2017

Fyn-Mediated Regulation of Protein Kinase A

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Undergraduate Honors Thesis

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Abstract:

Protein kinases are enzymes important for signal transduction in the regulation of cellular processes. The cAMP-dependent protein kinase A (PKA) has been previously reported to regulate the activity of the Src family kinase Fyn, an event important for cellular migration. This study aimed to characterize the reciprocal interaction, in which Fyn regulates PKA. In addition to our preliminary, unpublished findings that Fyn phosphorylates the PKA catalytic subunit at Y69 to increase its catalytic activity, we have shown through co-immunoprecipitation that Fyn physically associates with PKA in HEK293 cells. Quantitative mass spectrometry and subsequent biochemical validation shows that PKACα undergoes enhanced binding to a complex of centrosomal and Golgi-localized A-kinase anchoring proteins when Fyn is overexpressed, independent of Fyn kinase activity. Fyn was found in this complex, as well, implicating its involvement as an adaptor protein. Co-immunoprecipitation experiments with various Fyn alleles demonstrated the dispensability of the Fyn SH3 domain and the functioning SH2 domain in binding to PKACα. GST-fusion proteins containing either of these domains were also unable to enrich PKACα from HEK293 lysates. Fyn was found to bind PKACα independent of its association with the regulatory subunit, and preliminary data suggests that this interaction is direct through purified protein pulldown experiments. We hypothesize that this regulatory interaction activates PKA at the centrosome and Golgi apparatus, facilitating the potential phosphorylation of proximal substrates involved in cytoskeletal organization and mitotic processes.

Background:

I. Protein Kinases

Protein kinase enzymes have important functions in the transduction of intracellular and extracellular signals to produce a cellular response. They add an inorganic phosphate group to hydroxyl-containing serine, threonine, and tyrosine amino acid residues of their protein substrates, in a process known as phosphorylation. This post-translational modification can alter the configuration and activity of these substrates, allowing them to produce downstream effects. Cytosolic phosphorylation cascades are often responsible for mediating changes in cytoskeletal reorganization in the cytoplasm and gene expression in the nucleus due to receptor-ligand interactions at the plasma membrane. The cAMP-dependent protein kinase A (PKA) and Src family kinases (SFKs) have both been thoroughly investigated, and are each involved in a multitude of cellular functions. In addition to their central roles in regulating normal cellular processes, these kinases have both been implicated in the progression of diseases, including cancer (1,2). However, the underlying mechanisms of these cancer-promoting activities have yet to be fully elucidated, such as the involvement of PKA in cellular invasion and metastasis.

II. Cyclic AMP-Dependent Protein Kinase A

PKA is a heterotetrameric serine-threonine kinase consisting of two regulatory (R) and two catalytic (C) subunits, of which multiple isoforms exist: RI and RII, and Cα, Cβ, and Cγ. The C subunits are kept inactive when bound in the holoenzyme to a pseudo-substrate consensus sequence on the R subunits, and are released upon binding of cyclic adenosine monophosphate (cAMP) to the R subunits. This cAMP is produced from ATP by adenylyl cyclase, which is commonly activated downstream of G Protein-Coupled Receptors (GPCRs) by small G proteins

(3). The functional effectors of cAMP signaling also include the exchange protein directly activated by cAMP (Epac) family, which acts as a group of Rap guanine nucleotide exchange factors (4). PKA can also be activated by receptor tyrosine kinases (RTKs) through phosphorylation of its tyrosine 330 (Y330) residue (5). This phosphorylation event, mediated by platelet-derived and epidermal growth factors receptors (PDGFR/EGFR), increases the affinity of PKA-C for its protein substrates (5).

PKA is involved in many pathways of diverse cellular function, including metabolism, cytoskeletal rearrangement, transcriptional activation, proliferation, migration, and survival (6). PKA has been shown to mediate the cytoskeletal dynamics of neuronal cells necessary for directional growth cone extension through phosphorylation of Mena/Vasodilator-stimulated phosphoprotein (VASP) downstream of Netrin-DCC signaling, a chemoattractive receptor-ligand interaction (7). It regulates metabolic pathways by phosphorylating substrates such as phosphorylase kinase, which then activates glycogen phosphorylase to release free glucose (8). This provides an example of how hormones can regulate metabolism through induction of cAMP signaling. Changes in PKA activity are seen in mitosis, as well, a phenomenon that has been measured through the heightened phosphorylation of the fluorescent resonance energy transfer (FRET)-based biosensor AKAREV. This event occurs during cell division and in proximity to compact chromosomes during metaphase (9). However, the enzyme is under tight spatiotemporal regulation that leads to a decrease in its activity at the onset of anaphase. Deviation from an intermediate level of activity can cause defects in proper chromosome separation (9).

III. A-Kinase Anchoring Proteins

PKA activity and anchoring to particular subcellular domains have been shown to be necessary for cellular migratory processes, such as invasiveness in ovarian cancer cells (1). PKA

localization is mediated by A-kinase anchoring proteins (AKAPs), a class of scaffolding proteins known to interact with the R subunits. Binding occurs between the regulatory subunit binding domain of the AKAP and the dimerization/docking (D/D) domain of the R subunit, a product of the interactions between hydrophobic residues of alpha helices on each molecule (10). Many AKAPs are specific in binding to the R2 holoenzyme, but others are considered dual-affinity. These are capable of binding R1 and R2 holoenzymes, albeit affinity for R2 has been shown to be higher in some cases. The R2 binding domain (R2BD) has a conserved secondary structure among R2-specific AKAPs, but the primary amino acid sequence shows more variability (10). Initially, the short peptide Ht31, derived from the R2BD of AKAP-Lbc, was used as an inhibitor of AKAP-PKA binding to assess the effects of PKA anchoring. Today, the use of computational biology has facilitated the development of enhanced isoform-specific inhibitors (11). RIAD and super AKAP *in silico* are short peptides that are able to bind to the D/D domains of R1 and R2 subunits, respectively, and prevent association with AKAP binding pockets. These inhibitors mimic the R subunit binding domains of the high affinity R1 and R2 AKAPs to compete with them for cytosolic holoenzyme binding.

AKAPs are able to create discrete, localized microenvironments of cAMP signaling through docking of GPCRs, adenylyl cyclases, cyclic nucleotide ion channels, and Epacs (10). The dual recruitment of the PKA holoenzyme and phosphatases that remove phosphate groups, as well as phosphodiesterases (PDEs) that cleave cyclic nucleotides, provides a means for negative feedback. PDE4D specifically targets cAMP to inhibit PKA activity, allowing for tight regulation of localized cAMP signaling. This form of negative regulation is seen in the binding of PDE4D family members to Myomegalin (also known as PDE4D Interacting Protein, or PDE4DIP), and PKA-mediated phosphorylation of the family member PDE4D3 at serine 54 has

been shown to enhance its phosphodiesterase activity (12). The activity of PDE4D in AKAP9 complexes is responsible for lower concentrations of cAMP at the interphase centrosome, and this microdomain is abolished during mitosis, possibly due to Erk-mediated inhibition of PDE4D3 downstream of mitogen signaling (13). This results in an increase in PKA activity during mitosis. AKAPs are also known to colocalize components of different signaling pathways, such as PKC and GSK3, to allow cross talk and regulation (10).

AKAPs influence diseases such as cardiac hypertrophy, as specific pro-hypertrophic and anti-hypertrophic AKAPs direct PKA activity by recruitment of different downstream effector proteins (14). AKAPs have also been shown to have positive or negative regulation of cancer progression. AKAP12, or Gravin, is seen to have a tumor suppressor function due to its deletion in certain cancers, and the promotion of a cancerous phenotype when it is genetically knocked out (10). Conversely, Pericentrin overexpression induces a cancerous phenotype. This centrosomal protein colocalizes with AKAP9 and has been shown to be important for proper chromosomal separation, as well (10). AKAP9 functions to promote the G1/S cell cycle transition through Cdk2 recruitment to the centrosome, and its depletion prevents centrosome duplication, which may require PKA recruitment (15). This AKAP has increased expression in colorectal cancer, as caused by overexpression of the long non-coding RNA MALAT1 and its downstream effect on splicing factor activity (16). Recently, a novel compound targeting breast cancer cells has been found to displace AKAP9 from the centrosome, resulting in disrupted microtubule organization, inhibited migration, and apoptosis (17).

IV. Src Family Kinases

The second family of kinases of interest to this study are the SFKs, a family of non-receptor tyrosine kinases that similarly possess diverse cellular functions. The SFK members

have a conserved set of Src homology (SH) domains, comprised of kinase domain (SH1), phospho-tyrosine binding (SH2), proline-rich region binding (SH3), and N-terminal membrane association (SH4) regions. The SH2 and SH3 domains participate in intracellular binding interactions that promote an autoinhibitory state. The SH2 domain binds a C-terminal phosphorylated tyrosine residue, while the SH3 domain binds a proline-rich linker region in the middle of the protein. This conformational regulation ensures a high level of binding specificity, as there must be high affinity for a new binding partner to overcome the moderate affinity of intramolecular interactions. SFKs can be activated downstream of RTKs, by binding to a phospho-tyrosine on the intracellular domain of the receptor, or can be activated by GPCRs via an alternative mechanism (18). SFKs can attain an open conformation and subsequent activation through dephosphorylation of a C-terminal inhibitory tyrosine residue necessary for its autoinhibitory conformation, phosphorylation of a separate tyrosine residue in the kinase domain activation loop, and binding to phospho-tyrosine residues on other proteins (19). The oncogenic properties of SFKs were discovered earlier on, with the identification of v-Src, a variant lacking the C-terminal tyrosine responsible for negative regulation of the kinase (2). Accordingly, SFKs are overexpressed in some cancers, and are thought to contribute to cellular motility in part through control of cellular focal adhesions (20).

