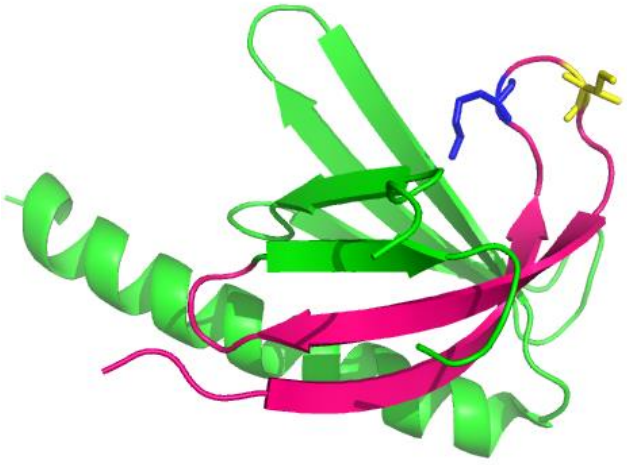
Chapter 3

Development of a PCC Agent Selective for the E17K Mutant Akt1 Protein



3.1 Introduction

3.1.1 The E17K Mutation in the Pleckstrin Homology Domain of Akt1

Akt1 kinase plays a critical role in the PI3K signaling pathway,[1] the activation of which is closely linked to tumor development and cancer cell survival[2]. The phosphorylation of regulatory amino acids (Ser474 and Thr308) on Akt occur through the localization of Akt to the cell membrane through its membrane-binding Pleckstrin Homology Domain (PH Domain). These phosphorylations activate the Akt protein, which can then activate many other downstream signaling pathways[2]. The recently discovered E17K mutation in the PH Domain of Akt1 results in an increased affinity for the phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3, or PIP3) substrate at the cell membrane (**Figure 3-1**)[3]. This switch from a negatively charged glutamic acid to a positively charged lysine amino acid in the PIP3 binding pocket causes this mutant protein to have a four times higher affinity for the PIP3 substrate. This increased affinity causes the Akt1 to be bound to the cell membrane, and hence activated four times longer than in healthy, wildtype cells. Consequently, this deregulated recruitment of Akt1 to the cell membrane causes constitutive activation of the PI3K pathway, which has been shown to be sufficient to induce leukemia in mice[3]. The oncogenic properties of the driving E17K single point mutation make it a target for specific detection and inhibition.

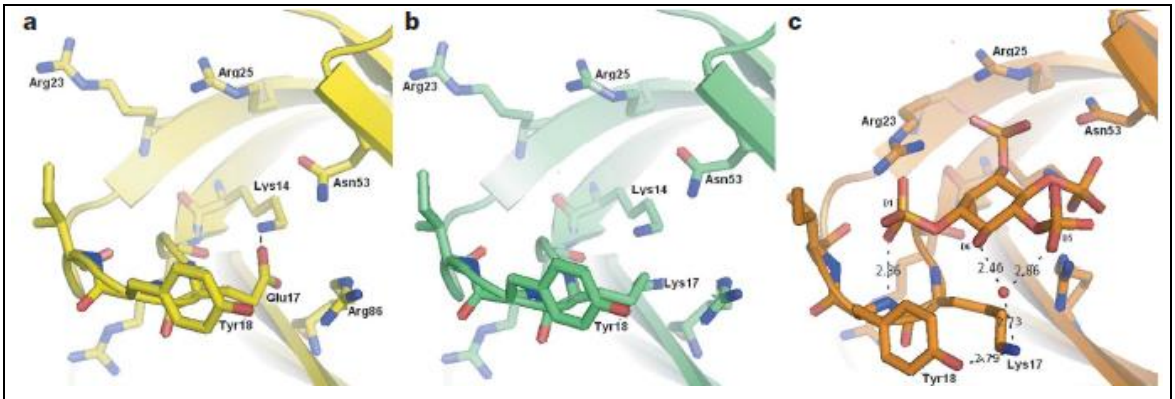


Figure 3-1: PH Domain Binding Pocket Changes upon E17K Mutation: *a.) Interaction between Lys14 and Glu17 in binding pocket of wildtype PHD, b.) repellent interaction between Lys14 and Lys17 in the binding pocket of the E17K mutant, and c.) new hydrogen bonds to water with the E17K mutation in complex with the PIP3 substrate.3*

3.1.2 A General Strategy for Targeting Single Amino Acid Point Mutations in Proteins

Targeting single amino acid point mutations in proteins is becoming a necessary step in the era of personalized medicine, and methods for the detection of these mutant protein biomarkers are highly desirable for guiding treatment decisions[4]. Thus, there is a need for an approach to identify small molecules that can be generally targeted against epitopes containing single amino acid point mutations, and can also potentially be developed into cell-penetrant inhibitors. Previously, a strategy was developed for targeting the phospho-epitopes by chemically synthesizing the surrounding chunk of protein and focusing the site of the *in situ* click screen by attaching an azide click handle to a phosphate chelating group.[5] This method has been generalized by directly substituting an alkyne click handle into the chemically synthesized peptide epitope. For this work, the peptide represents the epitope of Akt1 containing the E17K mutation, an attractive target due to the oncogenic nature of this mutation[3]. That target is subjected to an *in situ* click screen against an OBOC peptide library of 5-mers (comprehensive in 18 amino acids), each terminated in an azide presenting amino acid.

This generalized technique allows us to focus our PCC agent development to a location on the PH Domain that is adjacent to the E17K oncogenic mutation. The approach yielded a 5-mer peptide that exhibited a 10:1 selectivity for E17K Akt1 relative to wild-type (WT). We exploited the chemical flexibility and modularity of the PCC agent to append a dye and a cell penetrating peptide. The resultant ligand could selectivity image the E17K Akt1 protein in live cells, again with high selectivity relative to WT. The technique for epitope targeting described herein provides a general approach for the synthesis of small molecule peptides that are capable of selectively distinguishing between WT and mutant proteins in cancer. These small molecule peptides would be useful tools for disease detection assays, as well as provide a path towards the inhibition of their target proteins.

3.2 Materials and Methods

3.2.1 Akt1 PH Domain Expressions

Akt1 Pleckstrin Homology Domain DNA was purchased from DNA2.0, and the codons were optimized for expression in *E.coli*. The first 124 N-terminal amino acids from full-length Akt1 were used as the PH Domain DNA, and a 6-his tag separated by a thrombin cleavage site was added at the C-terminus of the protein for purification. In order to make the E17K mutant of the PH Domain, the glutamic acid in position 17 was mutated to a lysine via QuikChange (Stratagene), following all of the manufacturer's protocols. The DNA was synthesized in a pJexpress 414 vector containing an ampicillin resistant gene to be expressed in *E.coli* cells. Protein expression was performed by the Protein Expression Center at Caltech using their standard bacterial expression protocol, and purified via Ni-NTA column. The proteins expressed in this manner were used for the pull-down assays confirming the anchor binding via immunoprecipitation assays, and for the biligand screens. These PH Domain proteins were unsuitable for long-term storage under a large variety of tested conditions, so a GST tag was added to hopefully improve the long term stability.

For that reason, the DNA from DNA 2.0 was amplified out of the pJExpress vector using polymerase chain reaction (PCR) to insert the restriction enzyme sites EcoRI and NotI for insertion into a pGEX-4T-1 vector containing a GST tag. The primers used were:

5' - AGAGAATCCATGTCCGACGTCGCGATCGTAAAGGAAGGG – 3'

5' - TCTGCGGCCGCTTAGTGGTGATGATG – 3'

Both the wildtype and E17K mutant DNA were amplified out of the pJExpress vector, restriction enzyme digested, and ligated overnight into a pGEX-4T-1 vector that attached an N-terminal GST tag to the PH Domain protein. BL21-DE3-pLys cells were transformed with the DNA, confirmed correct via sequencing. An overnight starter colony from each protein was grown in 5mL LB + 100 µg/mL Amp overnight. 4mL of this starter culture was used to inoculate 500mL of LB+Amp, and grown to mid-log phase. The cultures were inoculated with 1mM IPTG and grown

5 hours at 28°C. The cells were spun down for 10 minutes at 8,000 RPM and lysed with lysis buffer (1x TBS, 1mM DTT, 1mg/mL Lysozyme, 1% Triton-X), and left for 30 minutes on ice before flash freezing in liquid nitrogen. Upon thawing on ice, the lysate was sonicated for 5 minutes, then centrifuged for 30 minutes at 10,000 RPM to remove cellular debris. The supernatant was then purified on a HisPur Co column (Pierce) using the recommended protocol. These GST-tagged proteins were used to confirm the biligand binding via immunoprecipitation assays, and for the triligand screens. They were also used to obtain the full ELISA curves of all three ligands. These proteins, however, were also not suitable for long term storage and needed to be re-expressed for all assays.

The imaging experiments required that the PH Domain protein be expressed in mammalian cells and have a GFP tag for visualization. Because of this, Akt1 DNA with codons optimized for use in mammalian cells was obtained from InvivoGen as a pUNO-hAKT1 plasmid. The DNA was mutated via QuikChange as before so that both a wildtype and E17K version were on hand. The primers used to clone the DNA from this vector into a TOPO C-terminal GFP mammalian vector (Life Technologies) were:

5’ – AAGATGGGGATGAGCGACGTGGCT – 3’

5’ – TCCCCGACCGGAAGTCCATCTCCTC – 3’

Cloning into the TOPO vector was performed by following all of the manufacturer’s recommended instructions. Because the GST-PH Domain proteins expressed in *E.coli* were still not stable for long term storage, this DNA was used to express the PH Domain in mammalian cells to test the storage suitability of this recombinant fusion protein. The expressions were performed by transfecting a suspension culture of HEK-293-6E cells with XtremeGene HD by the Protein Expression Center at Caltech following their standard protocols. These proteins were not purified,

and were used as-is out of cell lysates. This protein was used in triligand pull-down and inhibition assays, and was still not stable for long term storage.

