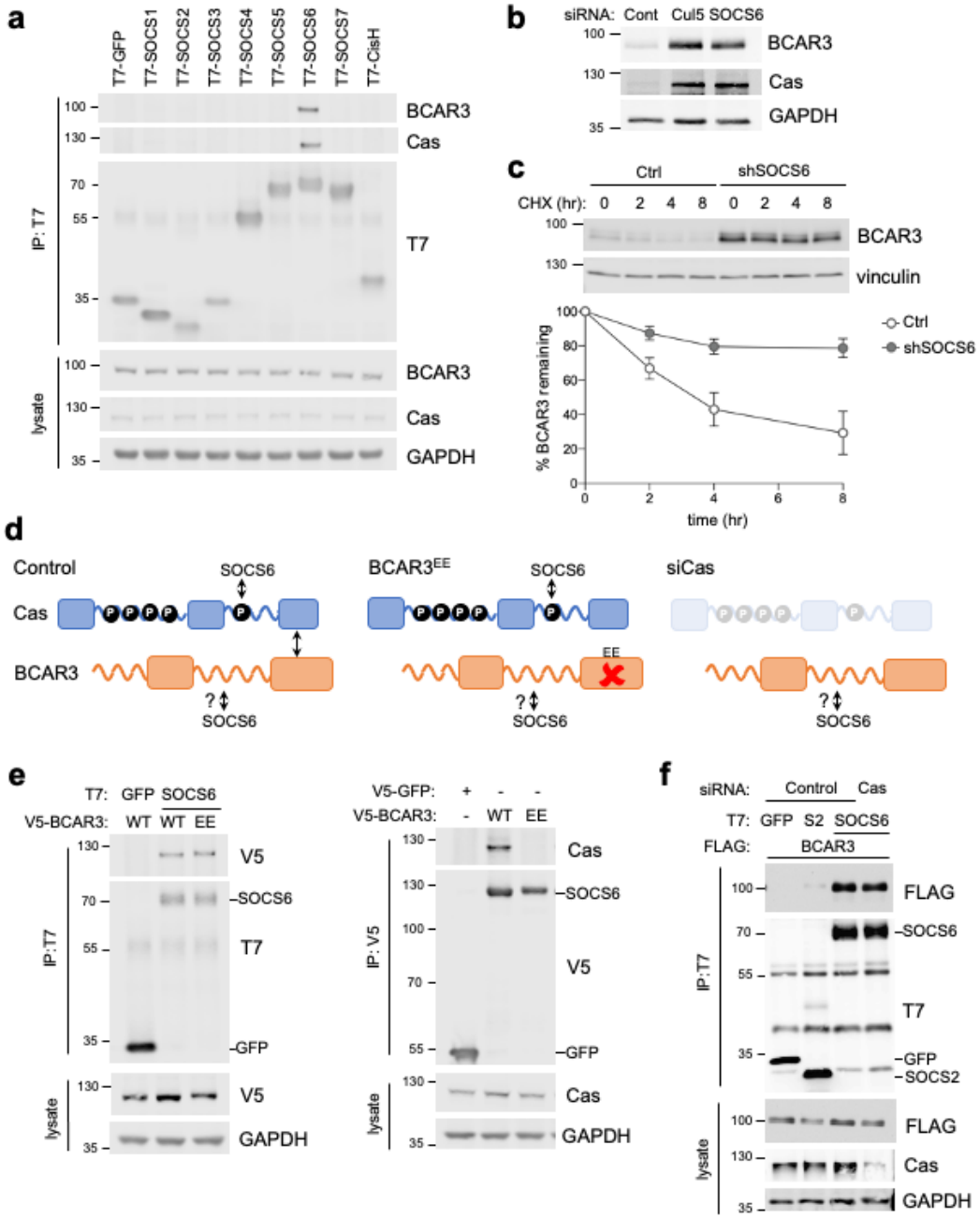

Figure 3. SOCS6 regulates BCAR3 stability independently from Cas. (a) SOCS6 binds BCAR3 and Cas. HeLa cells were transfected with T7-tagged GFP or SOCS proteins and treated with pervanadate for 30 minutes before lysis. Lysates were immunoprecipitated with T7 antibody and immunoprecipitates and lysates were immunoblotted with antibodies to BCAR3, Cas and T7. (b) SOCS6 regulates BCAR3 protein level. MCF10A cells were transfected with control, Cul5 or SOCS6 siRNA and analyzed by immunoblotting. (c) BCAR3 degradation. MCF10A control and SOCS6-deficient cells were treated with cycloheximide (CHX) for various times. Representative immunoblot and quantification. Mean±SEM; n=3 biological replicates. (d) Strategy for testing whether SOCS6 binding to BCAR3 requires Cas. (e) BCAR3 LxxE/RxxE mutation inhibits binding to Cas but not SOCS6. Left panel: HeLa cells were

