NEW APPROACHES FOR CONTROL AND VISUALIZATION OF PROTEIN
CONFORMATION IN LIVE CELLS.

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ABSTRACT

(Under the direction of Klaus M. Hahn)

The organization of protein activity in space and time is an essential but poorly understood aspect of cellular signaling. This has begun to change with development of molecular tools that enable visualization or control of protein activity with high spatial and temporal precision. Visualization has been accomplished with small protein domains (affinity reagents) that bind selectively to active proteins, while control has been achieved with light-responsive domains that change a target protein’s localization or active site exposure following irradiation with specific wavelengths of light. However, currently available methods suffer from limitations that have prevented their application to many important biological problems. This dissertation describes two strategies to support broader application of tools for visualization and control of protein activity in live cells. Suitable affinity reagents are not available for many proteins, and even when they are, they generally require extensive optimization and can perturb their target protein’s functionality. In the first study, I detail a new method to generate affinity reagents for large multi-domain proteins by leveraging a high affinity and highly specific protein-peptide interaction. This method provides a streamlined approach to generating absolutely specific affinity reagents with minimal perturbation for a wide range of target proteins. We also demonstrate how our approach can be applied to visualize protein conformation at the single-molecule level. A major challenge in developing light-controlled protein analogs is achieving
proper positioning of the attached light-responsive domain to effectively block a target protein’s active site. In the second study, I demonstrate how an engineered protein scaffold can be used to help position light-responsive domains to effectively control target protein activity.
To my family, especially Quyen Tang, Ann Stone, Orrin Stone, and Rachel Stone.

Your love and support made this possible.
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LIST OF ABBREVIATIONS

ADF    Actin Depolymerizing Factor
AID    Autoinhibitory Domain
BCR    B Cell Receptor
C      Celsius
CFP    Cyan Fluorescent Protein
CFP1   Colony Stimulating Factor 1
CO2    Carbon Dioxide
CSK    C-terminal Src Kinase
DH Domain Dbl Homology Domain
DIC    Differential Interference Contrast
Dlg1   Drosophila Disc Large Tumor Suppressor
DPBS   Dulbecco's Phosphate-Buffered Saline
ECM    Extracellular Matrix
EGF    Epidermal Growth Factor
EGFR   EGF Receptor
EM     Electron Microscopy
ePDZ   Enhanced PDZ Domain
F-actin Filamentous Actin
FACS   Fluorescence-activated Cell Sorting
FAK    Focal Adhesion Kinase
FBS    Fetal Bovine Serum
FCS  Fluorescence Correlation Spectroscopy
fMLP  N-Formylmethionine-leucyl-phenylalanine
FRET  Förster Resonance Energy Transfer
GFP  Green Fluorescent Protein
GPCR  G-protein Coupled Receptor
GTP  Guanosine Triphosphate
h  hour
HCl  Hydrochloric Acid
HDX  Hydrogen Deuterium Exchange
HEK293  Human Embryonic Kidney Cells 293
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG  Isopropyl β-D-1-thiogalactopyranoside
KD  Equilibrium Dissociation Constant
kDa  Kilodalton
LED  Light Emitting Diode
LEGI  Local Excitation Global Inhibition
LIM Domain  Lin11, Isl-1 & Mec-3 Related Domain
LIMK  LIM domain kinase 1
LOV2 domain  Light-Oxygen-Voltage domain
m  Meter
MEF  Mouse Embryonic Fibroblast
MEM  Minimum Essential Media
mL  Milliliter
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MTLn3</td>
<td>Metastasis to Lung Number 3 Rat Cell Line</td>
</tr>
<tr>
<td>NaBH4</td>
<td>Sodium Borohydride</td>
</tr>
<tr>
<td>NHE1</td>
<td>Sodium/Hydrogen Exchanger 1</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>nM</td>
<td>Nanomolar</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NT</td>
<td>Non-transfected</td>
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<tr>
<td>PA-FP</td>
<td>Photoactivatable Fluorescent Protein</td>
</tr>
<tr>
<td>PA-GFP</td>
<td>Photoactivatable GFP</td>
</tr>
<tr>
<td>PALM</td>
<td>Photoactivated Localization Microscopy</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Database</td>
</tr>
<tr>
<td>PDZ Domain</td>
<td>PSD95, Dlg1, and Zo-1 Related Domain</td>
</tr>
<tr>
<td>PI(4,5)P2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase C-gamma</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>Post-PA</td>
<td>Post-photoactivation</td>
</tr>
<tr>
<td>Pre-PA</td>
<td>Pre-photoactivation</td>
</tr>
<tr>
<td>PSD95</td>
<td>Post Synaptic Density Protein</td>
</tr>
<tr>
<td>PSF</td>
<td>Point Spread Function</td>
</tr>
<tr>
<td>Rcf</td>
<td>Relative Centrifugal Force</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartate Integrin Binding Motif</td>
</tr>
<tr>
<td>RhoGEF</td>
<td>Rho Guanine Nucleotide Exchange Factor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>RNAi</td>
<td>RNA Interference</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SASA</td>
<td>Solvent-accessible Surface Area</td>
</tr>
<tr>
<td>sCMOS</td>
<td>Scientific Complementary Metal Oxide Semiconductor</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SH2</td>
<td>Src Homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src Homology 3</td>
</tr>
<tr>
<td>SIM</td>
<td>Structured Illumination Microscopy</td>
</tr>
<tr>
<td>smFRET</td>
<td>Single Molecule FRET</td>
</tr>
<tr>
<td>SPT</td>
<td>Single Particle Tracking</td>
</tr>
<tr>
<td>SSH</td>
<td>Slingshot Homolog</td>
</tr>
<tr>
<td>SspB</td>
<td>Stringent Starvation Protein B</td>
</tr>
<tr>
<td>STED</td>
<td>Stimulated Emission Depletion</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen Tyrosine Kinase</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-associated Macrophage</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total Internal Reflection Fluorescence</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated Phosphoprotein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>YF</td>
<td>Src Active Mutant Tyr527 to Phe</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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</table>
Zdk  
Z-ffibody Targeting LOV2 Dark State