Fyn is one of the nine SFK members that is thought to be ubiquitously expressed, along with Src and Yes. As with other SFKs, Fyn has an N-terminal domain that regulates its membrane association via glycine myristoylation and cysteine palmitoylation, although its localization can also be cytosolic or nuclear (21). Two important tyrosine phosphorylation sites within the enzyme are Y420, its activating site that can be targeted through transphosphorylation, and the negatively regulatory Y531 site that is targeted by C-terminal Src kinase (Csk) for

phosphorylation and by protein tyrosine phosphatases such as CD45 for dephosphorylation (21). Fyn is involved in basic cellular processes in hematopoietic and neural lineages. It functions downstream of T-cell receptor signaling in tandem with the SFK Lck while colocalized in lipid rafts (22). In neural cells, Fyn functions in Trk transactivation downstream of adenosine receptors (18). It also phosphorylates the deleted in colorectal cancer (DCC) receptor, which is a necessary event for netrin sensitivity and axon outgrowth in chemoattractive axon guidance (23). Fyn promotes oncogenic processes including proliferation, suppression of apoptosis, and migration in breast, prostate, brain, and other forms of cancer. For instance, Fyn and Src are shown to be effectors of EGFR-mediated cancer growth and migration in glioblastoma (24). Multiple downstream signaling molecules are regulated by Fyn, such as the kinases AKT and AMPK, cytoskeletal regulators, and cell cycle effectors (21). Furthermore, its mediation of tamoxifen-resistance in estrogen receptor-positive breast cancer has demonstrated its potential as a therapeutic target (21).

V. Cross-Regulation of Fyn and PKA

In the canonical sense, the pathways of PKA and SFK activation have remained separate. A known route of PKA-SFK cross-regulation is already known, in the activation of Fyn by phosphorylation of its N-terminal serine 21 (25). This signaling event is important for cell migration and the focal adhesion targeting of Fyn, and the corresponding phosphorylation of serine 21 on Src has been shown to drive Src activation and cancer cell migration downstream of β-adrenergic receptors (26). More recently, the Deming lab has demonstrated an interaction in the opposite direction, in which the SFK members Fyn and Src phosphorylate PKA at tyrosine 69 (Y69) (unpublished data). The Deming lab has shown that this post-translational modification increases PKA-C catalytic activity *in vitro*, and Fyn overexpression appears to upregulate

phosphorylation of the specific proteins containing the PKA substrate consensus motif RRXS/T **(Figure 1)**. These two phosphorylation events demonstrate the capability of one enzyme to promote the downstream signaling effects of the other, a phenomenon that may be relevant in connecting their involvement in cancer progression. The identification of Y69 phosphorylation in multiple tumor samples through the online database Phosphosite further suggests that this event is a potential mechanism for oncogenic activity (27).

Over the past year, I have collected further preliminary data on a separate regulatory interaction between Fyn and PKA. This work was inspired by the preliminary observation that Fyn overexpression increased PKA substrate phosphorylation independent of Fyn kinase activity. Overexpression of a kinase dead mutant lacking catalytic activity induced a set of subtle increases at specific molecular weights, similar to the effect seen with wild-type Fyn **(Figure 1)**. We hypothesized that there was a physical interaction between the proteins, which I demonstrated through immunoprecipitation (IP) of PKACα and western blot analysis for the presence of Fyn. This result led us to hypothesize that Fyn could regulate the binding of PKA to other proteins, as well. To test this theory, a large-scale experiment was conducted in human embryonic kidney 293 (HEK293) cells, involving stable isotopic labelling of amino acids in cell culture (SILAC) and quantitative mass spectrometry. IP of PKACα and analysis of peptide ratios between control cells and those overexpressing Fyn revealed numerous proteins to have enhanced binding to PKACα in the presence of Fyn. Of the 41 binding partners identified, 40 were found to be Fyn-dependent **(Figure 2)**. While several novel binding partners were identified, three of those that exhibited the most enhanced binding in Fyn-transfected samples were known AKAPs: AKAP9, Myomegalin, and CDK5RAP2. Interestingly, Fyn promoted interaction of PKA-C with these AKAPs in a kinase-independent manner, as no significant

difference in binding was detected in a quantitative comparison of PKA binding partners between cells overexpressing either wild type or a kinase dead mutant of Fyn. This suggested that Fyn is acting more as an adaptor protein than a traditional kinase in this molecular phenomenon.

To better understand the mechanism of Fyn-mediated regulation of PKA, the binding interaction between the two must be further characterized. This study has aimed to map the domain of Fyn that mediates association with PKA and identify the protein in the complex to which Fyn most directly binds. It was originally hypothesized that Fyn binds indirectly through AKAP9, Myomegalin, or CDK5RAP2, given the results of our SILAC experiment. However, it remained possible that Fyn could bind to PKA directly. PKA-C contains a proline rich region that is conserved among AGC kinases (28), that could potentially act as a site for SH3 domain binding. There are also tyrosine residues on PKA-C available for phosphorylation and subsequent SH2 binding, such as Y330 and Y69. We have sought to answer these questions through co-immunoprecipitation (co-IP) experiments and purified protein pulldowns. This information will aid in the characterization of this hypothetical protein complex, the assembly of which may facilitate the phosphorylation of a particular subset of PKA substrates in an AKAP-dependent manner, and facilitate cellular processes such as migration and proliferation.

Methods:

This project involved the use of recombinant DNA and was authorized under the IBC protocol #: 08-031.

Cell Culture & Transfection: Human embryonic kidney 293 (HEK293) cells were grown in a 37°C humidified chamber, in Dulbecco's Modified Eagle Media (DMEM) with added 10%

fetal bovine serum (FBS) and 1% L-glutamine. 3.0 x 10^6 cells were plated onto 10 cm dishes and allowed to grow for about 20 hours. Cells were transfected with mammalian expression plasmids, either consisting of pcDNA3.1 empty vector or the same vector harboring coding DNA for various proteins, including wild type (WT) Fyn (Addgene), a kinase dead (KD) Fyn allele (Addgene), a mutant Fyn lacking the SH3 domain (ΔSH3) (Addgene), and a Fyn mutant in which Y185, Y214, and Y215 had been mutated to aspartate residues (Y3D) as described previously (29). Other constructs included YFP, PKACα-YFP, and Myc-tagged Myomegalin. Transfection took place in polyethylenimine (PEI), a common transfection reagent that induces endocytosis of DNA into the cell. 1 mg/mL PEI was used at a ratio of 3 μL PEI per 1 μg of DNA. 24 hours post-transfection, cells were serum-starved overnight in serum-free DMEM. The next day, cells were washed twice with 2 mL of ice cold 1X phosphate-buffered saline. Whole cell extracts (WCEs) were prepared by harvesting in 1 mL of NP40 lysis buffer (25 mM Tris pH 7.2, 137 mM NaCl, 25 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% IGEPAL), with freshly added reagents: 1 mM DTT, 1 mM Na_3VO_4, 1X HALT Protease inhibitor cocktail (Thermo Scientific), and 1 mM β-glycerophosphate. To determine protein concentrations, a bicinchoninic acid assay was performed using a Pierce BCA Protein Assay Kit (Thermo Scientific).

Western Blot: Proteins in 10% SDS-PAGE gels were transferred to a nitrocellulose membrane using a Trans-Blot Turbo RTA Transfer Kit (Biorad), and membranes were subsequently blocked in 1% BSA, 1X Tris-buffered saline + 0.1% Tween-20 solution (TBST), rocking for one hour at room temperature. 8% gels were used for co-IP of AKAP9, PDE4DIP, CDK5RAP2 with PKA-C-YFP. Primary antibodies including α-Fyn (Santa Cruz), α-PKACα (Santa Cruz), α-phospho-PKA substrate (Cell Signaling Technology), α-Tubulin (Cell Signaling

Technology), α-Myomegalin (Invitrogen), α-AKAP9 (Bethyl), α-CDK5RAP2 (Bethyl), α-GFP (Life Technologies), α-pY 4G10 (Upstate-Millipore), α- SFK pY416 (Cell Signaling Technology), α-PKAR2β (Santa Cruz), and α-PKAR1α (Cell Signaling Technology) were added in dilutions ranging from 1:2000 to 1:500, rocking at room temperature for one hour or overnight at 4˚C. Membranes were washed five times for five minutes each in 1X TBST. Secondary antibodies were added at a 1:5000 dilution for α-mouse-HRP (Jackson Immunolabs) and 1:10,000 for α-rabbit-HRP (Jackson Immunolabs) according to the source of the primary antibody, and were incubated with the membrane while rocking for one hour at room temperature. Light chain specific secondary antibodies (Jackson Immunolabs) were used for western blot as necessary, following immunoprecipitations. After five more identical washes, membranes were processed for development using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Co-immunoprecipitation: Two milligrams of protein from WCE from each sample were brought up to an identical volume with NP40 lysis buffer with added reagents and incubated overnight, rocking at 4˚C with two micrograms of either a protein-specific antibody or non-specific rabbit IgG. 25 μL of magnetic Dynabeads (Thermo Scientific) per sample were washed per manufacturer's instructions in 1 mL binding buffer (1X PBS, 0.01% Tween) and resuspended in 25 μL binding buffer per sample as needed, then added to each sample. These were rocked at 4˚C for two hours and put on a magnet before removing the supernatant. After five washes with 1 mL NP40 lysis buffer, the beads were resuspended in 1X Laemmli sample buffer. Samples were boiled for 5 minutes and run on a 10% SDS-PAGE gel for one hour at 200 volts before western blot analysis.