transfected with control vector or T7-SOCS6 and SNAP-V5-BCAR3^{WT} or SNAP-V5-BCAR3^{EE}. The transfected cells were treated with pervanadate and lysed. Lysates were immunoprecipitated with T7 antibody and immunoprecipitates and lysates were immunoblotted with V5 and T7 antibodies. Right panel: HeLa cells were transfected with control vector, SNAP-V5-BCAR3^{WT} or SNAP-V5-BCAR3^{EE} and lysed. Lysates were immunoprecipitated with V5 antibody and immunoprecipitates and lysates were immunoblotted with Cas and V5 antibodies. (f) SOCS6 binds BCAR3 in Cas-deficient cells. HeLa cells were treated with control or Cas siRNA and transfected with T7-SOCS6 and 3xFLAG-BCAR3. Lysates were immunoprecipitated with T7 antibody and immunoprecipitates and lysates were immunoblotted with FLAG and Cas antibodies.

Figure 4

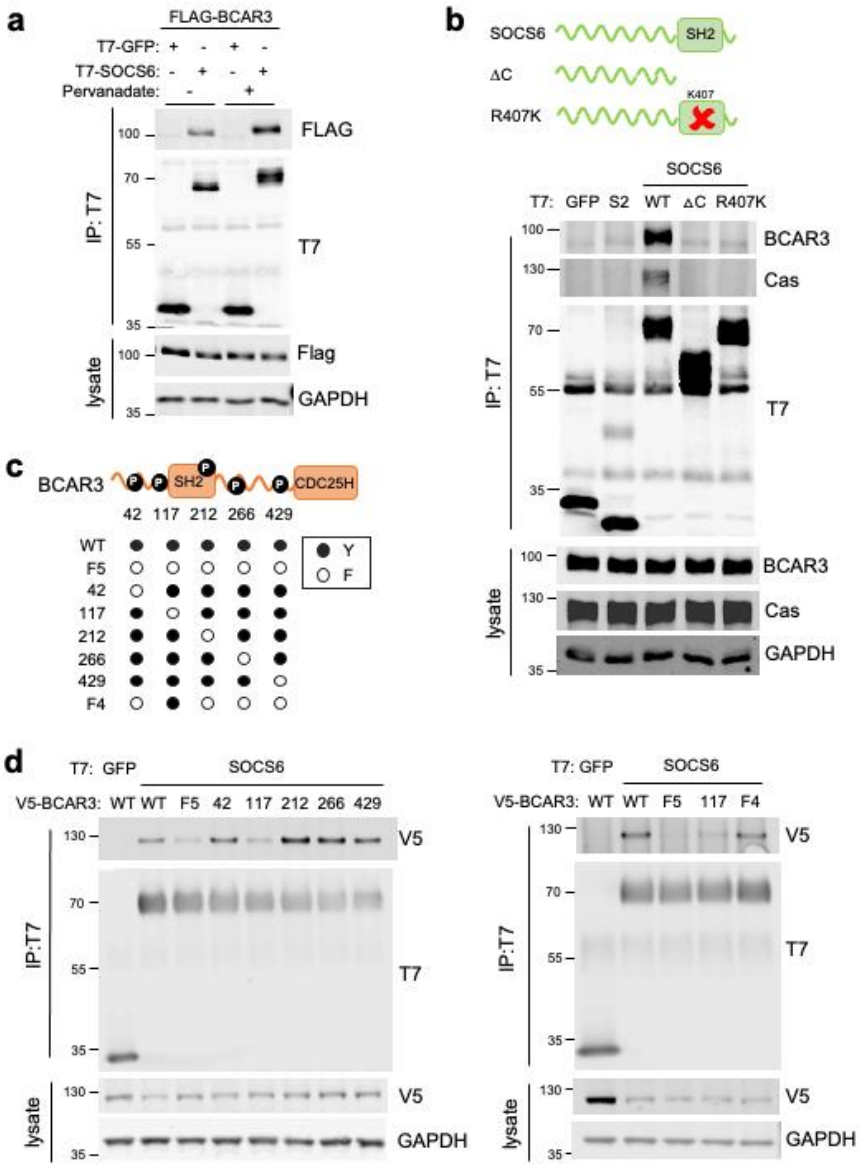

Figure 4. CRL5 binds BCAR3 pY117. (a) Phosphatase inhibition increases SOCS6-BCAR3 binding. HeLa cells were transfected with T7-GFP or T7-SOCS6 and 3xFLAG-BCAR3 and treated with pervanadate or vehicle. Lysates were immunoprecipitated with T7 antibody and immunoprecipitates and lysates were immunoblotted with T7 and FLAG antibodies. (b) SOCS6-BCAR3 binding requires the SOCS6 SH2 domain. Cells were transfected with control vector and T7-tagged SOCS constructs, treated with pervanadate and lysed. Cell lysates were immunoprecipitated with T7 antibody and lysates and immunoprecipitates were immunoblotted with BCAR3 and T7 antibodies. (c) $BCAR3^{WT}$ and phenylalanine (F) mutants. Residues Y42, Y117, Y212, Y266 