3.2.2 Design and Synthesis of Epitope-Targeting Anchor/Target Peptide

Epitope targeting for the point mutation of the PH Domain of Akt1 was accomplished by screening against a 33-mer peptide fragment derived from the N-terminus of the PH Domain, highlighted in **Figure 3-6,** that contained the E17K point mutation as well as a propargylglycine (Pra) alkyne click-handle substitution (I19[Pra]) for directing the *in situ* click reaction near the mutated site. The peptide fragment epitope sequence used in these studies was:

MSDVAIVKEGWLKKRGKY[Pra]KTWRPRYFLLKNDG

This 33-mer fragment was capped with an N-terminal biotin label for detection in the screen, and was purified on a prep-scale Dionex U3000 HPLC with a reverse-phase C4 column (Phenomenex). MALTI-TOF MS showed a peak for m/z = 4215.93 for the pure product, expected m/z = 4219.9.

3.2.3 CD Spectroscopy of 33-mer Target Peptide Epitope

Lyophilized powder of the 33-mer biotin-tagged target fragment that was used for screening was dissolved in 500µL of 1x PBS to a concentration of 0.5mg/mL. Concentrations were estimated by weight, and confirmed by A280 measurement on a NanoDrop. Experiments were performed using an Aviv 62 CD Spectrometer. The machine was purged for 20 minutes with N_2; then, the 1xPBS blank in a 500µL 1cm cuvette was added, and the machine was purged with N_2 for another five minutes. The spectra was acquired by taking three measurements/minute from wavelengths 199-250nM. The 33mer fragment sample was then added, purged for 5 minutes, and was measured exactly as the blank. The 33mer cuvette was then removed, and 500µL of

7.0M Guanadine-HCl (pH = 2.0) was added to denature the sample. This spectra was acquired as above.

To work up the data, the signal in **ΔA** from the sample was subtracted from the blank at each wavelength. Then the mean residue molar circular dichroism $\mathbf{\Delta\varepsilon_{MR}}$ was calculated from this readout using the number of residues in the fragment (33) and the concentration in mg/mL (0.5 for the folded sample, or 0.25 for the denatured sample, since it was diluted with Guanadine-HCl) using the equation: $\mathbf{\Delta\varepsilon_{MR} = \Delta A/((residue\ \#\ x\ concentration\ mg/mL)\ x\ l)}$[6]**.** The spectra were graphed by plotting this number against the wavelength.

3.2.4 Screen for Initial Anchor Ligand Peptide

Screens were performed using a library containing 100% Met coupled at the C-terminus for potential MALDI TOF/TOF sequencing[7]. The peptide library was a comprehensive 5-mer containing 18 unnatural D-amino acids, excluding Met and Cys due to stability reasons. The N-terminus consisted of an azide click handle with varying carbon chain lengths – 2 carbon, 4 carbon and 8 carbon – for in vivo click with the Pra on the target 33-mer epitope fragment. Screens were completed using with 300mg of dried library beads swelled at least six hours in 1x TBS (25mM Tris-Cl, 150mM NaCl, 10mM $MgCl_2$, pH = 7.5) buffer.

Preclear (**Figure 3-2a)**:

Swelled library beads were blocked overnight in 5% w/v dried non-fat milk in 1x TBS, then washed with 1x TBS three times. Five milliliters of a 1:10,000 dilution of streptavidin-alkaline phosphatase conjugate in 0.5% milk in TBS was added to the beads, and incubated shaking at room temperature for one hour. The beads were washed with a high-salt TBS buffer (1x TBS with 750mM NaCl) three times, then let shake in high salt buffer for one hour. The beads were then

washed three times with BCIP buffer (100mM Tris-Cl, 150mM NaCl, 1mM $MgCl_2$, pH = 9.0) and developed by adding 15mL BCIP buffer plus 13µL BCIP and 26µL NBT (Two part system, Promega). After one hour, the purple beads were removed by pipette and discarded. The remaining library beads were incubated in NMP for four hours to remove trace purple precipitate from the BCIP/NBT reaction, then were washed five times with methanol, five times with water, five times with TBS and blocked overnight in 5% milk.

Product Screen (**Figure 3-2b):**

Beads remaining from the preclear were washed three times with 1x TBS, then incubated with 5 mL of a 100 nM dilution of the 33-mer epitope target in 0.5% milk for either 5 hours or 12 hours to allow for an *in situ* click reaction to occur. The beads were then washed three times with 1x TBS, and incubated for one hour with a 7M Guanadine-HCl buffer (pH = 2.0) to remove all of the 33-mer epitope target that was not attached covalently to the beads. These beads were then washed ten times with 1x TBS, blocked for two hours in 5% milk, then incubated for one hour with a 1:10,000 dilution of streptavidin- alkaline phosphatase conjugate in 0.5% milk in TBS to detect for the presence of the 33-mer epitope target clicked to a bead. The beads were washed three times with a high-salt TBS buffer, then let shake in high salt buffer for one hour. Afterwards, the beads were again washed three times in BCIP buffer, and developed as per the preclear. Purple beads are removed from the screen via pipette, and are considered hit beads. These hits were incubated in the guanidine-HCl buffer to remove attached streptavidin, washed ten times with water, and sequenced via Edman degradation on a Procise CLC 494 system from Applied Biosystems. See **Table 3-2** for the 5-hour sequences and **Table 3-3** for the 16 hour, overnight sequences.

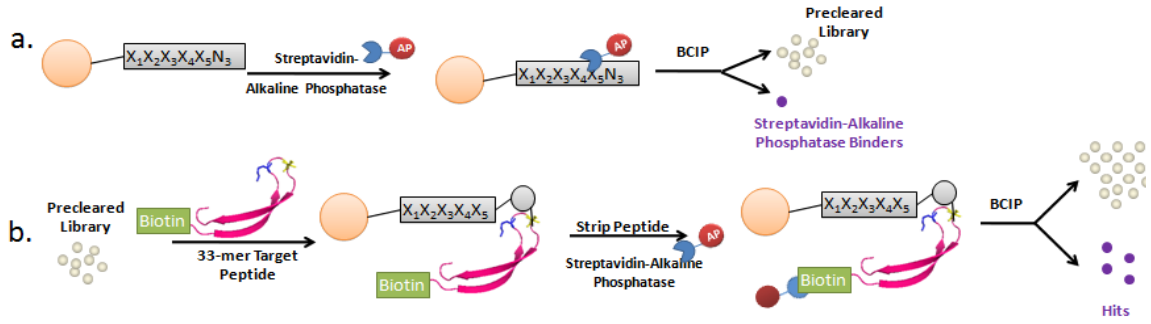


Figure 3-2: Screening Strategy for Anchor Ligand Determination *(a) Preclear: Library beads are incubated with streptavidin - alkaline phosphatase conjugate to remove any library beads that bind to this or the BCIP reagents. (b) Product Screen: Precleared library beads are incubated with the 33-mer target peptide containing an azide in situ click handle. The fragment catalyzes triazole formation between the alkyne on the 33-mer target and the azide on beads that contain peptide sequences that bind specifically to the 33-mer in a close enough proximity to the alkyne substitution for a click reaction to occur without copper. The unclicked peptide is then stripped from the beads, and the remaining covalently attached 33-mer is detected by streptavidin – alkaline phosphatase with BCIP development.*

3.2.5 Hit Library Bead Sequence Analysis

Hit sequences were segregated based on their hydrophobicity and sequence homology using principal component analysis. The algorithm analyzes a series of peptides via hydrophobicity and sequence homology, and graphs them on a 2D sequence map (**Figure 3-7**). Clusters of hits were circled, and one peptide from each cluster was scaled-up and tested for binding to both wildtype and E17K mutant PH domain. The ligands chosen for scale-up were: dqntr, ypwve, eefef, yleaf, and elnhy. Any ligand candidates that were difficult to call on the sequencing were not chosen for scale-up and testing.

3.2.6 Streptavidin-Agarose Immunoprecipitation (Pull-down) Assays for Binding Affinity

Pull-down assays were done on streptavidin agarose resin (Invitrogen). The resin was incubated with N-terminal biotinylated anchor peptide candidates identified via the principle component analysis seen in **Figure 3-7**. The anchor candidate-coated beads were then incubated with both wildtype and E17K mutant protein to compare the selectivity of the ligands as well as the binding ability.

Assays were performed using 50µL of streptavidin-agarose slurry (25µL resin) in Spin-X tubes (Sigma) to allow for the easy removal of the solutions. Resin was aliquotted into 14 tubes – six ligands plus a blank tested against two different proteins – then washed three times with 1x TBST (1x TBS + 0.1% Tween-20). Each set of tubes was incubated with a 10x excess of the appropriate biotinylated ligand to streptavidin binding sites in 200 µL 1x TBS or plain buffer for the blank. Ligand binding was done for one hour at room temperature, and then resin was washed three times with 1x TBS. Resin was blocked with 1x TBS with 5% BSA for two hours. The anchor-coated resin was then incubated with either wildtype or mutant expressed PH domain protein overnight (~16 hours) in cold room (4°C). Protein was spun out of tubes, and the resin was washed three times with high salt TBS, then incubated for five minutes in the high salt buffer. The resin was then washed three times with the 1x TBS buffer, and spun out to dry completely. Fifty µL of denaturing SDS gel loading buffer with 10% B-mercaptoethanol was added to the sample,s and they were incubated at 95° C for ten minutes to denature from the resin. The gel loading buffer was spun out of the Spin-X tubes, and the samples were run on an Any KD BioRad Premade Gel under denaturing conditions. Gel was transferred to nitrocellulose membrane, blocked for one hour in 5% milk (4°C), and western blotted[8]. Proteins were detected using rabbit polyclonal anti-Akt1 antibody (ab64148, Abcam) and an anti-rabbit HRP conjugated secondary anti-body (Cell Signaling), then developed with West Pico Chemilluminescent substrate (Pierce). Relative protein band sizes were analyzed to compare binding between the anchor candidates, and were used to determine selectivity for either wildtype or mutant PH Domain.