Zlock  
Zdk-mediated Locking of LOV2 Orientation

Zo-1  
Zonula Occludens-1 Protein

µL  
Microliter

µM  
Micromolar
CHAPTER 1  STUDYING CELL ADHESION AT THE MESOSCALE

Focal adhesion research has made tremendous progress over the past century. Much of this progress has been driven by advances in technology that have enabled researchers to ask new questions about the underlying structure and function of focal adhesions. Super-resolution and single-molecule microscopy, arguably the biggest such advance in the past decade, is providing unprecedented views of molecular organization in focal adhesions below the diffraction limit. However, currently available methods are limited to measuring protein localization and cannot detect changes in protein conformation. In chapter two, I describe a new method to visualize protein conformation at the single-molecule level in live cells, which will enable new studies of how protein conformation is regulated inside focal adhesions. Here I provide an overview of our current understanding of adhesions and how super-resolution and single-molecule methods has shaped that understanding.

1.1 Introduction to “mesoscale” biology

The term “mesoscale” has found wide use in a variety of scientific fields ranging from meteorology to solid-state physics, and more recently, biology. Mesoscale refers to phenomena of intermediate size and is thus necessarily ambiguous outside the context of a particular field of research. In the context of cell biology, mesoscale generally refers to phenomena and structures between 1 nm and 1 um – roughly the size of protein complexes on the lower end and subcellular organelles such as mitochondria on the upper end.¹ The adoption of the term has paralleled an
increasing realization among cell biologists that future progress in our understanding of fundamental cellular processes will require investigation of this little studied realm.\textsuperscript{2} This realization has been fueled by studies that have hinted at the existence of cellular organization on this scale and its importance in a range of cellular processes. For example, early studies of integrin ligand spacing found that focal contact and stress fiber formation required a minimum ligand spacing of 140 nm.\textsuperscript{3} Notably, this is orders of magnitude larger than individual proteins, and therefore indicates the existence of higher-order cellular structures that mediate recognition of integrin ligand spacing. While the existence of mesoscale cellular organization can be reasonably inferred from findings such as these, direct observation of these structures has generally been beyond the reach of traditional experimental approaches, and efforts to infer their specific characteristics have generally been unsuccessful due to the emergent nature of these phenomena. However, recent developments in microscopy have enabled pioneering studies into this realm, which have directly observed mesoscale cellular organization and structures with unanticipated characteristics.