and Y429 phosphorylation sites have been detected across a range of cell lines (www.PhosphoSite.org). (d) BCAR3 Y117 is necessary and sufficient for SOCS6 binding. HeLa cells were transfected with T7-GFP or T7-SOCS6 and SNAP-V5-$BCAR3^{WT}$ or YF mutants. Transfected cells were treated with pervanadate and lysed. Lysates were immunoprecipitated with T7 antibody and lysates and immunoprecipitates were immunoblotted with V5 and T7 antibodies.

Figure 5. Y117 and Cas regulate BCAR3 protein stability. (a) Y117F mutation increases BCAR3 protein level. SNAP-V5-BCAR3^{WT} and Y117F mutant MCF10A cells were induced with dox concentrations (ng/mL). Lysates were immunoblotted with BCAR3 antibody to detect endogenous and induced SNAP-V5-BCAR3. (b) Cas binds to BCAR3 Y117 and SH2 mutants. SNAP-V5-BCAR3^{WT} and mutants were induced in MCF10A cells with 10 ng/mL dox for 48 hours. Lysates were immunoprecipitated with V5 antibody and lysates and immunoprecipitates immunoblotted with Cas and V5 antibodies. (c) Cul5 regulates BCAR3 protein level through Y117. Representative immunoblot and quantification of BCAR3 protein in siRNA-treated, dox-induced SNAP-V5-BCAR3^{WT} and Y117F cells. Mean$\pm$SEM; n=3 biological replicates. ***p=0.0005 by t-test. (d) Cul5 regulates BCAR3 protein level through Cas binding but not the SH2 domain. As for panel (c), with dox-induced SNAP-V5-BCAR3^{WT}, R177K and EE cells. Vertical lines indicate different immunoblots, each run with its own wildtype control. Mean$\pm$SEM; n=3 biological replicates. **p<0.005 by t-test.