3.2.7 Point ELISAs with Anchor Ligand and 33-mer Epitope (Epitope Targeting Verification)

The 33-mer epitope used in screening was resynthesized without the alkyne click handle and with a 6-His tag as an orthogonal tag to the biotin on the anchor ligand. This tag was added after a PEG_5 on the N-terminus of the peptide, and was made and purified as was previously described. The mutant fragment had an expected m/z of 5160.72, observed MALDI-TOF MS m/z for [M+H] of 5161.61. The WT fragment has an expected m/z of 5161.72, and an observed MALDI-TOF MS for [M+H] of 5162.78.

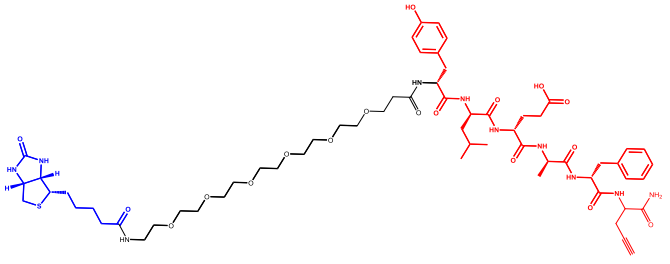


Figure 3-3: Biotin – PEG_5 – yleaf – Pra Anchor ligand: *As analyzed by MALDI-TOF MS, expected m/z = 1298.62, observed [M+Na] = 1319.89.*

For these assays, 100nM Biotin-PEG_5-yleaf-Pra (**Figure 3-3**) was immobilized for one hour on a Neutravidin-coated ELISA plate (Pierce). The plate was blocked in 5% BSA in 1xTBS for one hour, then again overnight at room temperature. The immobilized anchor was then incubated with either 1µM or 100nM wildtype 33-mer epitope or 1µM or 100nM E17K mutant 33-mer epitope for one hour. The plate was washed three times with 1xTBS + 0.1% Tween-20, and tapped dry. The epitope was then detected by a 1:1,000 dilution of an anti-his mouse mAb (ab18184, Abcam) for one hour, washed as above, and then detected with 1:10,000 dilution of an anti-mouse HRP-conjugated goat pAb (Abcam) for one hour. The plate was once again washed and developed with a 1:1 TMB substrate (KPL) for 15 minutes. To graph the data, the blank (epitope and antibodies binding to plate with no anchor ligand present) was subtracted from the triplicate sample values. The fraction bound was found by setting the highest value to 100% and normalizing the rest accordingly. The triplicate values were then graphed with their error bars, and the p-values were calculated by GraphPad using a two-way ANOVA test.

3.2.8 HPLC-Detected Immunoprecipitation (Pull-down) Assays (Epitope Targeting Verification)

Pull-down assays with the biotinylated anchor and his-tagged 33-mer epitope were performed to verify epitope targeting. As with the full-protein assays, the biotinylated anchor ligand was incubated for one hour with 50µL of streptavidin agarose slurry that had been washed three times with 1xTBS. The anchor ligand was washed out, and the resin was blocked for an hour in 5% BSA in 1xTBS. Two hundred µL of a 50µM solution of his-tagged 33-mer epitope in 1xTBS was added to the blocked resin, and this was incubated overnight (~16 hours) at 4°C. Because small peptide fragments like the 33-mer epitope are difficult to transfer to and detect on the nitrocellulose membrane as for a traditional Western blot, the amount of binding in these assays was detected via HPLC. In order to do this, the bound 33-mer peptide fragments were washed three times with 1xTBS + 0.5% BSA, and one time with 1xTBS. The resin was then incubated with 200µL of the 7M guanadine-HCl (pH = 2.0) buffer used to strip beads in the screen. The guanidine buffer was spun out of the beads in Spin-X tubes and injected onto a Beckman Coulter semi-prep HPLC with a reverse phase C18 analytical column. The peak seen on the HPLC illustrated how much of the 33-mer epitope bound to either the yleaf anchor or to blank beads.

3.2.9 Ligand-Directed Tosyl Labeling Experiments

For these assays, the yleaf anchor was appended with an N-terminal FMOC-piperidine-4-carboxylic acid (pip) as a linker on 300mg of rink amide resin in NMP using standard FMOC amino acid coupling techniques. The resin was equilibrated in anhydrous DCM, and 250µL of 3-(chlorosulfonyl)benzylchloride (tosyl) was added with 450µL of DIEA and shaken for 30 minutes at room temperature. Then, 250µL of 2-(2-(2-aminoethoxy)ethoxy)ethanol (EG), 450µL of DIEA and 19mg DMAP in anhydrous DMC were added and shaken overnight. The resin was washed and equilibrated in NMP, and 2eq Cy5 carboxylic acid (Lumiprobe) was coupled at 37°C overnight

using standard FMOC coupling techniques. The resin was washed, TFA cleaved and HPLC purified as usual; see **Figure 3-4** for image.

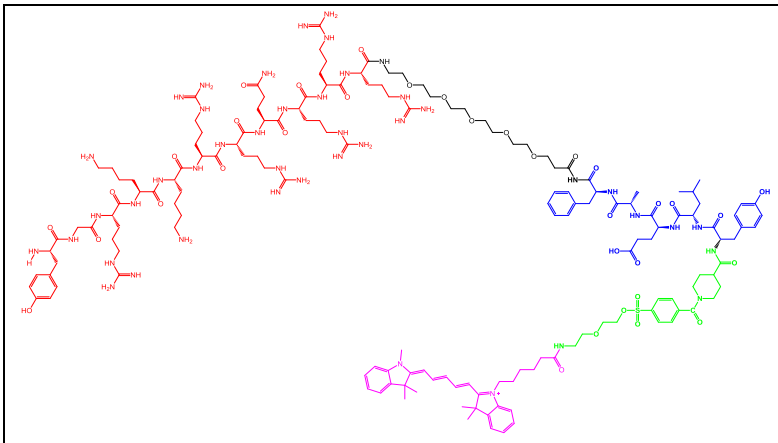


Figure 3-4: TAT – PEG$_5$ – yleaf – pip- tosyl – EG – Cy5: Labeling experiments were performed using a yleaf anchor ligand (blue) that was built onto a TAT peptide (red) with a PEG spacer (black). The labeling arm consisted of a pip spacer, tosyl labeling group, and EG spacer (green) with a Cy5 dye (pink) payload. MALDI-TOF MS, expected m/z = 3316, observed m/z [M+H} = 3317.50.

In order to label the protein, 50µL of full-length GST-E17K PH Domain from SignalChem was treated with 10x molar excess of the anchor ligand with the tosylate dye label and incubated for two days at room temperature. The mixture was lyophilized after two days, and then denatured by boiling in SDS-PAGE loading buffer. The labeled protein was run alongside an unlabeled control on an Any-KD gel (Biorad), then imaged on an Odyssey fluorescent gel reader (**Figure 3-13**). After confirming that labeling had occurred, the gel was stained with BioSafe Coomassie blue stain (BioRad), and the blue protein bands were cut out. The gel pieces were trypsin digested using the Pierce In-gel Digest Kit, following all of the manufacturer's instructions. The tryptic fragments from both the unlabeled and labeled protein digests were lyophilized to concentrate them, taken up in 2µL of 50% H_2O/50% Acetonitrile, and were analyzed by MALDI TOF MS.

Initially, analyses were performed by considering any peak that was present in the labeled protein sample that was not present in the unlabeled sample. The weight of the dye labeling arm – 552.3 g/mol – was subtracted from these peaks, and the corresponding tryptic fragment was located. This provided four potential fragment candidates that were all located near the 33-mer

epitope in the PH domain of the protein. Next, every MALDI peak in the labeled sample was analyzed by subtracting the weight of the dye label and comparing it to a potential tryptic fragment. One other fragment was identified using this method, and corresponded to the doubly labeled peak of one of the previously identified labeled fragments. These results confirmed multiple previous experiments done using LC/MS techniques that proved not strong enough to fragment the tryptic peptides into individual amino acids.

These tryptic peptide samples were then analyzed by MALDI TOF/TOF MS to identify the exact amino acid that contained the dye label. Only YFLLK was able to be successfully fragmented, and the TOF/TOF confirmed that the tyrosine was the label-containing amino acid. This confirms the results, seen in the original publication,[9] that only Y, H, and E nucleophilic amino acids are labeled using this technique. The remaining tryptic fragments all contain at least one of these amino acids, with the doubly-labeled fragment containing two.

The labeling sites were then plotted onto a Pymol image that combined the Akt1 protein (PDB ID: 3096) and the E17K PH Domain (PDB ID: 2UZR) with the N-terminal GST tag (PDB ID: 1UA5) that was present on the full-length protein from SignalChem that was used in these labeling assays. This Pymol-made fusion protein was used to approximate what the commercial protein looked like in solution and give an idea of the extent of the selectivity of this assay. The concentration of labeling sites only surrounding the epitope demonstrate the exclusive binding of this ligand in solution.