GST pulldown assay: Two milligrams of WCE from untransfected HEK293 cells were incubated with normalized levels of glutathione beads coupled to GST or GST-fusion proteins containing Fyn SH2 or SH3 domains produced in bacteria and purified as previously described (29). After rocking at 4˚C overnight, samples were centrifuged for 90 s at 10,000 rpm and most of the supernatant was removed. Five washes with 1 mL of NP40 lysis buffer were performed. Laemmli sample buffer was added and the beads were subjected to SDS-PAGE before western blot analysis.

Purified Protein Pulldown: 100 μL of glutathione beads (G-Biosciences) were resuspended in 1 mL of 10% BSA in 1X PBS and incubated for 2 hours, rocking at 4°C. The beads were split evenly between two tubes, resuspended in 1 mL NP40 lysis buffer and one tube of beads was incubated with purified, active GST-tagged Fyn A, (SignalChem) and rocked overnight at 4°C. 50 μL of supernatant was saved, and the remaining beads were washed with 1 mL lysis buffer. The beads were then resuspended in 1 mL lysis buffer and each tube was evenly split again into two tubes, which were all brought up to 1 mL with lysis buffer. One tube from each treatment was incubated with purified His-tagged WT PKACα and rocked for 2 hours at 4°C. 50 μL of supernatant was saved from the incubated solutions. The beads were then washed 5 times with 1% Triton X-100 lysis buffer (20 mM Tris-HCl pH 8.0, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 137 mM NaCl) and Lammli sample buffer was added. Each sample was boiled for 5 minutes, separated via SDS-PAGE, and blotted for PKACα and Fyn.

Densitometry: Adobe Photoshop CS6 was used to analyze band intensity in Fyn-PKA co-IP western blots. Scanned film blots were converted to grayscale and color was inverted. Mean intensity values were measured in sampling boxes of identical dimensions, and background signal was measured from an empty lane and subtracted from each value. Due to the overlap of

ΔSH3 Fyn bands with IgG heavy chain, the heavy chain pixel intensities for all other lanes were averaged and subtracted from that of the ΔSH3 band. Fyn IP levels were normalized to levels of Fyn in the lysate and to PKA levels in the IP. KD and ΔSH3 Fyn binding levels were expressed as fold increase relative to WT Fyn, which was set to 1. Values for WT, KD, and ΔSH3 Fyn were graphed on GraphPad Prism 7. A one-tailed paired t-test was performed between each set of treatments and included on the graph.

Results:

Validation of mass spectrometry determines Fyn-dependent AKAP binding

Biochemical validation of the mass spectrometry experiment in Figure 2A was pursued through co-IP experiments. HEK293 cells were transfected with YFP or PKACα-YFP, and pcDNA 3.1 empty vector or pcDNA 3.1 harboring WT Fyn **(Figure 3A)**. Lysates were immunoprecipitated for PKACα-YFP with an α-GFP antibody and blotted for PKACα and either AKAP9, CDK5RAP2, or Myomegalin. Each independent IP demonstrated enhanced enrichment of the respective AKAP with PKACα-YFP in cells overexpressing Fyn **(Figure 3B-D)**. While there is no visible AKAP9 or Myomegalin in the IP without Fyn overexpression **(Figure 3B, C)**, there is a more modest increase in the co-IP of CDK5RAP2 with PKACα-YFP in Fyn-transfected cells **(Figure 3D)**. However, this increase would become greater in magnitude when normalized to the levels of PKACα-YFP in the IP of each treatment. It is important to note that there are multiple isoforms of each of these AKAPs, with those of AKAP9 and Myomegalin having larger ranges of molecular weight. The size of the bands that co-immunoprecipitated with PKACα-YFP were consistent with those identified in specific regions of the SDS-PAGE gel in the experiment from Figure 2. These results provide support for the methods of the previous

experiment, and show that our IP experimental approach for isolating the protein complex of interest is feasible.

Fyn is in complex with Myomegalin and CDK5RAP2

Our preliminary data in Figure 2A and validation in Figure 3 demonstrated that Fyn overexpression enhances PKACα binding to the Golgi and centrosomal AKAP complex of AKAP9, CDK5RAP2, and Myomegalin. However, other than its interaction with PKA, there was no evidence that Fyn was directly associated with this complex. In testing this, endogenous CDK5RAP2 was seen to co-immunoprecipitate (co-IP) with Fyn in HEK293 cells **(Figure 4A)**. It is interesting to note that the reciprocal is not true; Fyn does not readily co-IP with CDK5RAP2, and the reason for this is unclear. It could be true that much more Fyn is immunoprecipitated with α-Fyn antibody relative to what is co-immunoprecipitated with CDK5RAP2, and a darker exposure blot is needed to visualize the protein in the latter sample. Meanwhile, CDK5RAP2 may be expressed at lower levels, so the ratio of its content in the CDK5RAP2 IP to that in the co-IP with Fyn is lower. It is also possible that the antibody for CDK5RAP2 targets a Fyn-binding site, so that CDK5RAP2 bound to Fyn cannot be isolated. Myomegalin also co-IPs with Fyn, and the same pattern is observed **(Figure 4B)**. In this case, Myc-tagged Myomegalin isoform 8 was used for transfection, and appears at a higher molecular weight than the endogenous CM isoform associated with Fyn, found closer to 250 kD. Fyn has been shown to associate with PDE4D4 through its SH3 domain (30), which may mediate indirect association with Myomegalin. These results raise the question as to whether Fyn binds to PKA directly or through one of these AKAPs.

Fyn binds to PKACα independent of its SH3 domain or PKA holoenzyme integrity

To begin to delineate the mechanism by which Fyn binds to PKA, the capacity of several Fyn variants to co-immunoprecipitate with PKACα was assessed. In addition to wild type (WT) Fyn, HEK293 cells were transfected with a kinase dead (KD) Fyn mutant to determine if binding could occur in a kinase-independent manner, as was suggested by our preliminary results. A truncation mutant lacking the Fyn SH3 domain (ΔSH3) was also introduced, to attempt to map the domain of Fyn involved in the binding interaction. Binding was maintained in each variant, indicating that the interaction is kinase-independent and the Fyn SH3 domain is dispensable **(Figure 5A, B)**. ΔSH3 Fyn, which runs near the Ig heavy chain at ~50 kD, shows significantly higher binding to PKACα than WT Fyn **(Figure 5A-C)**. The lack of an SH3 domain may prevent the autoinhibitory closed conformation of the protein, allowing for enhanced intermolecular binding interactions. However, the apparent enhanced binding of KD Fyn in Figure 5B has not been reproducible (n=5), and we believe the variability of binding may be more of a reflection of the total amount of KD Fyn transfected. For instance, in Figure 5A, KD is expressed at a higher level than WT Fyn. A lighter exposure was used for the Fyn IP blot to distinguish ΔSH3 from the IgG heavy chain, but endogenous and transfected WT Fyn binding is more apparent at darker exposures (data not shown).

We have evidence that Fyn can phosphorylate PKAC at Y69, which enhances its catalytic activity (unpublished data). We reasoned that phosphorylation of the C subunit by Fyn may facilitate release of the C subunit from the R subunit. To test this, we assessed whether the co-immunoprecipitations of PKA regulatory subunits with catalytic subunits was impacted by Fyn overexpression. When PKACα was immunoprecipitated from cells expressing WT or KD Fyn, both R1 and R2 subunits were found to be associated with it. However, expression of ΔSH3 Fyn

completely ablated PKACα binding to PKAR2β, and diminished its binding to PKAR1α, as seen by their lack of co-IP **(Figure 5B)**. This may be due in part to the possible hyperactivity of this variant as assessed by blotting for phospho-tyrosine in HEK293 cells overexpressing ΔSH3 Fyn **(Figure 5A)**. ΔSH3 Fyn appears to be hyperphosphorylated, and distinct substrates of various molecular weights have higher tyrosine phosphorylation levels. Hyperactivity has been previously reported in a Src mutant lacking its SH3 domain, as well (31). To assess if this increased activity is due to increased phosphorylation of the activation loop, we probed lysates with an antibody specific to the SFK Y416. This mutant may have slightly higher levels of Y416 phosphorylation in the activation loop compared to WT Fyn, while minimal phosphorylation is seen at this site in the KD **(Figure 5A)**. Furthermore, a slightly higher molecular weight band can be seen above the ΔSH3 mutant that may represent a hyperphosphorylated form. The difference in activity may also be due to the inability of mutant to form the autoinhibitory conformation, in which the SH3 domain is involved. However, without performing a kinase assay, it cannot be said that the increase in global tyrosine phosphorylation is not due instead to an inactivation of phosphatases.