Figure 6

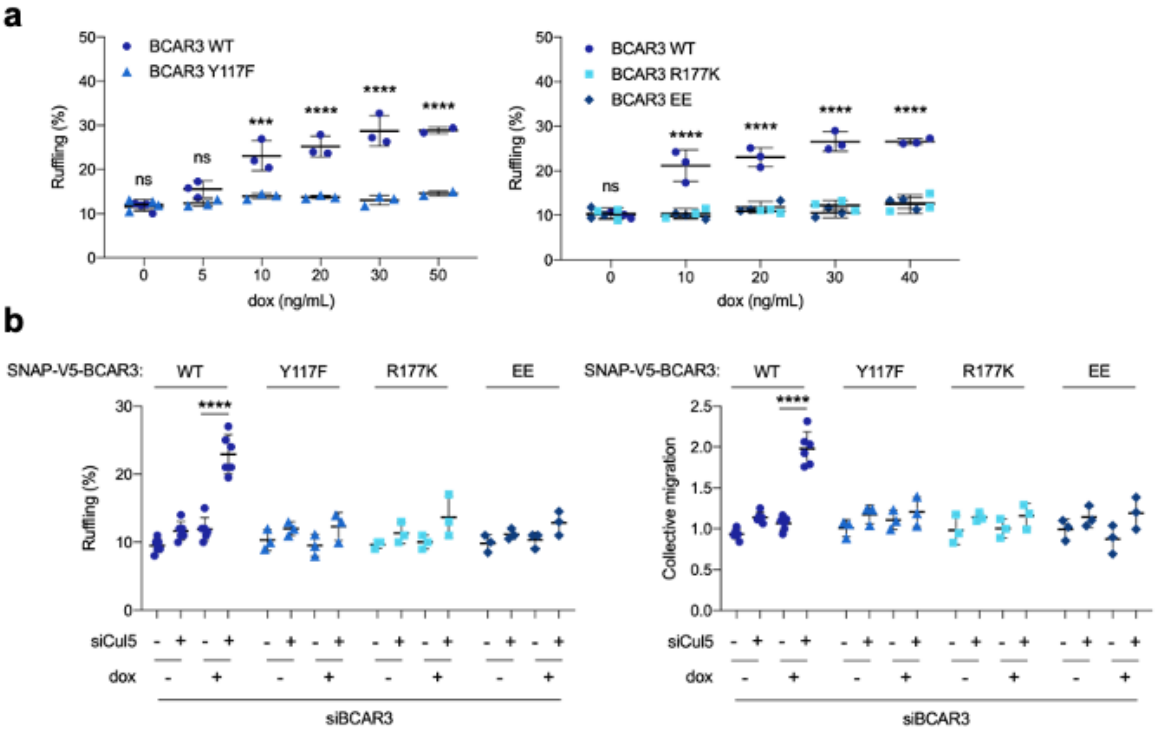

Figure 6. Lamellipodial ruffling and cell migration require multiple BCAR3 domains. (a) Over-expression. SNAP-V5-BCAR3^{WT} and mutant MCF10A cells were induced with various dox concentrations (ng/mL). Confluent monolayers were starved for EGF and wounded. Images of the wounds 6 hours later were used to count the percentage of ruffling cells. Mean±SEM; n=3-6. ***p=0.0001 and ****p<0.0001 (One-way ANOVA). (b) Rescue of ruffling and migration. SNAP-V5-BCAR3^{WT} and mutant MCF10A cells were treated with BCAR3 siRNA and control or Cul5 siRNA and induced with 10 ng/mL dox. Confluent monolayers were placed in EGF-free media and wounded. (Left) Percentage of ruffling cells at 6 hours. Mean±SEM of >250 cells per condition from n=3-6 experiments. ****p<0.0001 (One-way ANOVA). (Right) Relative migration at 12 hours. Mean±SEM; n=3-6 biological replicates, each with 8-12 technical replicates. ****p<0.0001 (One-way ANOVA).