3.2.10 Details of the MALDI-TOF Analysis of Tryptic Peptide Fragments

All of the peaks from the MALDI-TOF spectra of the labeled tryptic digests were analyzed for their potential to contain a dye label. The MALDI spectra were manually calibrated to ensure the least possible error. Each peak was then analyzed by zooming in on the spectra on the

computer and obtaining the exact mass for the monoisotopic peak, which is recorded as “MALDI peak” in **Table 3-1** below. The mass of the dye, 552.37g/mol, was subtracted from this peak, and it was compared to the closest possible theoretical tryptic digest fragment (“Digest”). The “expected” mass of the digest plus the dye was calculated and subtracted from the observed mass, “MALDI peak,” and the absolute value of this difference was recorded in “P/M 1.” The peak area was obtained from the MALDI data and added to the spreadsheet as “Peak Area” to allow for a cutoff (4500) of any peaks that looked to be within the noise. Any peak below this value is shown in red italics, and was not considered for this study. Any peak that was within 0.1% of the mass of the expected digest mass was considered to be within error of the instrument, was considered a hit dye-labeled fragment, and colored blue in the table. There were no more new peaks seen using this method than were discovered by looking for peaks that grew in from the unlabeled MALDI to the labeled MALDI. The labeled sites seen in this MALDI-TOF experiment were all seen previously in at least 2 LC/ESI-MS experiments attempting to identify the labeled region.

Table 3-1: Excel Table of Tryptic Fragment Analysis

MALDI Peak	Peak - dye	Expected	Digest	Peak Area	P/M 1	Corresponding Fragment
1053.15	*500.78*	*1051.6349*	*499.265*	*4296.69*	*1.5151*	
1090.15	537.78	*1114.6007*	562.2307	5813.86	24.4507	
1118.11	565.74	*1114.6007*	562.2307	12649.91	3.5093	
1142.16	*589.79*	*1132.6993*	*580.329*	*4217.63*	*9.4607*	
1179.14	*626.77*	*1173.6565*	*621.287*	*4393.2*	*5.4835*	
1194.14	641.77	*1201.732*	649.362	5139.51	7.592	
1202.16	*649.79*	*1201.732*	*649.362*	*4103.69*	*0.428*	
1234.66	682.29	*1234.7826*	682.4126	8193.47	0.1226	YFLLK
1300.08	747.71	*1303.7273*	751.3573	6445.8	3.6473	
1302.09	*749.72*	*1303.7273*	*751.357*	*4496.81*	*1.6373*	
1308.09	755.72	*1303.7525*	751.3825	5926.62	4.3375	
1320.57	768.2	*1320.7691*	768.3991	7886.31	0.1991	EGWLHK
1440.11	887.74	*1447.8246*	895.4546	6406.74	7.7146	
1475.16	922.79	*1477.9158*	925.5458	10131.17	2.7558	
1493.13	940.76	*1477.9158*	925.5458	9276.21	15.2142	
1499.13	*946.76*	*1507.814*	*955.444*	*4112.05*	*8.684*	
1515.1	962.73	*1507.814*	955.444	4687.71	7.286	
1567.65	1015.28	*1565.8591*	1013.489	7907.73	1.7909	
1639.2	1086.83	*1645.9403*	1093.57	21961.13	6.7403	
1707.53	1155.16	*1701.0101*	1148.64	12923.9	6.5199	
1791.09	1238.72	*1795.9606*	1243.591	5200.25	4.8706	
1802.79	1250.42	*1800.0105*	1247.641	8149.76	2.7795	
1851.79	*1299.42*	*1841.9813*	*1289.61*	*4331.77*	*9.8087*	
1995.47	*1443.1*	*1957.1459*	*1404.78*	*4368.22*	*38.3241*	
2212.04	1659.67	*2213.208*	1660.838	95735.94	1.168	EEWTTAIQTVADGLK
2225.51	1673.14	*2213.208*	1660.838	17712.89	12.302	
2233.95	1681.58	*2213.208*	1660.838	12256.12	20.742	
2284.12	1731.75	*2344.242*	1791.872	5711.7	60.122	
2306.92	1754.55	*2344.242*	1791.872	6553.24	37.322	
2344.23	1791.86	*2344.242*	1791.872	4506.1	0.012	EAPLNNFSVAQCQLMK
2383.46	1831.09	*2362.2571*	1809.887	8608.79	21.2029	
2406.7	*1854.33*	*2362.2571*	*1809.89*	*4338.6*	*44.4429*	

The peak at ~2212 was not seen on the unlabeled mass spec, but is seen on the labeled fragment, and was considered a hit. The peak at 2211 is also, however, a common mass seen for trypsin. We do see this particular unlabeled fragment fly in the MALDI-TOF MS (1659), and know that this is a site that can be labeled, based on the ESI-MS experiments that were conducted with a biotin and not Cy5 labeling arm (which therefore have a different labeled mass) that this is a site that can be labeled. In attempting to zoom in for the monoisotopic mass, we see a broad peak with no clearly-identifiable mass peak – unlike all of the other peaks in the spectrum, which showed the distribution of masses very clearly. This led us to believe that we are, in fact, seeing this peak labeled in the MALDI, especially since this site was seen as labeled by the ESI, and that the MALDI spectra is showing an overlap of the trypsin peak with the labeled fragment. The ESI labeling experiments were done using the biotin labeling arm, so this mass did not overlap with trypsin in these experiments, which confirms this. We just cannot exactly call this mass in the MALDI due to the similarity of this peak to that of trypsin.

3.2.11 Images of Anchor Ligand in HEK-293T Cells Expressing PH Domains

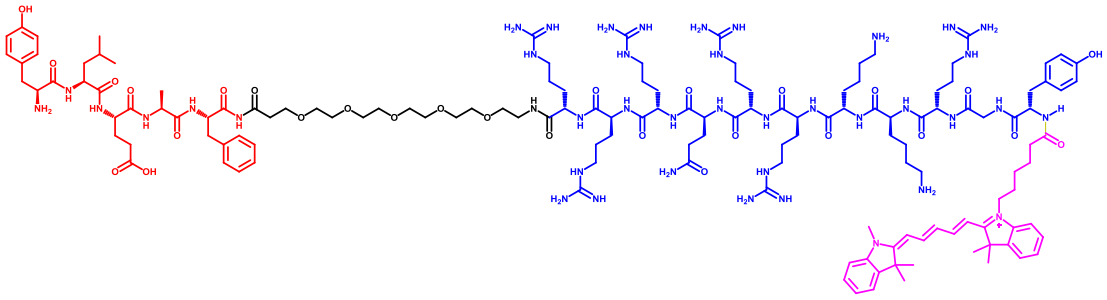


Figure 3-5: yleaf – PEG$_5$ – TAT – Cy5: *The yleaf anchor ligand (red) was appended with a TAT cell penetrating peptide (blue) and a Cy5 dye (pink) separated by a PEG5 linker (black). MALDI-TOF MS, expected m/z: 2937.72, observed m/z = 2937.83.*

These experiments were designed to visualize the dye-labeled anchor ligand in cells overlapping with the GFP-labeled PH Domain proteins. For this reason, the yleaf anchor ligand was synthesized with an N-terminal PEG$_5$, TAT (YGRKKRRQRR), and Cy5 dye (**Figure 3-5**). GFP-

tagged protein DNA was also cloned as described above. HEK-293T cells were grown in DMEM media supplemented with 10% FBS (both Invitrogen), 100x non-essential amino acid solution (Sigma), and PenStrep antibiotic (Invitrogen). Once the cells reached ~80% confluency, they were treated with trypsin to remove from the plate and split into a 12-well flat bottom cell culture plate with a D-poly-lysine (BD) coverslip at approximately a 50% confluency in 1mL total volume. The cells were allowed to attach to the coverslips for approximately 24 hours, then were transfected to express either wildtype GFP-PH domain or E17K mutant GFP-PH domain proteins using XtremeGene HD transfection agent at a ratio of 3:1 transfection agent to DNA. Several wells were left untreated as no protein blanks. The cells were given 24 hours to express protein. They were then serum starved for one hour in DMEM media prepared as above, but without the FBS. After one hour, the Cy5-labeled anchor was added to the wells to a final concentration of 50nM. As the HEK-293T cells express endogenous Akt1 protein, this level was adjusted to give the lowest background signal possible. The protein blank cells were also incubated with 50nM of the yleaf anchor to ensure that binding was due to the presence of the E17K mutant protein. A blank of PEG_5-TAT-Cy5 was also added to wells expressing either wildtype or E17K mutant to ensure that ligand binding was due to the presence of the yleaf anchor. After a one-hour incubation with the peptide, the cells were washed once in serum starved media, then incubated thirty minutes in serum starved media to wash out any excess peptide. During this time, the cells were also treated with 10µg of Hoescht 33342 dye to stain the nuclei. After the thirty-minute period, one well of each wildtype or mutant protein with peptide was activated with PDGF for 10 minutes. The cells were then washed twice with cold PBS buffer, fixed with 10% Neutral Buffered Formalin Solution (Sigma) and glued onto microscope slides. Images were taken on a Zeiss LSM 510 Meta NLO with Coherent Chameleon confocal microscope. A 40x Plan-apochromat lens was used. The laser

intensity and gain were fixed for all pairs of images between wildtype and mutant samples to ensure that the differences seen were not artificially created.

3.3 Results and Discussion

3.3.1 In situ Click Epitope-Targeted Screening Strategy for E17K PH Domain-Specific Ligand

Using FMOC SPPS peptide synthesis techniques,[10] a peptide epitope representing residues 1-32 of the E17K PH Domain of Akt1 was synthesized. From the crystal structure (PDB ID: 2UZR), these residues form a β-sheet around the E17K mutation (blue). The epitope fragment was appended with an N-terminal PEG_5-biotin to serve as a detection handle when screening. This manual synthesis of the epitope allowed for an I19Pra substitution (Pra – propargylglycine) to provide an alkyne click handle on the most proximal side-chain residue to the E17K mutation. Following chromatographic purification, and characterization via mass spectrometry, HPLC and circular dichroism, the modified epitope was ready for screening.