Since PKA R2 subunit binding is also dispensable for ΔSH3 Fyn binding to PKAC, this suggests that the interaction likely occurs independent of any AKAPs. However, as the activity and interactions of the ΔSH3 mutant are not fully characterized, it was necessary to see if this effect proved to be true in the WT, as well. This was accomplished by artificially dissociating the PKA holoenzyme via stimulation with 30 μM Forskolin. The result showed a clear ablation of PKAR2β co-IP with PKACα, while WT Fyn remained bound **(Figure 6)**. This mimicked the effect of ΔSH3 Fyn transfection, demonstrating the ability of the WT to bind free C subunit. A triple phosphomimetic mutant (Y3D), with three tyrosines mutated to aspartates within the Fyn

SH2 domain, was seen to bind as well. These phosphorylation sites have been shown to repel SH2-binding partners (29). Thus, it appears that Fyn binding to PKA occurs independently of traditional SH2 and SH3 binding interactions.

Fyn SH2 and SH3 domains alone are insufficient to bind PKACα

To further validate the dispensability of the SH3 domain for binding and determine the role of the SH2 domain in the interaction with PKACα, GST pulldowns were performed with fusion proteins containing the isolated Fyn SH2 and SH3 domains. While our previous results showed binding to be independent of Fyn kinase activity, it remained possible that phospho-tyrosine binding could occur at a site targeted by a different tyrosine kinase, such as RTKs that target Y330 (5). Neither of the Fyn domain fusion proteins were sufficient to enrich PKACα from HEK293 lysate, as the PKACα bands in the pulldown lanes were seen at only background levels relative to the lysate **(Figure 7)**. A band running just above PKACα was obtained in the GST-Fyn SH3 pulldown, but this was found to be non-specific binding carried over from bacterial purification, as the band was observed in the mock pulldown lacking lysate, as well (lanes 3 and 6). The phospho-tyrosine-binding capacity of the GST-Fyn SH2 domain was tested to show the functionality of the fusion protein, and interestingly the GST-Fyn SH3 domain was found to be associated with a distinct tyrosine-phosphorylated subset of binding partners, as denoted by their distinct molecular weights. This demonstrates the differential interactomes of each Fyn domain that mediate its association with diverse cellular signaling pathways. In the future, each domain should be tested for binding to known Fyn SH2 and SH3 domain partners as positive controls. The insufficiency of the SH2 and SH3 domains to bind to PKA-C suggests that binding may depend on a different area of the protein, such as the kinase domain, the N-terminal domain, or a linker region. The SH2 domain may also be needed in combination with one of

these segments. Further experimentation involving a series of Fyn truncation mutants is necessary to map the region of Fyn that confers binding.

Fyn may bind directly to PKACα

We showed that full-length Fyn is able to bind PKACα, but whether this interaction is direct or indirect was still to be determined. Utilization of purified protein pulldowns assays allowed for investigation of this question, using His-tagged PKACα and GST-tagged Fyn. While the His-pulldowns performed resulted in high background binding of Fyn to the His-select Dynabeads alone (data not shown), preventing visualization of any Fyn enrichment by PKACα, we were able to show the reciprocal with GST-pulldown experiments. Incubation with GST-Fyn enriched His-PKACα compared to incubation with glutathione beads alone, which displayed a small amount of background binding **(Figure 8)**. This preliminary result suggests a direct interaction between the proteins, and that Fyn is not binding through an AKAP, but to the holoenzyme itself or at least the free C subunit, as seen in Figure 4. Future experiments using GST-coupled glutathione beads as a negative control would help eliminate the possibility that PKA is being enriched by the GST tag alone. These results could be further validated by far-western blot, in which one purified protein would act as a probe for the other after renaturation on a nitrocellulose membrane.

Discussion:

Here we report a novel form of crosstalk between the PKA and SFK pathways in which Fyn not only phosphorylates PKAC directly at Y69, but binds to it directly and enhances its association with a centrosomal and Golgi-localized multi-AKAP complex. Our use of Fyn variants in co-IP experiments has demonstrated the dispensability of the SH3 domain for Fyn to

co-IP with PKACα, as seen by the increased affinity for ΔSH3 Fyn in this interaction. Furthermore, neither GST fusion protein containing the Fyn SH2 or SH3 domain was sufficient to enrich PKACα from HEK293 lysates. If these domains are not involved in the interaction, the binding site could map to the N-terminal domain, the kinase domain, or one of the linker regions between the Src homology domains. Construction of a set of Fyn deletion mutants for use in co-IP experiments could provide an effective means of identifying the region involved in PKA binding. It is also possible that the SH2 and SH3 domain functions are dispensable, but that a structural motif formed by the overall tertiary structure of the full-length kinase is needed. Forskolin treatment of HEK293 cells caused a dissociation of PKAR subunits from PKAC, but Fyn co-IP was retained, suggesting that Fyn is not binding indirectly through PKAR or an AKAP. Interestingly, this effect was recapitulated with ΔSH3 Fyn overexpression. This mutant appears to cause PKA holoenzyme dissociation, potentially through Y69 phosphorylation. Finally, Fyn binding to PKACα was preliminarily found to be direct through purified protein pulldown assays.

This study was initiated to further characterize the regulatory influence that Fyn has on PKA, in which Fyn overexpression leads to an increase in PKA substrate consensus site phosphorylation and the enhanced binding of specific proteins to PKACα. AKAP9, Myomegalin, and CDK5RAP2 form a complex that localizes to the Golgi apparatus and centrosome. Here, they participate in microtubule nucleation and organization. This process is mediated by recruitment of γ-tubulin ring complexes (γ-TURCs) in the pericentriolar matrix by CDK5RAP2 and Myomegalin, from which the growing microtubule polymer can be extended (32). These regulatory processes of the microtubule cytoskeleton are important for spindle formation during mitosis, and Golgi Apparatus reassembly after mitosis (32). While there are several Myomegalin

isoforms of diverse function, co-immunoprecipitations and western blot analysis have identified the CM isoform of about 250 kD to preferentially associate with PKA in cells overexpressing Fyn. CM Myomegalin regulates microtubule nucleation, as opposed to the 160 kD EB isoform that regulates dynamic microtubule growth through interaction with EB1, a +TIP protein (33). Genetic knock down of Myomegalin has been shown to inhibit microtubule growth and cell motility (33). Given the role of PKA in cell motility, and the binding of PKA to these AKAPs, it is possible that Fyn-mediated regulation of PKA-AKAP binding promotes cytoskeletal reorganization, an important component of metastatic activity. The direction of PKA activity towards specific substrates could mediate this response, as AKAPs are known to bring different effector proteins into proximity through anchoring effects (10). This event has implications in the contribution of each kinase to a migratory phenotype, and would further define their role in cancer metastasis. The influence of PKA and Fyn on microtubule organization may also affect chromosomal segregation through spindle formation, and other mitotic events that are initiated at the centrosome.

The apparent sequestration of PKA to the centrosomal AKAP complex of AKAP9, Myomegalin, and CDK5RAP2 appears to be mediated by Fyn binding. It is possible that the enhancement of PKA substrate phosphorylation seen to correlate with Fyn overexpression is also caused in part by this process. A multifaceted activation of PKA could be caused by the additive effects of Y69 phosphorylation on PKACα and a lowering of the PKA cAMP-activation threshold. PKA holoenzyme anchoring to AKAP9 induces autophosphorylation of serine 114 on the R2 subunit by the C subunit, which decreases the level of cAMP needed to achieve a given level of PKA activity (13). It can also be hypothesized that this interaction contributes to the

enhanced migratory phenotype observed in cells with centrosomal amplification (34), if these cells are enriched for an active PKA-AKAP9 complex.