1.2 Cellular adhesion at the mesoscale

Cell-matrix adhesion is a valuable lens through which to explore the importance of mesoscale biology. However, it is important to review the past few decades of adhesion research to better understand how and why the mesoscale has emerged as the next frontier in cell adhesion research. Cell-matrix adhesion is largely mediated by the integrin family of transmembrane receptors, which serve as the primary link between the cytoskeleton and the extracellular matrix. Integrins serve as signaling hubs that sense and transmit information about the surrounding environment via interaction with numerous intracellular proteins, which connect integrin
signaling to a wide array of biological processes including cell survival, proliferation, motility, and differentiation. Misregulation of adhesion is also a factor in many disease states. Mutations in classical adhesion proteins such as vinculin, integrin, tensin, FAK, parvin have been implicated in numerous diseases affecting almost all bodily systems including the immune, cardiovascular, and digestive systems. Additionally, numerous cancers and neurological disorders have been associated with defects in cellular adhesion.

1.3 Adhesion research at the molecular-level

Historically, cell adhesion has been studied across several scales ranging from the molecular to the cell to the whole organism. Early biochemical and immunofluorescence studies in the 1980’s and 1990’s identified several cytoskeletal proteins that bound to integrin and colocalized with adhesions, laying the groundwork for the idea that adhesions are macromolecular signaling platforms that link the extracellular matrix to the actin cytoskeleton. More recently, proteomic studies of isolated focal adhesions have identified over a thousand different proteins associated with adhesions. Interestingly, adhesion’s constituent proteins differ depending on cell type and culture conditions, hinting at the incredible diversity and complexity of adhesions. Bioinformatic analysis of the data from these proteomic studies has yielded functional and protein-protein interaction maps, which highlighted the fact that many interactions between adhesion components are mutually exclusive. This hinted at further diversity in focal adhesions and painted a picture of adhesions as large-scale protein interaction networks that are dynamically modulated depending on cellular context, which enable the cell to respond to a variety of external stimuli. These studies are characteristic of the molecular-scale approach to
understanding adhesion, where the focus is on identifying the specific molecules that comprise adhesions and how they interact with each other.

1.4 Adhesion research at the cell-level

Adhesion has also been studied at the level of the cell, with approaches that rely heavily on immunofluorescence and fluorescent protein tagging strategies (reviewed in a later section) to localize specific components of adhesions in live and fixed cells. A series of pioneering studies in the 1990’s and 2000’s discovered the dynamic nature of adhesions and their incredible molecular and morphological diversity in different subcellular contexts. They found that adhesions are formed at the leading edge of a migrating cell in a process linked to integrin activation and clustering. Initially these early adhesions (nascent adhesions) are relatively small and contain few components.\textsuperscript{15,16} However, a select population of these nascent adhesions mature into focal adhesions at lamellipodia-lamellum interface in a tension-dependent process.\textsuperscript{17} In contrast to adhesion formation, disassembly occurs both at the cell front and rear, and appears to be differentially regulated depending on subcellular context.\textsuperscript{18,19} Adhesion disassembly occurs when upstream signaling by kinases, phosphatases, and proteases alter the relative rate of adhesion-component association and disassociation to favor disassembly.\textsuperscript{20} The process of adhesion formation, maturation, and disassembly is characterized by a marked change in the composition and morphology of adhesions. Adhesions form posterior to an actin-, VASP-, and alpha-actinin rich region in the lamellipodia. These nascent adhesions appear as tiny foci (500 nm – 1 µm) with high levels of phosphotyrosine, β3-integrin, talin, paxillin, and low levels of vinculin and FAK.\textsuperscript{21} Adhesion maturation is associated with elongation and a loss of phosphotyrosine and paxillin.\textsuperscript{22,23} During maturation, adhesions become linked to actin cables.
that mediate contractility during cell migration. Tensin and zyxin, which are notably absent from newly formed adhesions, are highly enriched in mature fibrillar adhesions. While these studies provided a wealth of information on the molecular content and evolution of different types of adhesions, the specific organization and orientation of the proteins found in adhesions largely remained a mystery. A notable exception were several studies that utilized Förster resonance energy transfer (FRET) to measure the distances between proteins in adhesions. One such study found that FAK is found in adhesions in close proximity to paxillin and p130Cas. While these studies could not discern the precise organization of these proteins, they provided further evidence for the existence of mesoscale organization and structure in focal adhesions.