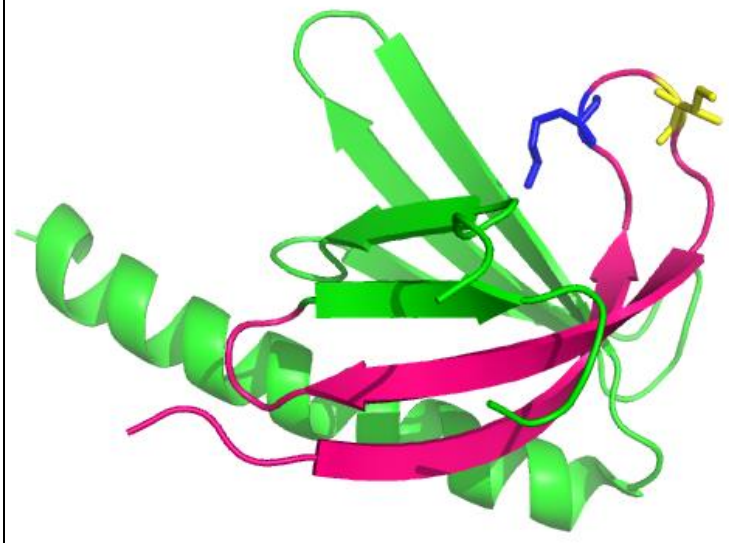


Figure 3-6: Design of Screening Target Epitope: *PH Domain (green) with highlighted screening target epitope (pink). The epitope was designed to surround the E17K mutation shown in blue. The amino acid in position 19 was substituted with a propargylglycine (Pra) alkyne-containing amino acid (yellow) for focusing the site of the in situ click screen.*

A single generation in situ click screen can yield ligands with a high selectivity for the target. Hits from such a screen are those library elements that are covalently coupled to the synthetic epitope through a triazole linkage. The *in situ* click reaction itself is low yielding[12], but the biotin handle on the synthetic epitope permits enzymatic

amplification of those hit beads using a colorimetric streptavidin-linked alkaline phosphatase assay. The basic screening strategy is shown in **Figure 3-2**. Out of the 1.5 million library members that were screened against the alkyne-containing 33-mer E17K PH Domain fragment, only 21 beads (0.0014%) showed the presence of the covalently coupled epitope. These beads were sequenced using Edman degradation (

Table *3-2*, Table 3-3). The hits were segregated based on their hydrophobicity and sequence homology using principal component analysis (**Figure 3-7**). Based upon this analysis, five ligands that represented the diversity of hits (**circled in Figure 3-7**) were scaled-up with a biotin tag and tested for binding to both E17K and WT full-length PH Domain. These hits were dqntr, ypwve, eefef, yleaf, and elnhy. Here, the lowercase sequence letters indicate that the amino acids that comprise the peptide are non-natural D-stereoisomers.

Table 3-2: Hit Sequences from Anchor Screen against 33-mer peptide epitope (5hr click screen):

Az2	G	v	e	k	f
Az8	y	h	e	w	f
Az4	i	s	e	y	e
Az2	p	h	w	l/k	f
Az8	d	l	l	t	f
Az4	a	r	s	d	f
Az8	f	k/l		G	t
Az8	f	e	i	q	
Az8	e	e	p	d/n	f

Table 3-3: Hit sequences from Anchor screen against 33-mer peptide fragment (16hr screen):

Az4	e	e	f	e	f
Az8	f	e	e	a	i
Az2	e	l	n	h	y
Az2	h	a	r	h	q
Az2	h	e	w	v	t
Az4	n	w	y	a	w
Az4	n	l	v	p	n
Az2		r	r	r	f
Az4	a	l	n	s	k
Az8	p		a	y	h
Az2	n	r	y	v	r
Az8	y	l	e	a	f

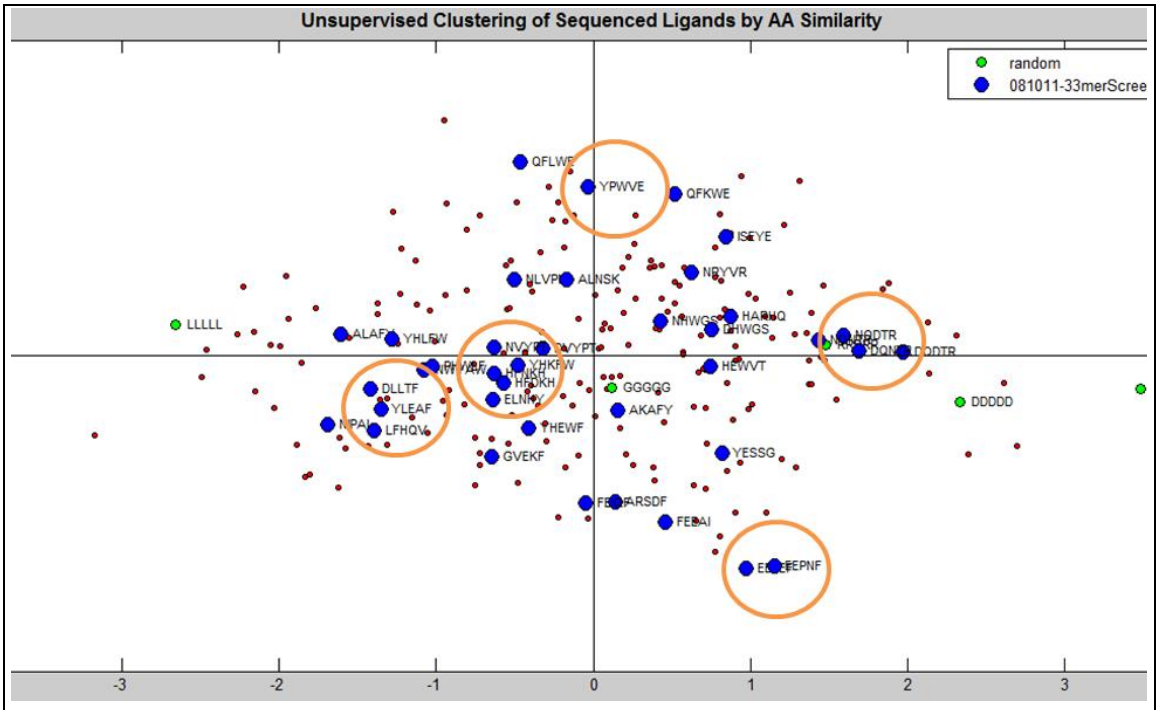


Figure 3-7: Clustering of Anchor Sequence Ligands by AA Similarity: *Hit sequences from the anchor screen were segregated based on their hydrophobicity and sequence homology using principal component analysis. Circled clusters indicate regions where a peptide was selected and scaled-up as a possible anchor sequence. The potential anchor sequences that were tested are: dqntr, ypwve, eefef, yleaf and elnhy.*

Streptavidin – agarose immunoprecipitation assays (**Figure 3-8**) were used to probe for the ability of the anchor candidates to recognize and bind to the proteins in buffer. One ligand

candidate showed a distinctively stronger binding to the E17K protein relative to the WT, seen in Lane 5. This peptide sequence, "yleaf," (**Figure 3-3**) was carried forward for additional investigations. Two out of the four other candidates, though, also showed a preference for the E17K mutant protein. One candidate showed a preference for the WT protein, and one candidate showed no strong binding to either fragment. This result was to be expected, because it is possible for a ligand to bind to the fragment in a way that is not directly accessible on the surface of the full protein. It can also be hypothesized that the three fragments that showed a preference for the E17K fragment bound at or around the site of the mutation, and the peptide that shows binding to the WT protein must bind away from the mutation.

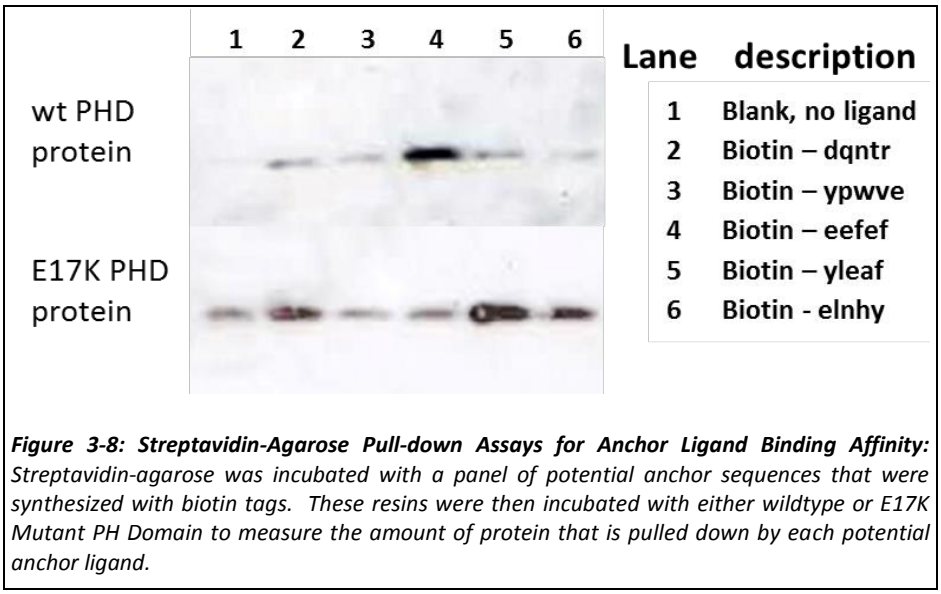


Figure 3-8: Streptavidin-Agarose Pull-down Assays for Anchor Ligand Binding Affinity: *Streptavidin-agarose was incubated with a panel of potential anchor sequences that were synthesized with biotin tags. These resins were then incubated with either wildtype or E17K Mutant PH Domain to measure the amount of protein that is pulled down by each potential anchor ligand.*

3.3.2 CD Spectroscopy of 33-mer Target Peptide Epitope

In order to determine whether the epitope used in the screen would retain the secondary structure of the full protein, CD spectroscopy was performed (**Figure 3-9**). The biotin-tagged, alkyne-containing fragment that was used as a screening target was tested, and the resultant spectra do show the presence of secondary structure. This result is confirmed by the

disappearance of this structural signature upon the addition of a denaturing guanidine-HCl buffer. More importantly, the characteristic dip at 217nM of the blue, fully-natured fragment spectra is the signature of a β-sheet, which is the expected structure of this part of the full protein. We can assume, then, that this fragment is maintaining a structure similar to that of the natured protein, even with the incorporation of the click handle. Therefore, peptide binding to the E17K fragment should see a surface similar to that of the full-length PH Domain protein. The messiness of the spectra from 200 – 210 nm could be due to the biotin label that has been attached to the fragment, or be due to the absence of the rest of the protein, causing random coiling or unfolding.