Figure 6 – figure supplement 1

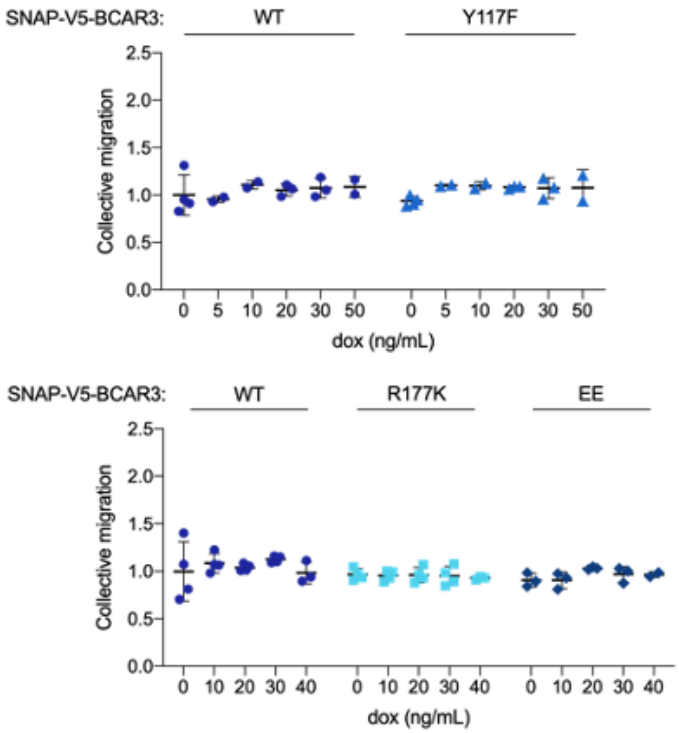

Figure 6 - figure supplement 1. Over-expression of wildtype or mutant BCAR3 does not induce cell migration. SNAP-V5-BCAR3^{WT} and mutant MCF10A cells were induced with various dox concentrations (ng/mL). Confluent monolayers were starved for EGF, scratched and allowed to migrate. Relative migration was measured after 12 hours. Mean±SEM; n=3 biological replicates each with 8-12 technical replicates. p>0.05 (One-way ANOVA).

Figure 7

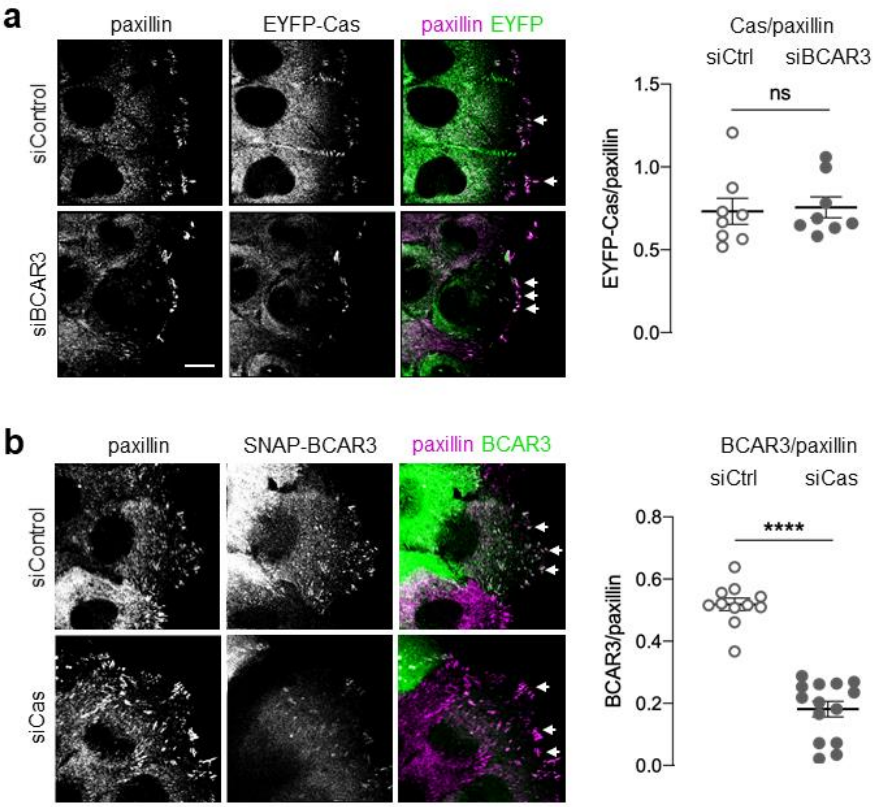

Figure 7. Cas recruits BCAR3 to adhesions. (a) Cas localization does not require BCAR3. MCF10A cells expressing low levels of EYFP-Cas were treated with control or BCAR3 siRNA and allowed to reach confluence. Confluent monolayers were placed in EGF-free media and wounded. After 6 hours, cells were fixed and stained for EYFP-Cas and paxillin. (Left) Maximum intensity projection images. Arrowheads: adhesion sites at the leading edge. Scale bar: 10 µm. (Right) Mean fluorescence intensity of EYFP-Cas relative to paxillin in adhesion sites at the leading edge. Mean±SEM; n=8 cells from 2 biologically independent experiments. p>0.05 by unpaired t-test. (b) BCAR3 localization requires Cas. SNAP-V5-BCAR3^{WT} MCF10A cells were treated with control or Cas siRNA and induced with 10 ng/mL dox. Confluent monolayers were placed in EGF-free media and wounded. After 4 hours, cells were treated with cpJF549-SNAP ligand for one hour. One hour later, they were fixed and stained for paxillin. (Left) Maximum intensity projection images. (Right) Mean fluorescence intensity of SNAP-BCAR3 relative to paxillin in adhesion sites at the leading edge. Mean±SEM; n=11-14 cells from 2 biologically independent experiment. ****p<0.0001 by unpaired t-test.