Adhesion formation is predominantly regulated by Src and FAK, as well as the scaffold proteins talin and paxillin. Adhesion tyrosine phosphorylation is associated with adhesion formation and turnover, whereas dephosphorylation is associated with maturation and stabilization. For example, paxillin phosphorylation by Src/FAK is required for focal complex formation and also stimulates lamellipodial protrusion. Studies utilizing cross-correlated fluctuation analysis have shown that nascent adhesions form containing tetrameric complexes of FAK and paxillin in a 1:1 stoichiometry, and that formation of these complexes requires paxillin phosphorylation by Src/FAK. In addition to tyrosine phosphorylation by Src/FAK, adhesion formation also depends on the scaffolding of adhesion components by talin and paxillin. Recently, several groups have begun to examine the mesoscale organization of Src. One used a combination of time-resolved fluorescence fluctuation spectroscopy and super-resolution microscopy to examine the organization of Src in different plasma membrane domains including adhesions. They found that a small fraction of Src forms transient clusters with an average size of 10 – 80 nm. Another group analyzed the single-molecule dynamics of a series of Src mutants
and found that mutations in specific Src domains differentially affected its mobility in adhesions vs the rest of the plasma membrane.\textsuperscript{32} They also found that Src kinase activity was important for its diffusive properties in adhesions, but not other areas on the plasma membrane. Another group used SPT to directly visualize the Src-family kinase Lck, which is expressed exclusively in hematopoietic cells, as it exchanged between the plasma membrane and the cytosol.\textsuperscript{33}

1.5 Evidence for the existence and importance of mesoscale organization in adhesions

Studies of adhesions at the molecular and cellular level have demonstrated that adhesions are macromolecular assemblies containing a diverse array of different proteins that interact with each other in a variety of manners. And while quantitative proteomic or data mining approaches have been useful for generating hypotheses about how these proteins interact to regulate adhesions, they cannot predict the subcellular location, duration, strength, and functional outcome of their interactions. Therefore, it is critical to be able to resolve the structure and dynamics of focal adhesions at the scale of its constituent proteins. The importance of understanding focal adhesion organization at the scale has been highlighted by several studies that have found that nanoscale spacing and density of integrin ligands is an important determinant of adhesion function. One study utilizing nanopatterned fibronectin dots of various size found that changes in dot size on the order of hundreds of nanometers produced profound differences in cell shape and commitment to differentiation in epidermal stem cells.\textsuperscript{34} Another study utilized nanoparticles coated with RGD peptide (integrin recognition motif) and varied integrin ligand spacing from 30 to 120 nm, which found that melanoma cells attachment and spreading was optimal at a ligand spacing of 60 nm, with sharp reductions in cell attachment and spreading with either lower or higher ligand spacing.\textsuperscript{35} Findings such as these supports the
existence of precise nanoscale structures that mediate recognition of integrin ligands and suggests that resolving these structures may provide new insights into adhesion function.

1.6 Development of super-resolution and single-molecule microscopy

In the past decade, tremendous progress has been made toward the goal of directly observing nanoscale and mesoscale structures in their native cellular contexts. This progress has stimulated an incredible interest in this area and a large number of studies have been published, which have been extensively reviewed elsewhere.\textsuperscript{36} For the purposes of this introduction, we will focus on methods used extensively in the field of adhesion research.