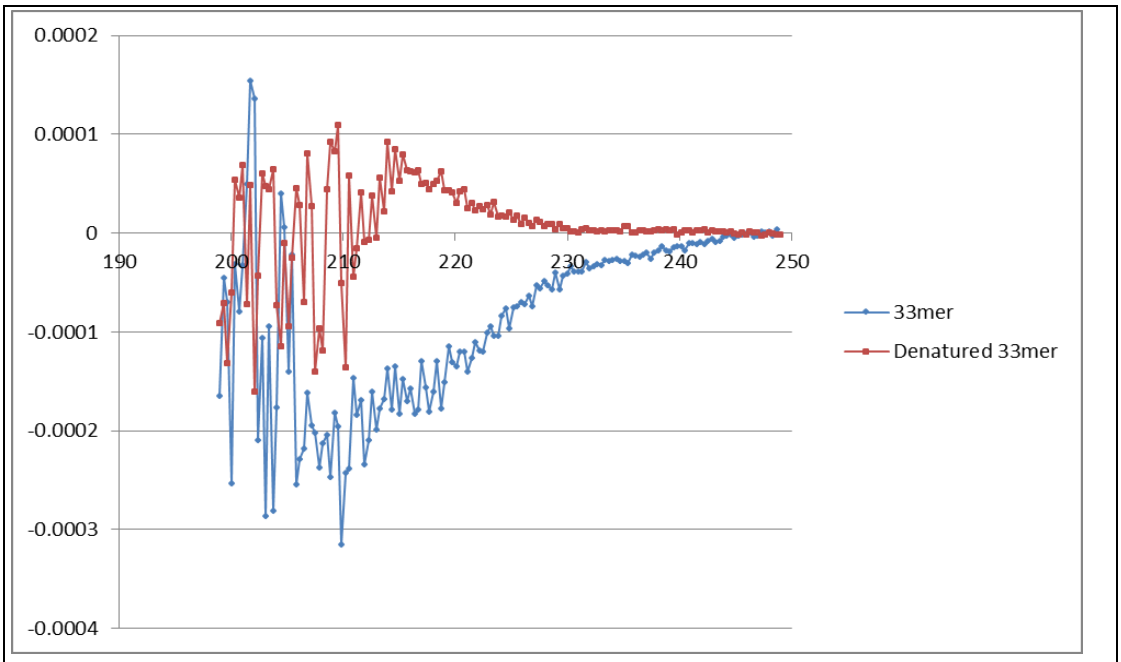


Figure 3-9: CD Spectra of 33mer Epitope Fragment used in Screening: *The blue spectra indicates the 33mer target fragment that was used in screening; the red indicates the denatured epitope.*

3.3.3 Verification of the Epitope Targeting Strategy

The biotin-modified yleaf peptide (**Figure 3-3**) was subjected to a variety of binding assays against the synthesized WT and E17K 33-mer PH Domain fragments prepared without the biotin label and alkyne click handle. These assays were instead labeled with an N-terminal 6-His tag in

order to have an alternative detection handle to the anchor ligand. First, the yleaf peptide was used in immunoprecipitation assays to pull-down either the WT or E17K mutant 6His-tagged 33-mer peptide fragments, as opposed to the full-length proteins that were used to initially validate the candidates. Typical immunoprecipitation assays involve western blotting to estimate the amount of protein binding, but peptide fragments are too small to be consistently captured or quantified on a blot. Because of this, the amount of peptide epitope precipitated in these assays was quantified via injection onto an analytical HPLC. These unique immunoprecipitation assays further confirmed preferential yleaf ligand binding to the E17K mutation relative to the WT epitope (**Figure 3-10**). As an assay control, another candidate ligand that, in initial testing, did not exhibit preferential E17K binding to the full protein (lane 4, **Figure 3-8**), was tested, and yielded consistent results to the full-protein pull-down assays (**Figure 3-8**). The first immunoprecipitation assays demonstrated that the anchor bound selectively to the full-protein, and these HPLC-detected pull-downs confirm that the binding and the selectivity are due to the interaction between the anchor and 33-mer epitope.

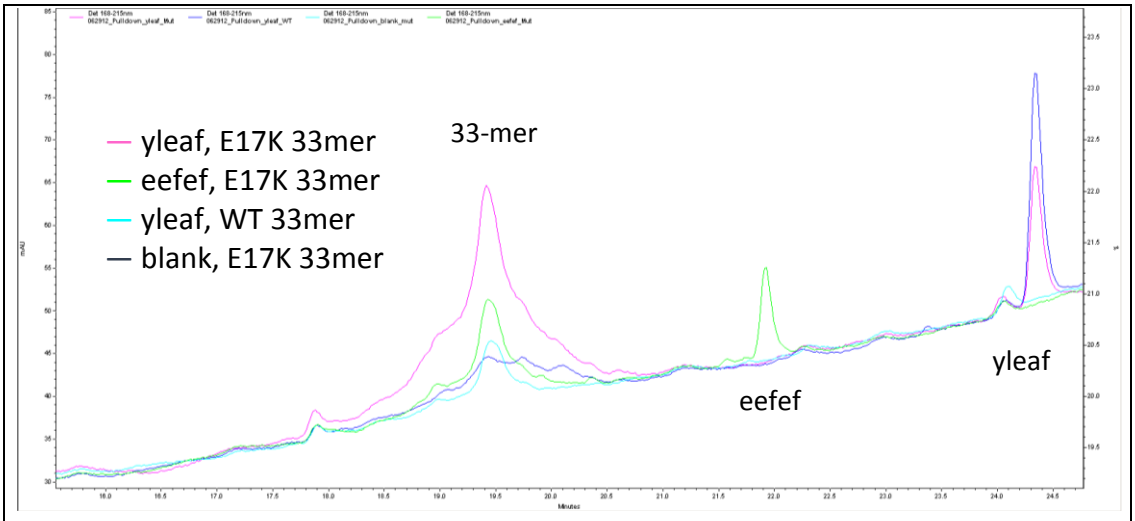


Figure 3-10: HPLC-detected Immunoprecipitation Assay for Epitope Targeting Verification. *The HPLC traces show the quantity of the 33-mer fragment that was pulled-down in the assay. The anchor ligand, yleaf, pulls down the most E17K 33-me,r and the least WT 33-mer. The amount of pull-down by the eefef discarded anchor candidate demonstrates the validity of the assay. This corroborates what was seen in the full-protein pull-downs in Figure 3-8.*

The selectivity of the yleaf peptide for the E17K 33-mer epitope was also tested in an ELISA assay format. For these assays, the WT or E17K 33-mer peptide fragments were captured using the PEG-biotin-modified yleaf ligand immobilized on a Neutravidin-coated plate. The yleaf ligand exhibited significant selectivity for the E17K fragment over the WT across a 100 nM – 1 µM concentration range (**Figure 3-11**), further demonstrating the binding of this ligand to the specific epitope of interest and providing additional validation of the epitope targeting strategy.

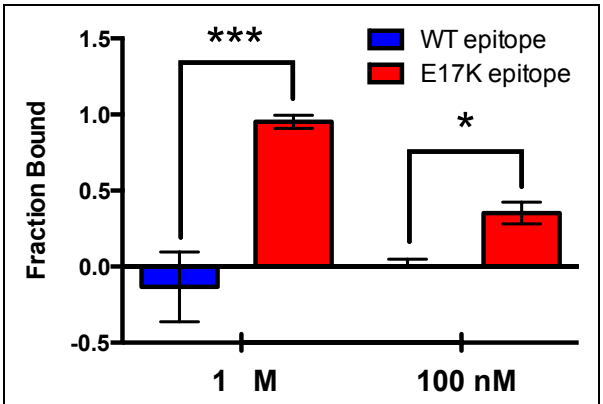


Figure 3-11: ELISA Assay Verification of Epitope Targeting. *The WT and E17K 33-mer fragments were incubated with the yleaf anchor immobilized on an ELISA plate. Strong preference for the E17K fragment is demonstrated.*

3.3.4 Ligand-Directed Labeling Experiment to Confirm Epitope Targeting and Ligand Selectivity

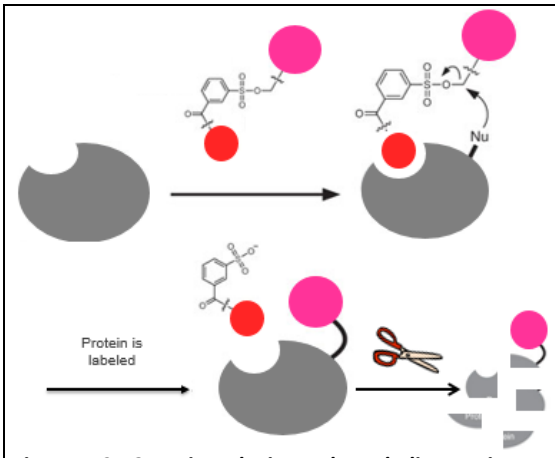


Figure 3-12: Ligand-Directed Labeling Diagram, *modified from Tsukiji et al9. The anchor (red) with the Cy5 dye (pink) attached through an electrophilic tosyl group. The reaction with nucleophilic side-chains on the protein can indicate where the ligand is binding. These sites can be confirmed through trypsin digestion and MS.*

The selectivity of the yleaf anchor ligand was further verified using the directed labeling technique reported by Tsukiji *et al*[9]. The approach yields information relative to the binding location of the ligand on the protein target. For this method, a payload is attached to the N-terminus of the targeting yleaf ligand through an electrophilic tosylate linker. Upon ligand binding to the protein target, the payload is transferred onto the protein through a nucleophilic S_N2 reaction with proximal nucleophilic amino acid side chains (**Figure 3-12**). The protein can then be trypsin-digested, and the identity of the fragments containing the payload can be mapped on the protein surface using mass spectrometry (MS). Thus, the site of ligand binding can be estimated. The assay also serves as an independent validation of the immunoprecipitation and ELISA binding assays discussed above.