Figure 8

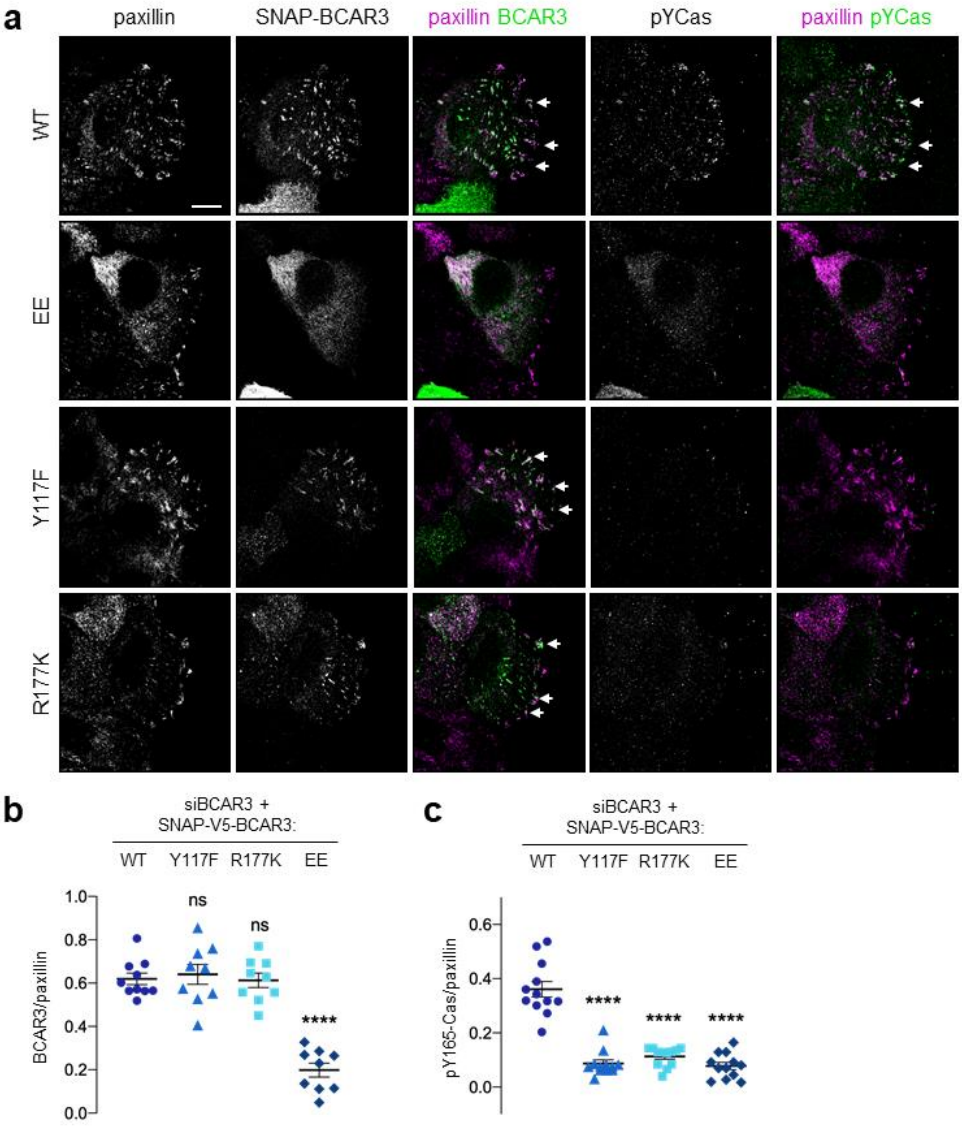

Figure 8. BCAR3 stimulates Cas phosphorylation in adhesions, dependent on BCAR3 Y117 and SH2 interactions. (a) Wildtype or mutant SNAP-V5-BCAR3 MCF10A cells were treated with control or BCAR3 siRNA and induced with 10 ng/mL dox. Confluent monolayers were placed in EGF-free media and wounded. After 4 hours, cells were treated with cpJF549-SNAP ligand for one hour. One hour later, cells were fixed and stained for adhesion marker paxillin and pY165-Cas. Maximum intensity projection. Scale bar = 10 µm. (b) Mean fluorescence intensity of SNAP-V5-BCAR3 relative to paxillin in adhesion sites at the leading edge. Mean±SEM. ****p<0.0001 (One-way ANOVA). (c) Mean fluorescence intensity of pY165-Cas relative to paxillin in adhesion sites at the leading edge. Mean±SEM. ****p<0.0001 (One-way ANOVA).