1.7 Fluorescence Correlation Spectroscopy

An early advance came with the development of fluorescence correlation spectroscopy (FCS), which measures fluctuations in fluorescence intensity from a focused laser spot in a live cell that arise due to molecules diffusing through the laser beam.\textsuperscript{37,38} This technique has been used extensively in the context of adhesions to investigate the dynamics of individual adhesion proteins and small complexes in live cells. One recent study found that the integrin adhesome is extensively preassembled in the cytosol into small complexes of at most three proteins.\textsuperscript{39} While FCS has provided a wealth of information on interactions between adhesion proteins, it relies on averaging many molecules and cannot track individual molecules, and it cannot precisely localize populations of molecules to uncover potential high order structures.
1.8 Single-Molecule Tracking

In contrast to FCS, single particle tracking (SPT) enables tracking of individual molecules to generate diffusive trajectories that can reveal important biological information about the tracked molecule. An important milestone in SPT was the first detection of a single fluorescent molecule at room temperature.\(^4\) This set the stage for future use of fluorescent molecules as tags to visualize proteins and other biological molecules, both in vitro and in live cells. An early example used fluorescently-labeled lipids to visualize the diffusion of individual lipid molecules in the plasma membrane of live cells.\(^4\) The development of green fluorescent protein (GFP) and other related fluorescent proteins provided another importance advance by enabling genetic labeling of proteins with fluorescent tags.\(^4\) GFP was later discovered to have switching behavior that enabled fluorescence to be turned on and off with UV light.\(^4\) Mutagenesis was used to enhance this property resulting in a GFP derivative (PA-GFP) that underwent a 100-fold increase in fluorescence upon UV stimulation.\(^4\) Further developments in photoactivatable fluorescent proteins, as well as improved optical and detection technology, enabled researchers to photoactivate a small subset of labeled proteins and thereby track them individually, greatly simplifying the process of SPT in live cells.\(^4\) One study utilized photoactivatable fluorescent proteins to track individual integrin molecules as they diffused within focal adhesions.\(^4\) They found that integrins underwent cycles of free diffusion and immobilization, and that immobilization was dependent on actin binding proteins.

1.9 Super-resolution Microscopy

The development of SPT enabled visualization of individual molecules in live cells, which provided important information about the behavior of proteins in their native context.
However, an important limitation of SPT is that it can only visualize a small fraction of the total population. As such, it is generally unable to uncover higher order structures that arise from the entire population. Studies utilizing fluorescent proteins revealed the dynamic partitioning of adhesion components into different subcellular locations, but detailed information about their organization inside individual adhesions remained lacking due to poor spatial resolution. This changed with the development of super-resolution microscopy, which enabled spatial resolution in optical imaging beyond the diffraction barrier originally described by Abbe.\textsuperscript{48} In the mid-2000’s, a number of techniques were published that described imaging with resolution beyond the diffraction barrier including: stimulated emission depletion (STED), structured illumination microscopy (SIM), and photoactivated localization microscopy (PALM).\textsuperscript{49} Similar to SPT, PALM relies imaging individual fluorophores at low density such that they appear as diffraction limited spots, which can be super-localized (2 – 25 nm) using the known point spread function (PSF) for the microscope or approximated with a Gaussian function for simplicity.\textsuperscript{50}

In the original paper describing PALM, super-resolution images of the focal adhesion protein vinculin revealed web-like networks inside of adhesions that were not resolvable in diffraction-limited TIRF images. In a paper published shortly after, the same group found that several pairs of adhesion proteins that appeared to co-localize by conventional microscopy were in fact spatially segregated with very little overlap.\textsuperscript{51} Another paper determined the orientation of talin dimers relative to vinculin in live cells, an initial example of how super-resolution can provide cellular context to protein-protein interactions described in biochemical studies.\textsuperscript{52} While PALM achieved remarkable resolution in the x-y axis, resolution in the z-axis remained limited. This changed with the introduction of interferometric photoactivated localization microscopy (iPALM), which combined PALM with single-photon, simultaneous multiphase interferometry.
to achieve sub-20-nm localization of proteins in three-dimensions. Application of iPALM to focal adhesions discovered that adhesion proteins are highly organized in 3D and different proteins partition into discrete layers from membrane proximal proteins such as integrin and paxillin, to more distal proteins alpha-actinin and VASP. The regulation of vinculin was further studied with iPALM, which was found to translocate upwards during activation in a process linked to talin. These studies and others have started to understand how individual adhesion molecules organize themselves inside adhesions, which should help drive a deeper understanding of how interactions between adhesion components, originally identified in biochemical and proteomic studies, give rise to the macromolecular assemblies we call adhesions.