Considering the diverse aspects of Fyn-mediated regulation of PKA, a potential mechanism for increased PKA activity can be hypothetically modeled **(Figure 9)**. Direct binding of Fyn to PKACα may facilitate Y69 phosphorylation, which has been shown to increase PKA catalytic activity (unpublished data). While it is not known which phosphorylation event occurs first, PKA also activates Fyn through S21 phosphorylation, an event necessary for targeting to focal adhesions and for cell migration (35). It is possible that phosphorylation at Y69 on PKAC leads to S21 phosphorylation on Fyn due to activation or proximity, or vice versa. Fyn also recruits PKA to the Golgi and centrosomal AKAP9 complex, providing a second means of modification. This PKA in complex with AKAP9 may have an increased autophosphorylation of S114 of PKAR2, which lowers the cAMP threshold necessary for activity (13). While the resulting hyperactive PKA would be subjected to negative regulation by PDE4D in complex with AKAP9, the potential would remain for increased phosphorylation of proximal substrates functioning in cytoskeletal regulation and proliferation, events that are important in cancer progression. If these downstream events do occur, this model would provide an underlying mechanism for the promotion of these tumorigenic cellular processes by Fyn and PKA, as well as a putative explanation for the correlation of PKAC Y69 phosphorylation identification with cancer sample phosphoproteomic analyses. Identification of substrates in complex with AKAP9, Myomegalin, or CDK5RAP2 that are differentially phosphorylated with Fyn overexpression will provide insight to which pathways Fyn and PKA are targeting in this signaling microdomain.

Further experiments are necessary to validate certain aspects of this cellular phenomenon. The redistribution of PKACα binding towards AKAP9, Myomegalin, and CDK5RAP2 with Fyn

overexpression has been shown through quantitative mass spectrometry and co-IP experiments in HEK293 cells, but it would be beneficial to visualize this change in localization through immunofluorescence. It would be hypothesized that Fyn overexpression would cause an increase in colocalization of AKAP9 and PKACα, as measured by overlapping fluorescence of antibodies tagged with different fluorophores. For this regulatory event to be considered biologically relevant, the results will also have to be recapitulated in multiple cell lines. Mouse embryonic fibroblast cells null for Src, Yes, and Fyn (SYF cells) would be an interesting candidate, as they would provide a negative control of Fyn-deficient background. Transfecting SYF cells with Fyn would allow for assessment of PKA association with Golgi and centrosomal AKAPs with or without Fyn, rather than in conditions of Fyn overexpression. For translational relevance, similar experiments should be performed in cancer cell lines. Knock down of Fyn in cancer cells that express Fyn at high levels, such as the U87 glioblastoma cell line (24), could test the necessity of Fyn expression for enhanced PKA binding to Golgi and centrosomal AKAPs.

In addition to confirming altered PKA localization, cellular immunofluorescence-based experiments will allow analysis of changes in PKA activity downstream of Fyn. HEK293 cells and other lines can be transfected to express the AKAR reporter, which undergoes fluorescence resonance energy transfer when its PKA substrate consensus sequence is phosphorylated. AKAR phosphorylation causes a conformational change that brings its CFP domain within proximity of its YFP domain, and CFP-excitation induces YFP-emission. We would hypothesize from our results that Fyn overexpression will increase PKA activity, as measured by the ratio of YFP:CFP emissions. The advantage of this type of experiment is in the visualization of PKA activity changes in discrete cellular locales, information that is lost in the homogenization that occurs during cell lysis. Therefore, it can be observed whether PKA activity is increasing throughout the

cytoplasm, specifically at the centrosome and Golgi apparatus, or at another AKAP-tethering site such as the plasma membrane.

To assess the downstream cellular phenotypic effects of the interaction between Fyn and PKA, it would be interesting to perform cellular proliferation and migration assays. The question of whether Fyn-mediated centrosomal PKA activity influences either of these processes could be tested by Fyn overexpression and then experimental disruption of PKA anchoring to AKAP9. Cells transfected with or without Fyn could be transfected or treated with an AKAP inhibitor such as sAKAPis or StHt31, in plasmid or peptide form. Choice of inhibitor delivery types may be influenced by accessibility to AKAPs, as ectopically applied membrane-associated inhibitors may be limited in their contact with centrosomal and Golgi-localized AKAPs. If Fyn alone is sufficient to promote proliferation or migration, it will do so independent of PKA anchoring. However, if it is needed in tandem with centrosomal PKA, then the AKAP inhibitor may attenuate the proliferation or migration rate in conditions of Fyn overexpression. If this regulatory pathway is seen to influence a cellular phenotype, then understanding the underlying mechanism will require identification of substrates that demonstrate Fyn-enhanced PKA phosphorylation. This could be accomplished via global phosphoproteomic analysis using quantitative mass spectrometry, as done for PKA binding proteins in this study, or through mass spectrometry of substrates found in complex with PKACα and AKAP9 via IP.

The interplay between Fyn and PKA signaling pathways carries significance beyond centrosomal protein interactions. Each kinase is involved in diverse cellular processes, and the potential for regulatory crosstalk downstream of canonical GPCR and RTK transduction pathways extends the reach of the interaction to a wider spectrum of effector proteins. Fyn activation could feed into activation of PKA effectors, and vice versa. In the process of axon

outgrowth, PKA and Fyn have already both been implicated in Netrin-mediated chemoattraction. PKA mediates cytoskeletal reorganization for growth cone dynamics via anchoring to ERM proteins downstream of DCC receptor activation, causing downstream phosphorylation of VASP (7). However, DCC activation and neural outgrowth require phosphorylation of DCC by Fyn (23). It is plausible that Fyn facilitates relocalization of PKA to the cell membrane through binding to the C subunit, where the R subunits are then anchored to ERM proteins upon Fyn phosphorylation of DCC. This interconnectivity will be of interest to those studying the roles of either kinase in diverse cell types, such as T cells, where the role of Fyn in T cell receptor signaling (22) may warrant the investigation of PKA function in this process.

Our results indicate the existence of a novel regulatory interaction between PKA and Fyn that occurs through phosphorylation and a physical interaction. Fyn is able to increase PKA substrate phosphorylation and association with specific binding partners, including centrosomal and Golgi-localized AKAPs. We hypothesize that this event may be the root of the many reports of PKAC Y69 phosphorylation within tumor samples, and that it begins to define the role each kinase holds in cancer progression, specifically migration. We have begun to characterize the molecular binding interaction between the two kinases, and we aim to delineate the downstream effects on cellular processes in future experiments.

Acknowledgments:

This work was supported by the Arnold & Mabel Beckman Foundation, the UVM Office of Undergraduate Research "Undergraduate Research Opportunities Program Grant", and the College of Arts & Sciences "Academic Programs for Learning and Engagement Award" (to SAB), as well as a Scientist Development Grant from the AHA, and the Lisa Steele Endowed Professorship in Health Sciences (to PBD). We thank the Vermont Genetics Network and NIH INBRE for funding of proteomic studies.

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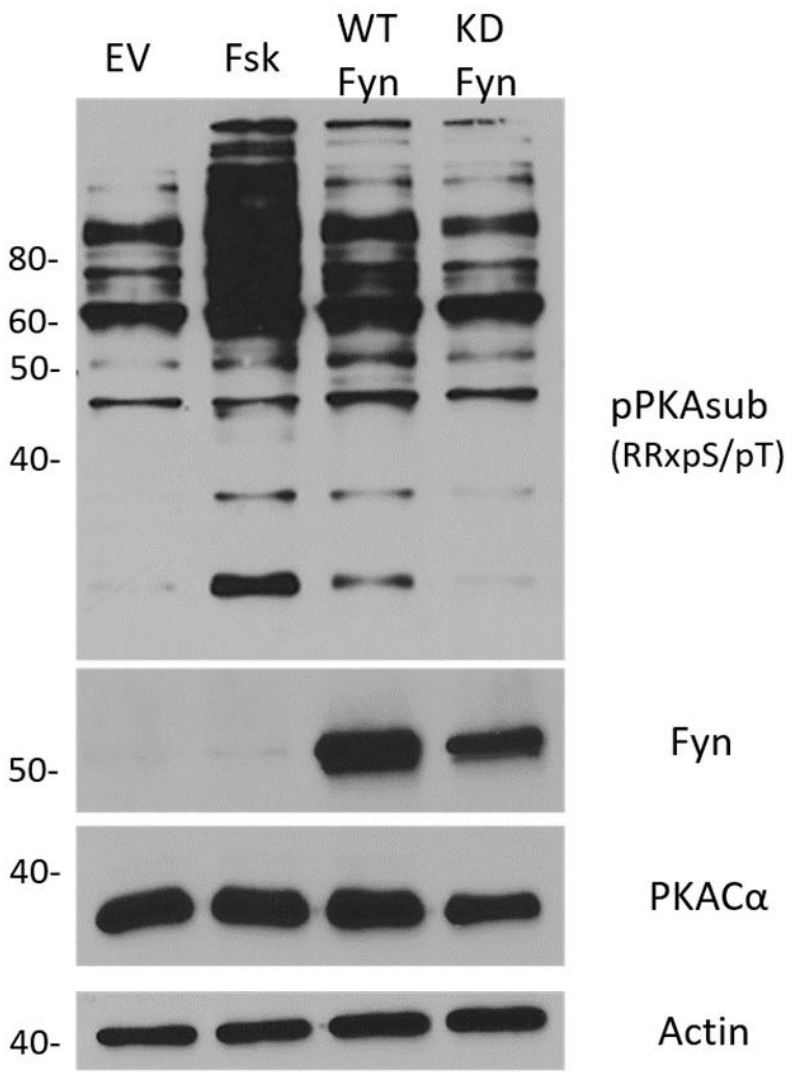

Figure 1. Fyn overexpression increases PKA substrate phosphorylation independent of Fyn kinase activity. HEK293 cells were unstimulated, stimulated with Forskolin at 30 μM for 15 minutes, or transfected with mammalian expression vectors encoding wild type (WT) or kinase dead (KD) Fyn. Cells were serum starved after 24 hours and harvested after 48 hours. Phosphorylated PKA substrate levels in lysates were monitored via western blot analysis. Experiment performed by Jacqueline Mann.