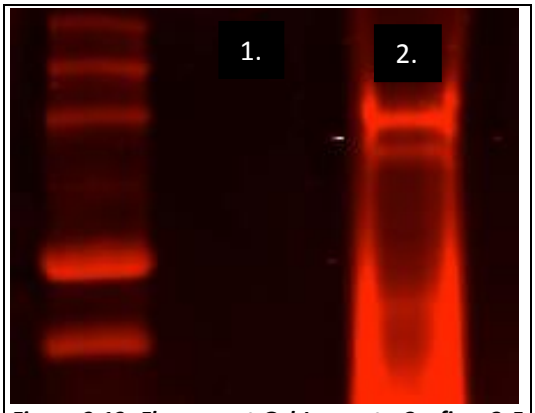


Figure 3-13: Fluorescent Gel Image to Confirm Cy5 Labeling. *Lane 1 shows the control protein with no label, and Lane 2 shows the labeled protein. The large band corresponds to the labeled GST-E17K-Akt1.*

For the assay, yleaf was modified at the N-terminus to contain a tosylate linker attached to a Cy5 dye molecule to enable easy identification of the labeled and digested protein fragments (**Figure 3-4**). A Glutathione S-Transferase (GST)-Akt1(E17K) protein (SignalChem) was incubated with the Cy5-appended yleaf peptide. The labeling of the protein target was initially confirmed by visualization on a fluorescent gel reader

(**Figure 3-13**). The labeled protein and an unlabeled control were then trypsin-digested from the gel, and were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (**Figure 3-14**). Five peaks appeared in the MS of the labeled protein that were not present in the unlabeled protein digests, which all corresponded to an expected trypsin fragment plus the weight of the linker and dye.

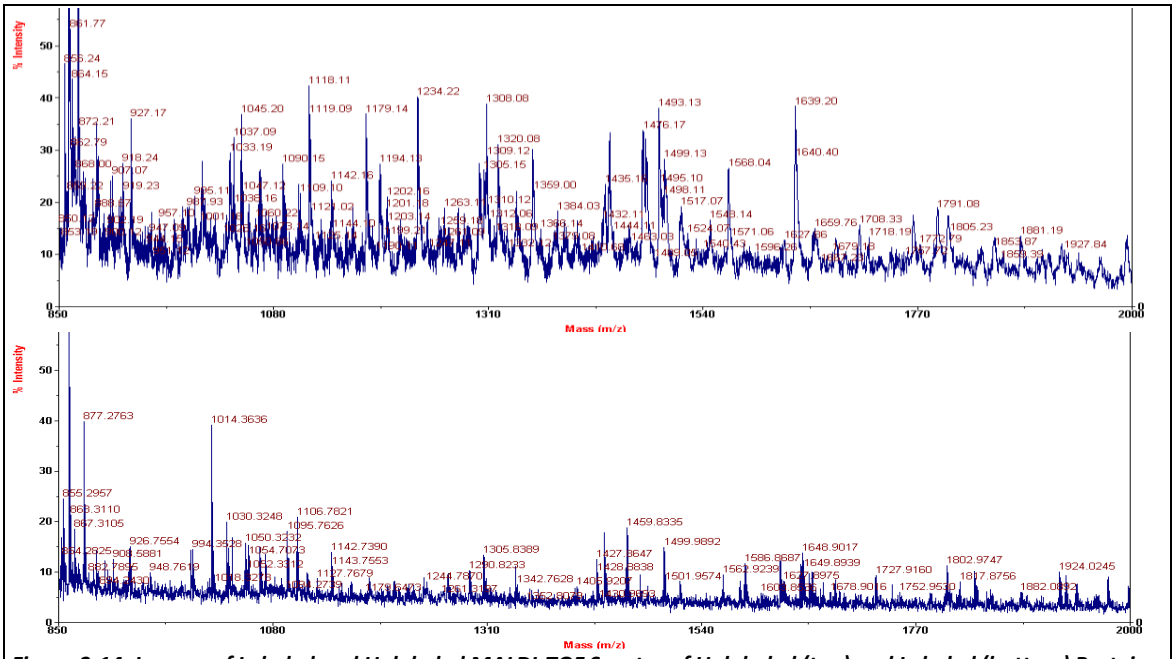


Figure 3-14: Images of Labeled and Unlabeled MALDI-TOF Spectra of Unlabeled (top) and Labeled (bottom) Proteins.

These peaks were then analyzed by MALDI TOF/TOF MS to extract sequence information for the labeled regions of the protein. All but one of the dye-labeled peptides were difficult to fragment, as is characteristic of cationic peptide labels[11]. The labeled digest YFLLK could be fragmented, and indicated the presence of the dye on the Y amino acid (**Figure 3-15**). This is consistent with the original literature on the labeling technique,[9] which showed that Y, E and H amino acids are the nucleophiles that can be labeled. The other labeled Akt1 fragments that were identified contain at least one of these amino acids. One fragment contains two such amino acids and, in fact, there were MALDI peaks corresponding to the masses of both the singly-and doubly-

labeled fragments. **Figure 3-16** shows the location of the labeled fragments in the PH Domain sequence, as well as the amino acids that should contain the label.

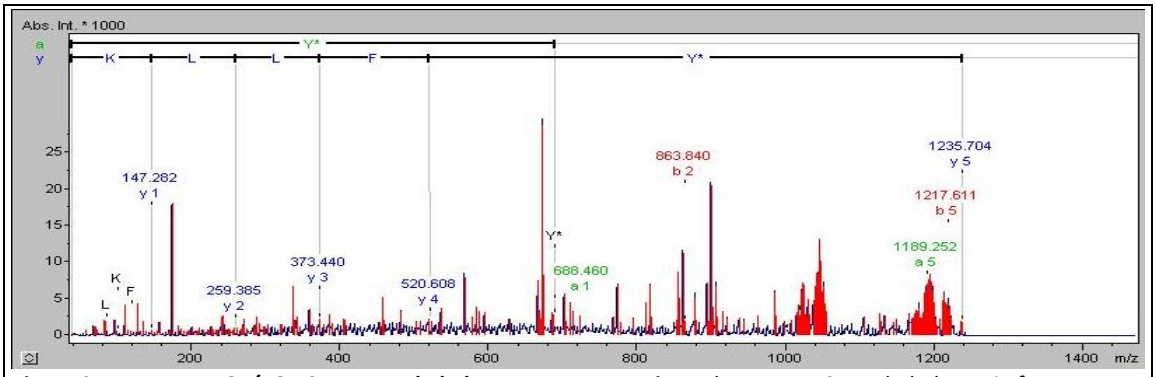


Figure 3-15: MALDI-TOF/TOF Cy5 Dye-Labeled YFLLK Fragmentation: The YFLLK – Cy5 Labeled trypsin fragment analyzed by MALDI-TOF/TOF MS. The fragments shown above demonstrate that the Cy5-dye is on the Y amino acid, which corresponds to the results found by authors of the original technique[9].

The labeling sites were then mapped on a composite crystal structure of GST (PDB ID: 1UA5) and Akt(E17K) (Akt PDB ID: 3096, E17K PDB ID: 2UZR) (**Figure 3-17**). All labeled sites surround the anticipated binding site of the yleaf ligand. A thorough search of the entire MALDI spectra was conducted to identify any other labeled fragments anywhere on the large protein, but none were found. Thus, this labeling experiment demonstrates that only sites around the expected N-terminal binding site of the yleaf ligand are labeled, confirming the very specific binding of the peptide ligand at the site directed by the epitope-targeted in situ click screening process.