1.10 Protein conformation and activation at the mesoscale

While single-molecule methods including SPT and super-resolution provided unprecedented views into the organization of adhesions in the mesoscale, they lacked a critical piece of information, protein conformation. A key finding of the past 50 years of biochemistry research is that proteins are dynamic creatures that are constantly undergoing structural rearrangements both large and small, which are integral to their biological functions and are commonly misregulated in disease states. A few recent studies have started to apply single-molecule methods to measure protein conformation. One study used iPALM to measure changes in integrin conformation by labeling its head domain with a PA-FP. The integrin head domain undergoes a conformational change upon activation that results in an extended conformation that places the head domain further away from the plasma membrane. By comparing the distance between a plasma membrane marker and the integrin head domain, they could detect conformation changes for individual integrin molecules. Another approach to measuring
conformation uses single-molecule FRET to detect the change in distance between two fluorophores attached to the same protein at different locations. However, smFRET in live cells is still impractical for most biological applications. The protein of interest must be site-specifically labeled at two different locations and microinjected into the cell, which can be technically challenging for many proteins and may perturb normal protein or cell behavior. In addition, the conformational change to be measured must be large enough to provide a sufficient change in FRET efficiency that can be detected in live cells, where signal-to-noise considerations prevent detection of small changes. Another approach to smFRET used a fluorescently labeled GTP analog to measure binding to the small GTPase Ras, which was fused to a fluorescent protein. However, most proteins do not have such ligands that can be used to detect particular protein conformations.
CHAPTER 2  THE COFILIN PATHWAY AND CANCER METASTASIS

2.1 Chemotaxis in biology and disease

Chemotaxis, the ability of cells to sense and direct their movement in response to extracellular gradients of chemoattractants, lies at the heart of many important biological processes and disease states.\(^6\) During development, chemotaxis facilitates the directed migration of individual or multicellular assemblies of cells required for organ formation and wiring of the nervous system.\(^6\) Misregulated chemotactic pathways drive the aberrant migration of tumor cells and leukocytes that cause cancer metastasis and chronic inflammation, respectively.\(^6\) In cancer, chemotaxis underlies each stage of metastasis – invasion, intravasation, extravasation, and resultant growth at distant sites.\(^6\) The dissemination of tumor cells gives rise to secondary tumors, which are the primary cause of mortality in 90% of cancer related deaths.\(^6\) Tumor cells chemotax in response to gradients of particular growth factors and chemokines, which can also be important for tumor cell growth and survival. An important chemoattractant with a demonstrated role in breast cancer invasion and intravasation is epidermal growth factor (EGF).\(^6\) During breast cancer metastasis, chemoattractant signaling facilitates the formation of alternating assemblies of tumor cells and tumor- associated macrophages (TAMs), also known as “streams.” Formation of these streams is dependent on a paracrine loop in which EGF secreted by TAMs enhances migration of EGF receptor (EGFR)-expressing tumor cells, and colony stimulating factor 1 (CSF1) secreted by tumor cells attracts CSF1 receptor (CSF1R)-expressing TAMs.\(^10\) Streaming tumor cells and macrophages migrate along extracellular matrix (ECM)
fibers toward blood vessels where TAMs facilitate the intravasation and consequent dissemination of tumor cells to distant sites.\textsuperscript{69}