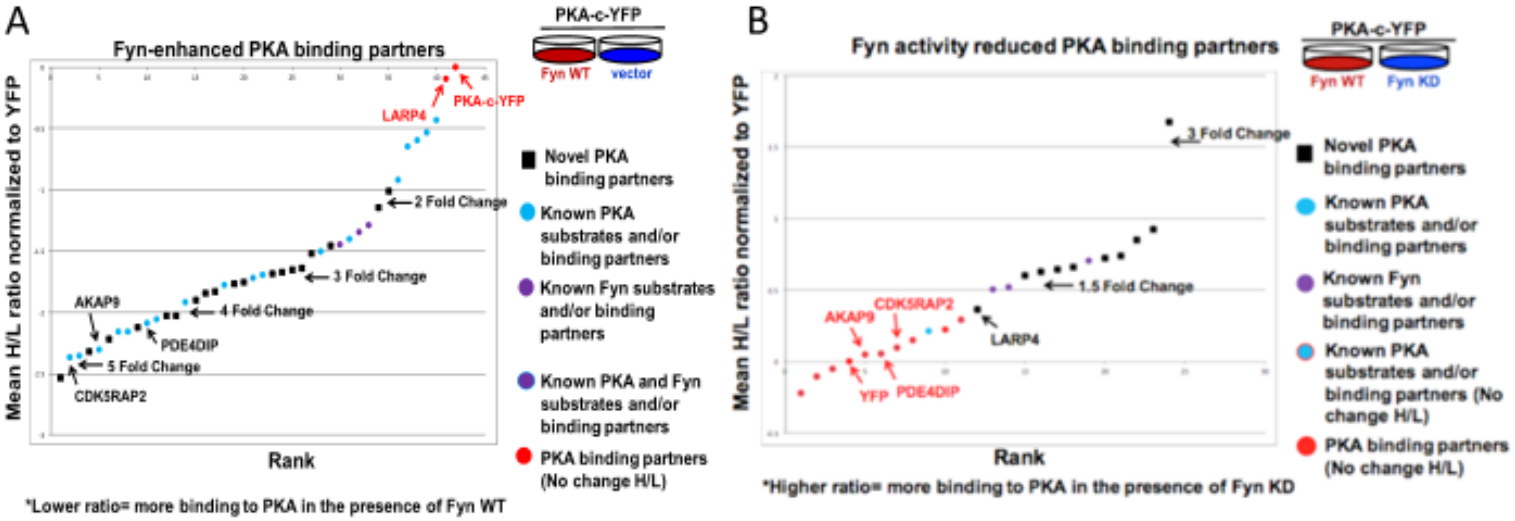

Figure 2. Fyn overexpression enhances PKA-C binding to intracellular proteins. A: A rank order plot of normalized peptide ratios between heavy and light treatments: HEK293 cells were grown in heavy or light SILAC media and transfected with YFP or PKACα-YFP and pcDNA 3.1 empty vector or WT Fyn. Cells were serum starved after 24 hours and harvested after 48 hours, before pooling heavy and light treatments (YFP/vector with YFP/Fyn, and PKA/vector with PKA/Fyn, respectively). Lysates were immunoprecipitated for YFP with α-GFP and separated with SDS-PAGE before trypsin digestion and processing for LC-MS/MS. All identified binding partners with a negative H/L ratio exhibit enhanced binding to PKA-C-YFP in Fyn-transfected samples. AKAP9, CDK5RAP2, and PDE4DIP are all AKAPs identified to be Fyn-dependent. **B:** A rank order plot of normalized peptide ratios between heavy and light treatments: HEK293 cells were transfected and processed as in **A**, but with YFP or PKACα-YFP and WT or KD Fyn. All identified binding partners with a positive H/L ratio exhibit enhanced binding to PKACα-YFP in KD Fyn-transfected samples. AKAP9, CDK5RAP2, and PDE4DIP were all found to bind independent of Fyn kinase activity. Plots were constructed by Dr. Marion Weir and the mass spectrometry experiment was performed by Jackie Mann, Dr. Marion Weir, and Dr. Paula Deming.

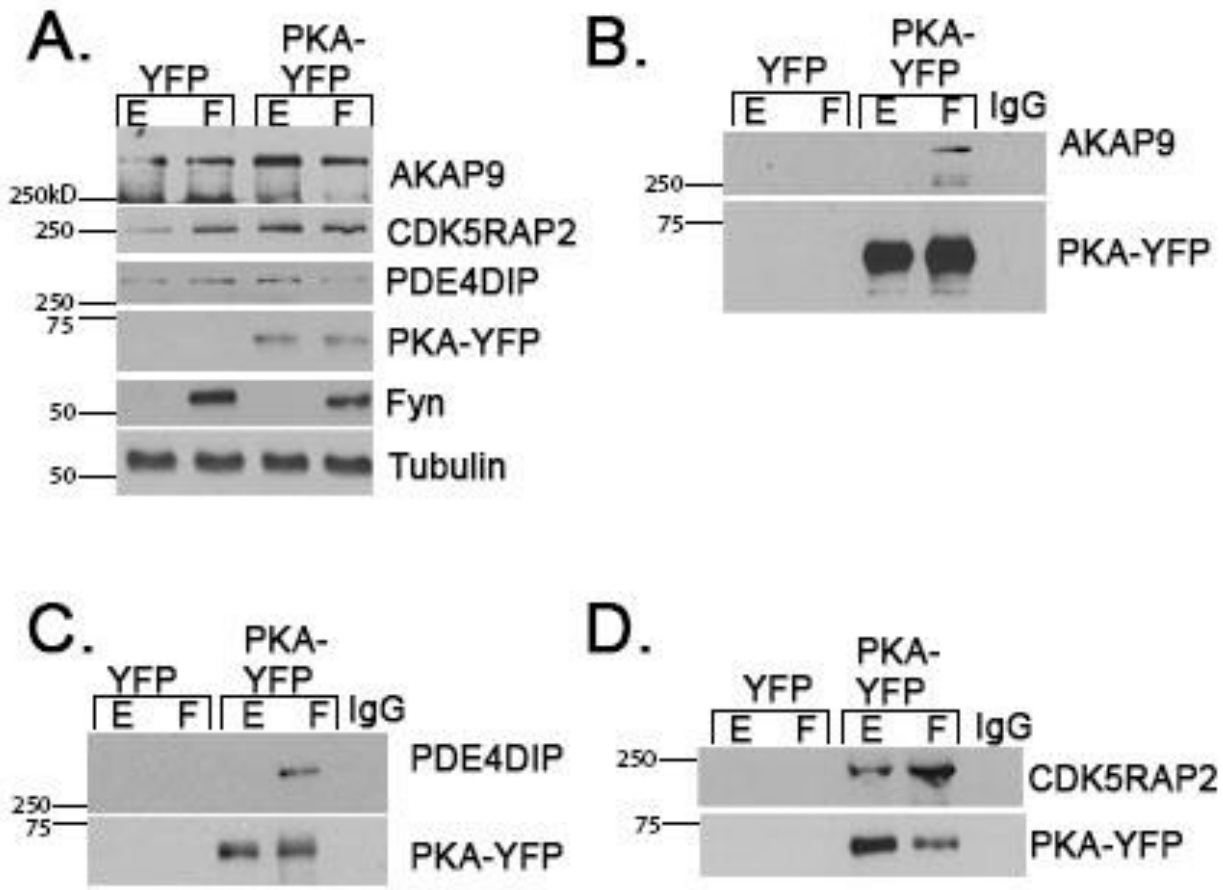

Figure 3. Validation of Fyn-dependent AKAP binding. A: HEK293 cells were transfected with YFP or PKACα-YFP, and pcDNA 3.1 empty vector or WT Fyn. Cells were serum starved after 24 hours and harvested after 48 hours. AKAP9, CDK5RAP2, PDE4DIP, PKACα-YFP, Fyn, and Tubulin levels were analyzed in lysates by western blot. **B-D:** Lysates from A were immunoprecipitated for YFP with α-GFP. The levels of PKACα-YFP and either AKAP9, PDE4DIP, or CDK5RAP2 were monitored via western blot. A non-specific rabbit IgG control was performed on pooled lysates from the four treatments.