MSDVAIVK|EGWLHK|R|GK|YIK|TWR|PR|YFLLK|
NDGTFIGYK|ER|PQDVDQR|EAPLNNFSVAQCQLMK|TER|
PR|PNTFIIR|CLQWTTVIER|TFHVETPEER|
EEWTTAIQTVADGLK|K|QEEEEMDFR|SGLVPR|GSAG

Figure 3-16: Trypsin-Digested Sequence of PH Domain Protein. *The red dashed lines show where the trypsin cuts are located. The fragments highlighted in red are the ones that showed labels, and the amino acids colored in cyan are the ones containing the labels.*

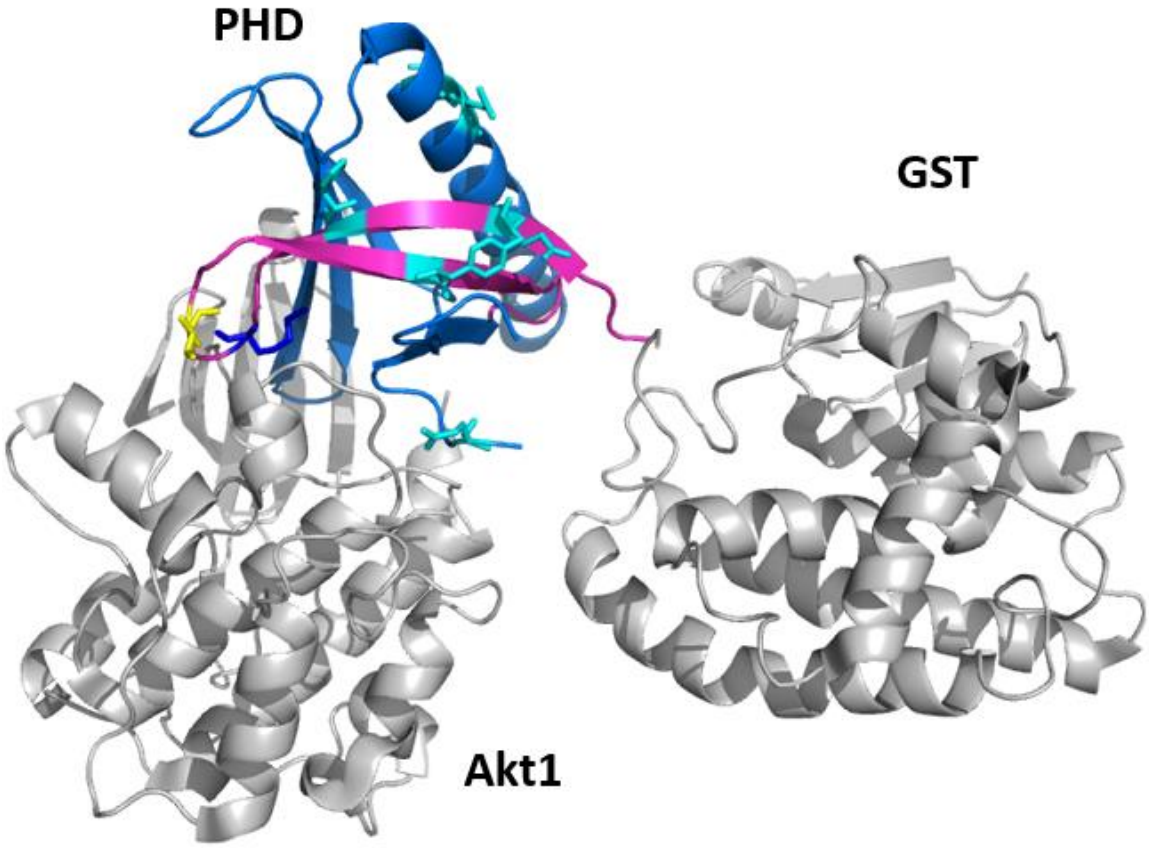


Figure 3-17: Compiled Crystal Structure of Fully Labeled Protein*. The cyan amino acids labeled in cyan are all clustered immediately around the pink 33-mer fragment of the blue PH Domain. No labeled sites were found in any other part of the large fusion protein.*

3.3.5 In Cell Imaging

Live cell-based assays can provide a demanding environment for demonstrating the selectivity of the yleaf PCC agent to the E17K Akt1. In addition, they also can demonstrate the value of a small, epitope targeted ligand relative to a similarly targeted antibody, since antibodies cannot enter live cells. To demonstrate target binding in live cells, HEK-293T cells were transfected to express GFP-tagged E17K or GFP-tagged WT PH Domain proteins. The yleaf ligand was then labeled with both a Tat cell-penetrating peptide and a Cy5 dye (**Figure 3-5**). The combination of the GFP label on the protein, and the Cy5 label on the dye, permitted the use of multi-color fluorescence microscopy for interrogating any spatial registry between the two

fluorescent labels. Live HEK-293T cells expressing these GFP-tagged proteins were exposed to varying concentrations of the modified yleaf ligand for one hour. The cells were then thoroughly washed with PBS to equilibrate the concentration, and fixed for fluorescence microscopy measurements.

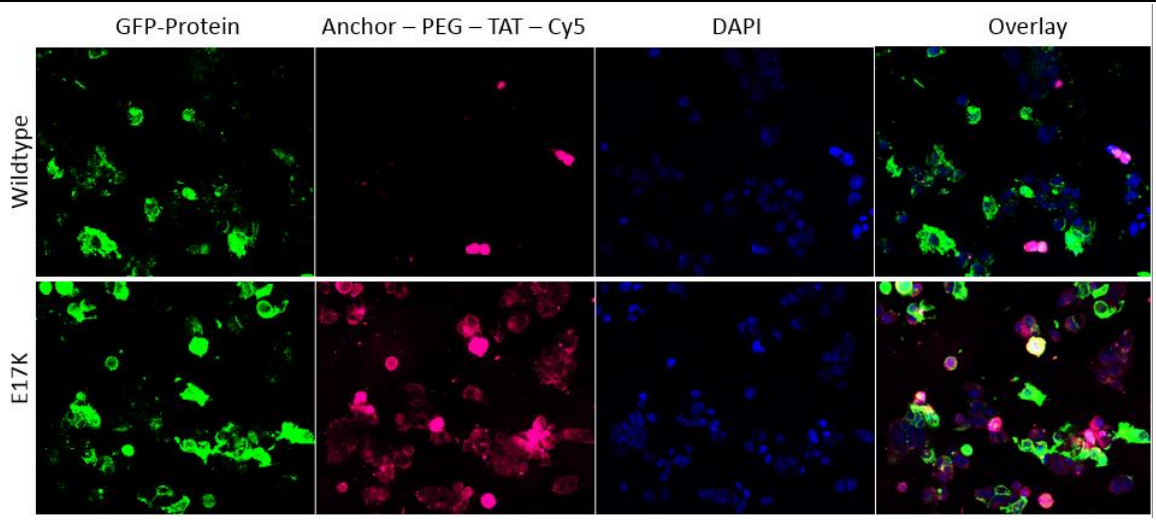


Figure 3-18: Images of Anchor Ligand in GFP-tagged WT and E17K PH Domain. *The green fluorescence shows the equivalent expression of the WT and Mut proteins in cells. The pink anchor ligand is distributed throughout almost all of the E17K – expressing proteins, but shows only background binding to the WT – expressing cells.*

Confocal microscopy images of the two differentially-expressing Akt1 PH Domain cells showed a consistent level of expression between the GFP-WT PH Domain and GFP-E17K PH Domain. However, the level of the PCC agent retained by the cells was substantially different (**Figure 3-18**). Nearly all of the cells expressing the mutant protein show some level of capture agent retention and demonstrate co-localization of capture agent and GFP-PH Domain protein (**Figure 3-19**). The GFP-WT cells, however, show very low levels of capture agent retention, and do not

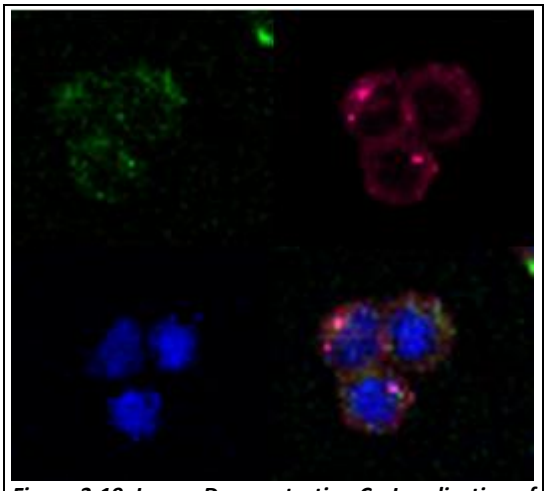


Figure 3-19: Image Demonstrating Co-Localization of Cy5 - *PCC Agent with GFP - tagged PH Domain proteins in cells.*

seem to have any co-localization of the two. These measurements demonstrate the selectivity of the E17K capture agent for its target within the demanding environment of live cells.

3.4 Conclusions

The *in situ* click-focused epitope screen for capture agent development presents a rapid strategy for discovering peptide ligands that bind to any site of interest on a protein surface. This method is not limited to conserved binding pockets, post-translational modifications, or structured regions of proteins. By only accepting hits wherein the target epitope catalyzed the formation of a covalent bond, it was ensured not only that the candidate ligands bound to the site of interest, but that they also bound tightly and in an exact orientation so that this triazole could form. In this way, the *in situ* click screen became not only a screen for ligand affinity, but also for ligand specificity. The peptide hits developed using this method are very specific for the exact location on the protein where the click reaction was centered. The PCC Anchor, yleaf, developed using this technique has demonstrated the ability to detect the E17K mutant PH Domain in conditions ranging from simple assays in buffer to complex imaging experiments in cells. Assays validating the peptide-peptide binding of the epitope target and the yleaf anchor ligand also highlight the exquisite selectivity of this ligand for the E17K mutation. This technology provides an ideal solution for the discovery of selective ligands to an area of interest on a protein surface, and demonstrates the ability to produce agents capable of distinguishing even the slightest change in protein structure – a single point mutation.

3.5 Acknowledgements

The labeling and imaging experiments shown were done with Blake Farrow. Steve Millward and Aiko Umeda assisted with the protein expression and cell culture. Ying Qiao Hee and Jeremy Work made many of the peptides used in these experiments. Bert Lai performed the

MALDI-TOF/TOF sequencing of the tryptic fragments. Jost Vielmetter, Angela Ho, and Sravya Keremane of the Protein Expression Center were indispensable in the expression of these proteins. Felicia Rusnak and Jie Zhou performed the trypsin digests and LC/MS for the protein labeling experiments. The advice from Mona Shahgholi on MS techniques and experimental set-up was essential to the success of the labeling experiments.

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