\section*{2.2 Chemotaxis facilitates directed cell migration}

During chemotaxis, cells interpret chemotactic gradients in their environment in order to convert their basal, generally random, migratory behavior into directed migration toward the source of chemoattractant. This involves three separate but related steps – chemosensing, polarization, and locomotion.\textsuperscript{70} Chemosensing refers to the ability of cells to detect extracellular gradients of chemoattractants and convert them into a polarized, amplified and sustained intracellular response. The LEGI (local excitation-global inhibition) model has often been used to explain how cells accomplish this task.\textsuperscript{71} According to this model, an unpolarized distribution of chemoattractant receptors results in greater receptor occupancy on the side of the cell facing the source of chemoattractant. Receptor occupancy triggers a fast, local excitatory signal and also a slow, global inhibitory signal, which results in polarization and asymmetric actin polymerization that drives cell locomotion toward the chemotactic cue.\textsuperscript{72} The most common chemoattractants are chemokines signaling through G-protein coupled receptors (GPCRs) and growth factors signaling through receptor tyrosine kinases (RTKs). Activation of these receptors by chemoattractants initiates signaling to mobilize the cytoskeletal machinery that mediates polarization and actin polymerization.\textsuperscript{72}
2.3 ADF/Cofilin family

The ADF/cofilin family of actin-binding proteins consists of actin depolymerizing factor (ADF), cofilin-1, and cofilin-2. In vertebrate tissue, cofilin-1 (hereafter referred to as cofilin) is the most abundant isoform and the only family member required for viability.\textsuperscript{73,74} Regulation of cofilin during chemotaxis is complex and involves a number of cell surface receptors signaling downstream, leading to the activation of several kinases, phosphatases, and phospholipases that regulate cofilin directly.\textsuperscript{75} Initially cofilin was thought to depolymerize actin filaments by enhancing the off-rate of actin monomers at the pointed end.\textsuperscript{76,77} However, a more detailed characterization of cofilin behavior in vitro demonstrated that cofilin’s mechanism is concentration dependent, with actin filament severing observed at lower concentrations, and de novo nucleation of actin filaments observed at higher concentrations.\textsuperscript{78}

2.4 The cofilin pathway in chemotaxis

It is generally accepted that the cofilin pathway plays an important role in chemotaxis; however, cofilin’s precise function remains controversial. Some studies have identified a role for cofilin in the early stages of chemotaxis as an essential component of the cell’s chemosensing machinery by setting the direction of cell migration by initiating asymmetric actin polymerization downstream of chemoattractant receptors.\textsuperscript{68,79} Other studies indicate that cofilin’s function is limited to disassembling and recycling F-actin to maintain a dynamic actin cytoskeleton during locomotion.\textsuperscript{80,81} Understanding cofilin’s function is difficult due to the range of perturbations employed in the relevant investigations. Studies that indicate cofilin functions in chemosensing generally utilized EGF stimulation, which acts locally on fast time scales.\textsuperscript{82} In contrast, studies that determined cofilin function is limited to recycling F-actin utilized genetic
perturbations such as overexpression and RNAi, which act globally on long time scales.\textsuperscript{83,84} Further complicating matters is the finding that either cofilin knockdown or overexpression of constitutively active cofilin result in F-actin stabilization.\textsuperscript{85,86} These observations imply that the timing, duration, and localization of cofilin activity determine the output of the cofilin pathway. Understanding the relative contribution of these factors necessitates the development of new tools capable of controlling cofilin activity with spatial and temporal precision in living cells. Genetic perturbations such as cofilin overexpression are inadequate due to the slow increase in cofilin they produce, and the lack of control over the timing of cofilin activation. Additionally, cofilin overexpression results in spatial reorganization and partial disintegration of F-actin networks at the leading edge that inhibits cell migration, despite an apparent increase in the overall rate of actin polymerization.\textsuperscript{85} This likely indicates that sustained cofilin activation places the cell in a non-physiological state that may bear little relevance to normal mechanisms of cell migration. In contrast, photocontrol of cofilin activity would enable cofilin activation at a precise location with seconds resolution, paralleling the dynamics of cofilin signaling, which can reach peak activity just one minute after activation.\textsuperscript{87}