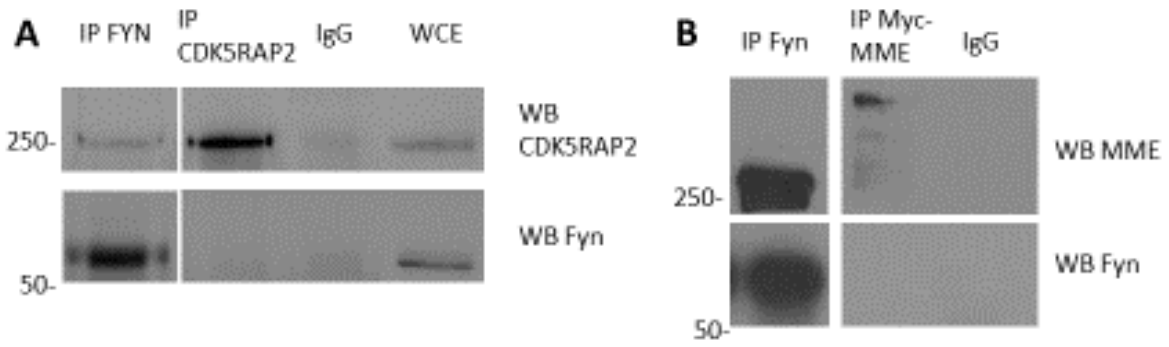

Figure 4. Fyn is associated with CDK5RAP2 and Myomegalin. HEK293 cells lysates underwent IP for endogenous Fyn and either endogenous CDK5RAP2 **(A)** or transfected Myc-tagged Myomegalin (MME) **(B)**. A non-specific rabbit IgG control was performed with combined lysates for each experiment. IPs were blotted for either AKAP and Fyn, as were the lysates in **A**. Blots are repurposed from a different experiment and cropped to include only specific lanes of Fyn and AKAP IPs that were run on a given gel.

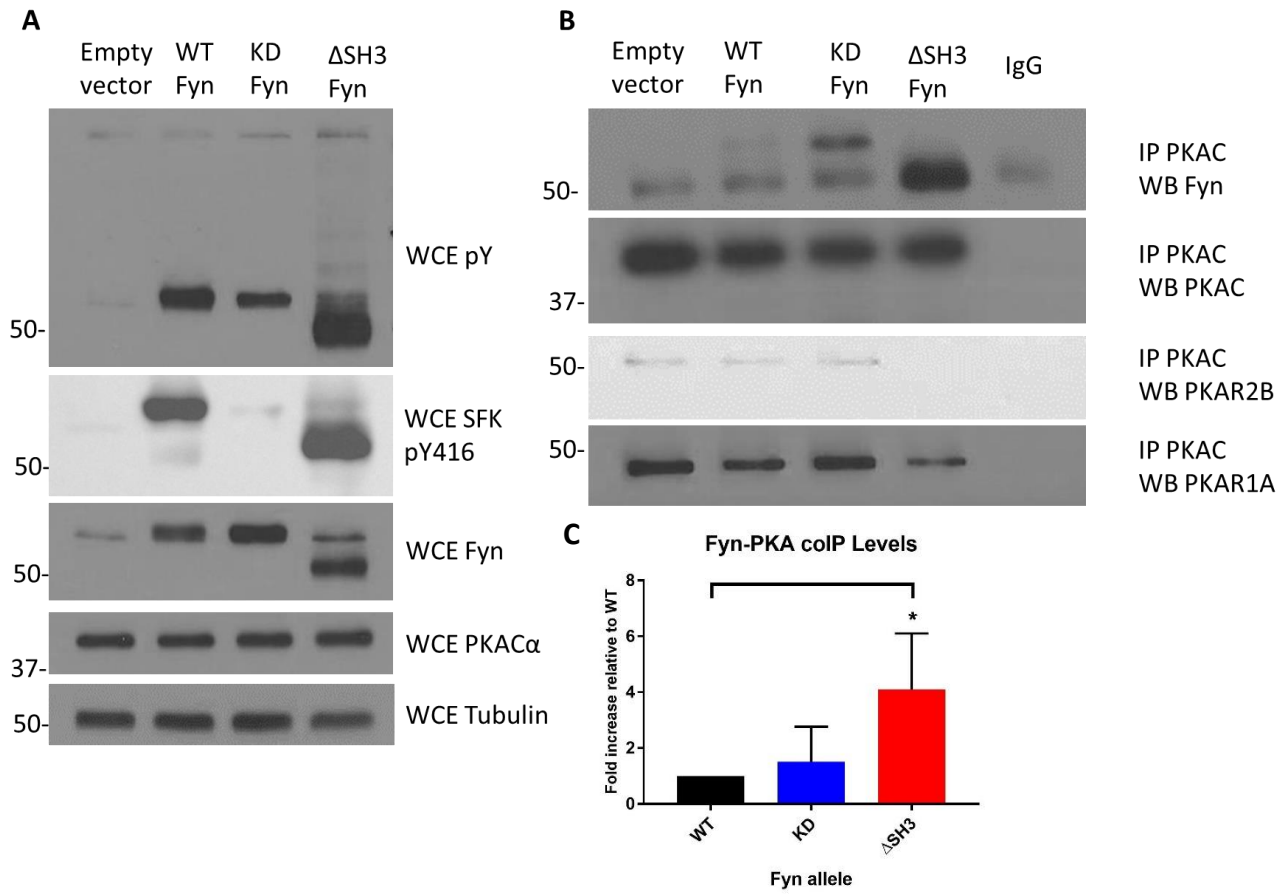

Figure 5. The Fyn SH3 domain is dispensable for PKA-C binding. HEK293 cells were transfected with pcDNA 3.1 empty vector, wild type (WT), kinase dead (KD), or delta-SH3 (ΔSH3) Fyn. Cells were serum starved after 24 hours and harvested after 48 hours. Lysates were blotted for Fyn, PKACα, phospho-tyrosine, Src family kinase phospho-tyrosine 416 (SFK pY416), and Tubulin **(A)**. Lysates were immunoprecipitated for PKACα and blotted for PKACα, Fyn, PKAR2β, and PKAR1α **(B)**. A rabbit IgG control was performed on combined lysates, with a fourth of the total from each treatment. Densitometry analysis was performed to determine levels of Fyn binding through normalization to Fyn levels in lysate and PKACα levels in the IP with Adobe Photoshop CS6 **(C)**. Levels of binding were measured relative to that of WT Fyn (set to 1). A one-tailed paired t-test was performed between each treatment (*: $p < 0.05$, WT n=5, KD n=5, ΔSH3 n=4).