Figure 8 – figure supplement 1

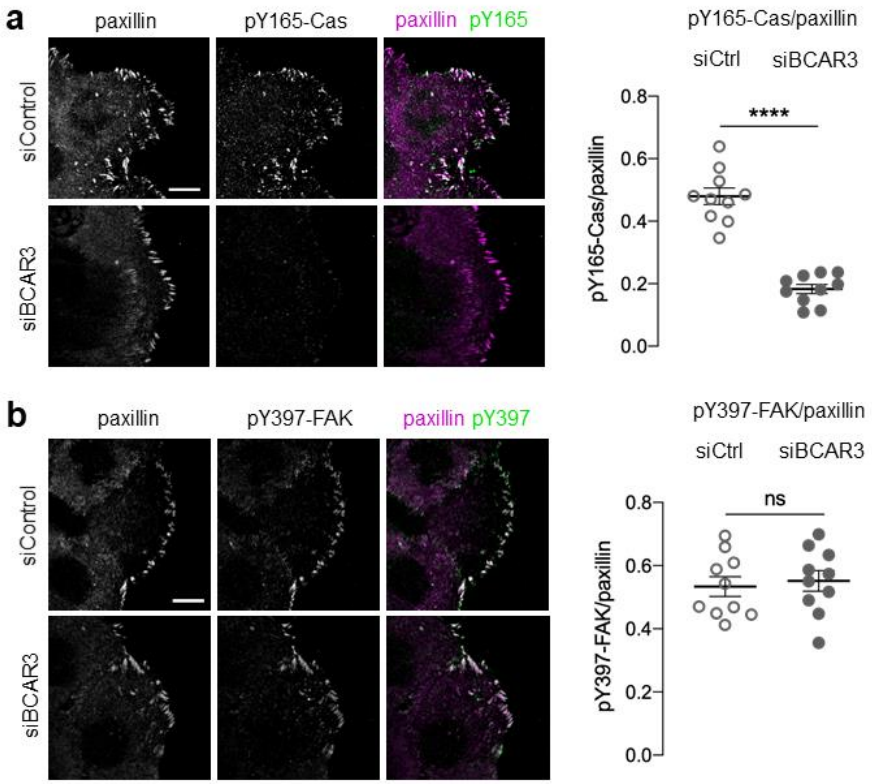

Figure 8 – figure supplement 1. BCAR3 is required for Cas tyrosine phosphorylation, but not FAK autophosphorylation. MCF10A cells were treated with control or BCAR3 siRNA. Confluent monolayers were placed in EGF-free media and wounded. After 6 hours, cells were fixed and stained for adhesion marker paxillin and (a) pY165-Cas or (b) pY397-FAK. (Left) Maximum intensity projection images. Scale bar = 10µm. (Right) Mean fluorescence intensity of (a) pY165-Cas or (b) pY397-FAK relative to paxillin in adhesion sites at the leading edge. Mean±SEM; n=10 cells from 1 experiment. ****$p<0.0001$ and $p>0.05$ by unpaired t-test.

Figure 9

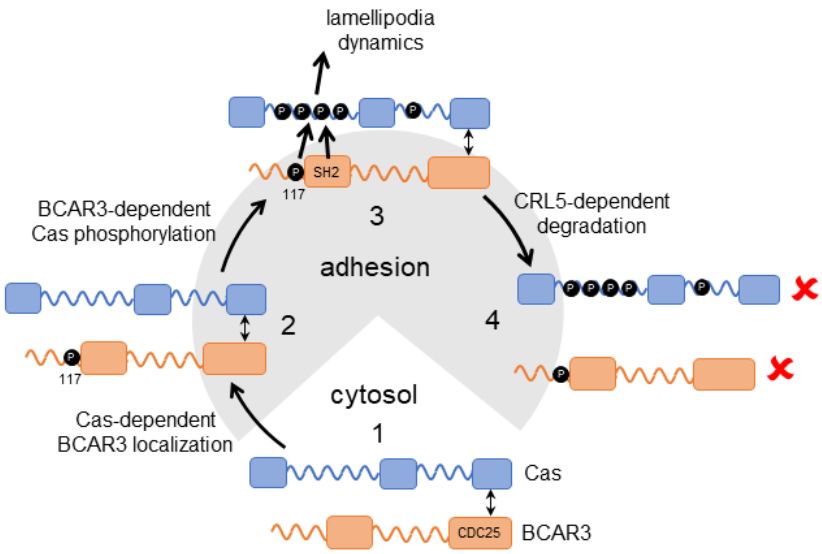

Figure 9. Model showing inter-dependence of Cas and BCAR3 for localization and activation. Step 1: A pre-formed BCAR3-Cas complex translocates to integrin adhesions through localization signals in the N and C terminal domains of Cas. Step 2: BCAR3, phosphorylated at Y117 by an unknown kinase, stimulates Cas phosphorylation in a process that also requires the SH2 domain, leading to activation of signaling pathways that stimulate lamellipodial protrusion and ruffling. Step 3: Signaling is terminated by the CRL5SOCS6-dependent ubiquitination and degradation of BCAR3, phosphorylated in their respective phosphodegrons. Remaining questions include the identity of the kinase that phosphorylates BCAR3 Y117, the timing of Y117 phosphorylation, and the mechanism by which BCAR3 pY117 and SH2 domain stimulate Cas phosphorylation.

Figure 9 – figure supplement 1

Protein	Residue	Sequence[a]	Evidence (reference) peptide[b]	mutant[c]	biology[d]
Kit	pY568	gnn**Y**VYIdptq	(1,2)	(1)	
PDGFR	pY579	ghe**Y**IYVdpmq	(2)		
Flt3	pY591	eyf**Y**VdFreye	(3)	(3)	(3)
Flt3	pY919	eei**Y**IiMqscw	(3)	(3)	(3)
Cas	pY664	med**Y**dYVhlqg		(4)	(4)
Dab1	pY198	dpv**Y**qYIvfea		(5)	(5-6)
BCAR3	pY117	tve**Y**VkFsker		(7)	(7)
Spot array[e]		xxx**Y**VYIxxxx IFF MWM WVV	(8)		

Figure 9 – figure supplement 1. Sequence alignment of SOCS6 binding sites.

[a] Local sequence aligned to phosphotyrosine (Y). Capital letters indicate residues at +1 to +3 positions that fit the consensus from the spot array.

[b] Phosphopeptide binds to SH2 domain in vitro

[c] Decreased binding of Y to F mutant protein in cells

[d] Y to F mutant is stabilized or gains function in cells

[e] A library of phosphopeptides randomized at positions +1 to +3 was selected by binding to the SOCS6 SH2 domain. Bound peptides were sequenced. Residues selected at each position are shown in decreasing order of preference.

References: (1) (Bayle et al., 2004) (2) (Zadjali et al., 2011) (3) (Kazi et al., 2012) (4) (Teckchandani et al., 2014) (5) (Feng et al., 2009) (6) (Simo et al., 2013) (7) this work, (8) (Krebs et al., 2002).

Supplementary Material

Supplementary Table 1. Quantification of phosphotyrosine peptides in control and Cul5-deficient MCF10A cells. Phosphopeptides whose abundance significantly differed between control and Cul5-deficient MCF10A cells in two separate experiments. Sheet 1: Experimental conditions, numbers of phosphopeptides quantified, and number of significant changes. Sheet 2: Phosphopeptides significantly increased in Experiment 1. Sheet 3: Phosphopeptides significantly increased in Experiment 2. Sheet 4: Phosphopeptides significantly increased in both Experiments (see also Figure 1 – Figure Supplement 1).