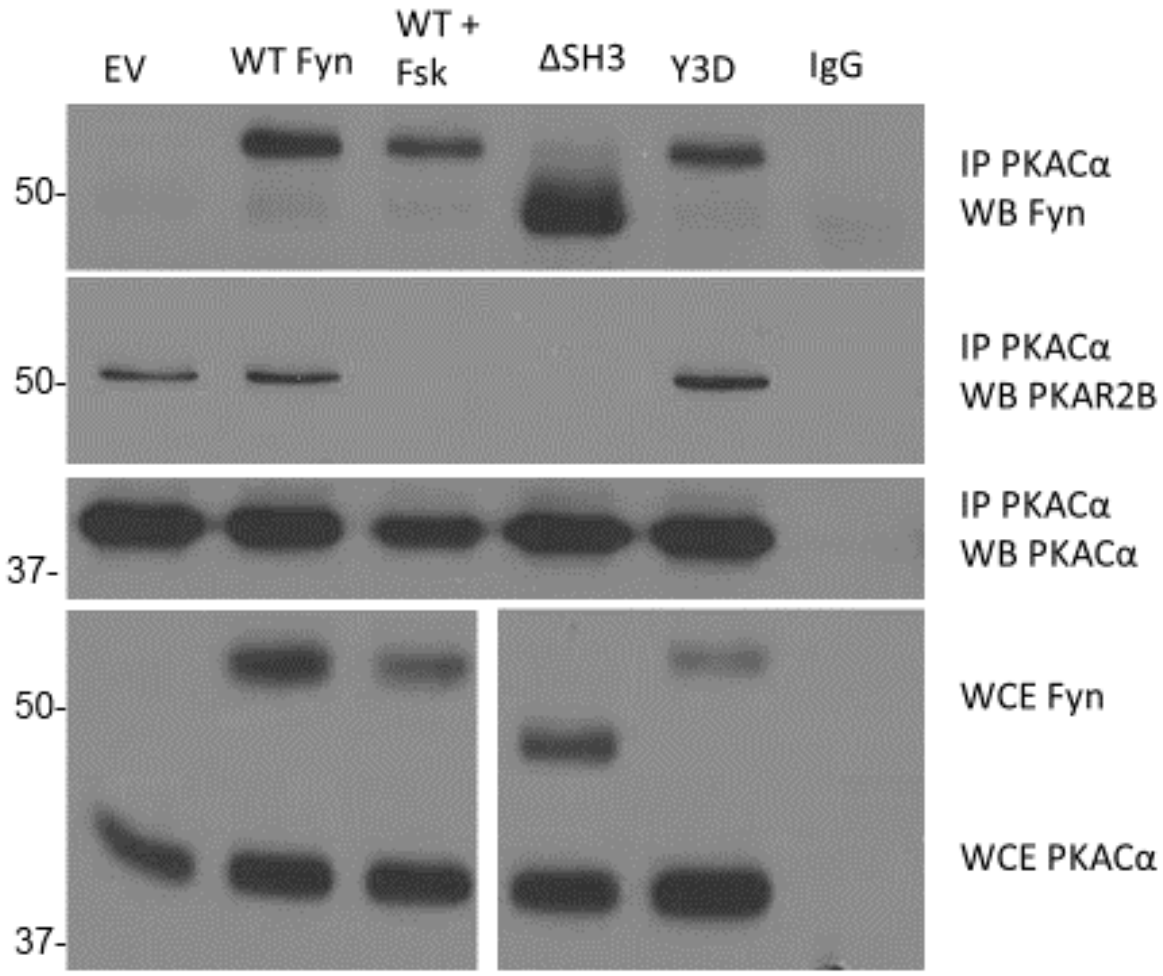

Figure 6. PKA holoenzyme integrity and Fyn SH2 domain function are dispensable for Fyn binding to PKA-C. HEK293 cells were transfected with pcDNA 3.1 empty vector, WT, ΔSH3, or Y3D Fyn (SH2 domain triple tyrosine to aspartate mutant). One WT Fyn-transfected sample was treated with 30 μM Forksolin and incubated for 15 minutes before harvest, and all were harvested after 48 hours. A rabbit IgG control was performed on combined lysates from each treatment. Lysates were immunoprecipitated for PKACα and blotted for PKACα, Fyn, and PKAR2β. Lysates were blotted for Fyn and PKACα. One lysate lane was excised, as it was not used in the final IP.

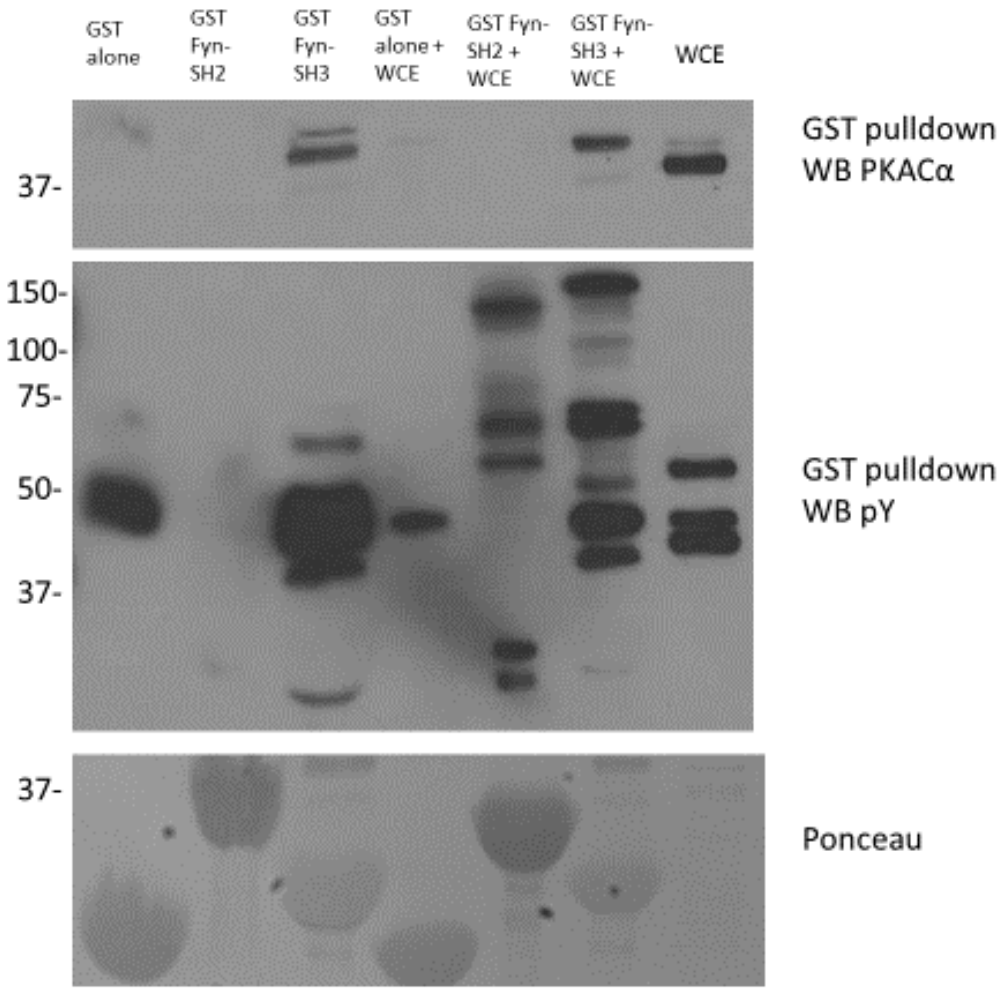

Figure 7. Fyn SH2 and SH3 domains alone are insufficient for PKA-c binding. Untransfected HEK293 cells were lysed and incubated with glutathione beads coupled to GST, GST-Fyn SH2, or GST-Fyn SH3. Beads and GST-fusion proteins were also run alone, without incubation with lysate, and 20 μg of lysate was run as well. A Ponceau stain was performed to determine the levels of each GST protein. Pulldowns were blotted for PKACα and phospho-tyrosine.

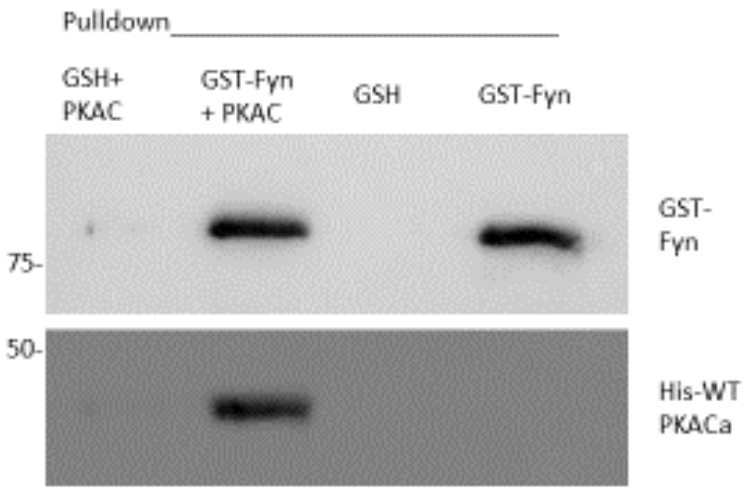

Figure 8. Purified Fyn appears to bind to PKA-C directly. Glutathione beads were blocked in 10% BSA for 2 hours, and incubated overnight with or without purified GST-Fyn. Samples were split and incubated for 2 hours with or without purified His-tagged PKACα. Pulldowns were resuspended in 1X Lammli sample buffer and separated via SDS-PAGE. Western blot analysis was performed for Fyn and PKACα.

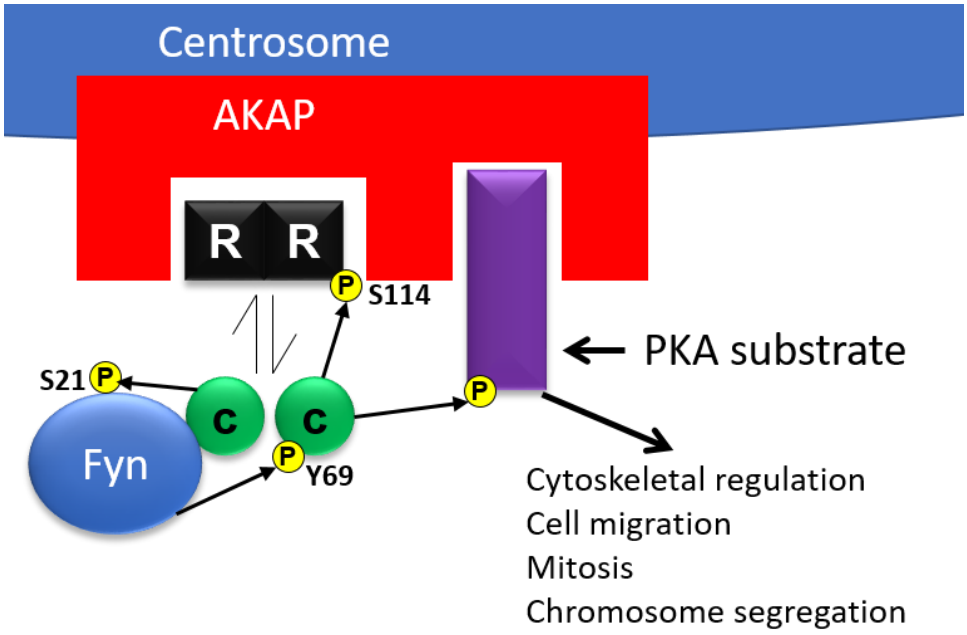

Figure 9. Fyn-PKA-AKAP signaling model. This hypothetical model is depicting Fyn directly binding to PKAC, facilitating PKAR2 anchoring to a centrosomal AKAP (AKAP9, Myomegalin, or CDK5RAP2). Fyn phosphorylates PKAC at Y69. Active PKAC phosphorylates Fyn at S21, PKAR2 at S114, and other substrates in proximity to the complex that could mediate various downstream cellular processes.