2.5 The cofilin activity cycle in cancer and inflammation

Over the past few decades, a model of cofilin’s role in chemosensing, termed the cofilin activity cycle, has been developed.\textsuperscript{79,82} For tumor cells, EGFR activation results in cofilin release from an inhibitory interaction with PI(4,5)P\textsubscript{2} at the plasma membrane (PM), mediated by phospholipase C-gamma (PLC\textsubscript{γ}).\textsuperscript{87–89} Release from the PM allows cofilin to bind to and sever actin filaments, generating free barbed ends for later polymerization by the Arp2/3 complex.\textsuperscript{90,91} Diffusion of active (unphosphorylated) cofilin is limited by LIMK-mediated phosphorylation,
which inactivates cofilin and releases a bound actin monomer. Subsequent cofilin dephosphorylation by the slingshot homolog (SSH) family of phosphatases replenishes the pool of PM-bound cofilin and completes the cycle. An additional level of regulation of cofilin activity is provided by the sodium/hydrogen exchanger 1 (NHE1) whose activation causes a rise in intracellular pH that enhances actin filament severing by cofilin. Its important to note that, depending on cell type, activation can occur at a different place in the cycle. In leukocytes, fMLP receptor activation results in Rac2-dependent cofilin dephosphorylation and consequent generation of free barbed ends at the leading edge. Despite these differences, the model predicts that for both tumor cells and leukocytes local cofilin activation at the cell edge initiates chemotaxis via production of free barbed ends that enhances later Arp2/3 complex-mediated actin polymerization.

2.6 The cofilin pathway drives metastasis in vivo

Invasion and intravasation of tumor cells in vivo has been shown to be directly related to the activity status of the cofilin pathway. Studies of EGF-mediated chemotaxis for single tumor cells in cell culture has led to the hypothesis that tumor cell chemotaxis during streaming in vivo is also initiated by cofilin activation. Cofilin activation in the leader cell of a stream likely sets the direction of migration for the entire stream via rely chemotaxis.

2.7 Chemically-caged cofilin

Cofilin has been caged previously by a chemical modification that rendered it inactive until irradiated with light. Activation of chemically-caged cofilin is irreversible and renders
photoactivated cofilin insensitive to endogenous regulation. Proper regulation of cofilin activity is critical to normal cell behavior, casting doubt on conclusions reached using chemically-caged, irreversibly activated cofilin. Microinjection is also technically demanding, which has limited adoption of the chemically caged cofilin. In contrast, a genetically-encoded caged cofilin will enable modulation of cofilin activity while maintaining endogenous regulation, and also avoid the need for microinjection, thereby facilitating broad adoption and application of this approach.
CHAPTER 3 A SYSTEM TO PROBE PROTEIN CONFORMATION AT THE ENSEMBLE AND SINGLE-MOLECULE LEVEL

3.1 Introduction

Linking protein structure with function has been a longstanding goal of biological research as it is critical to understanding both basic biology and disease states. Ideally, a protein’s structure could be determined along with its biochemical properties and cellular context at a particular moment. To this end, various methods have been developed to determine protein structure with their own advantages and limitations. X-ray crystallography and cryo-electron microscopy (cryo-EM) have produced near or at atomic resolution structures of numerous proteins but are limited to capturing static “snapshots” of protein’s structure.\textsuperscript{103,104} In contrast, nuclear magnetic resonance (NMR) and single-molecule Förster resonance energy transfer (smFRET) measure structural features of functional proteins in solution and therefore can capture structural dynamics underlying protein function, but generally cannot match the resolution of crystallography or cryo-EM.\textsuperscript{105,106}

NMR and smFRET have primarily been used to study proteins in vitro, but protein structure is known to be influenced by the numerous interactions proteins undergo in live cells.\textsuperscript{107} Therefore, the relevance of in vitro observations to in vivo protein behavior remains an open question. Further, in vitro studies preclude correlating protein conformation with cellular processes. While recent progress has been made toward adapting NMR and smFRET for use in
live cells, significant barriers exist to their broader application. NMR is limited to proteins of small size and generally requires high intracellular concentrations of isotopically labeled protein that is often much higher above endogenous levels. Further, ensemble-averaging inherent to in-cell NMR precludes detection of heterogeneous protein conformations and limits correlating protein conformation with cellular processes. While smFRET can measure extremely low concentrations of target proteins, it requires site-specific labeling with bright organic fluorophores that can be technically challenging and may interfere with protein function. Further, in-cell smFRET requires large changes in FRET efficiency, limiting the range of conformational changes that can be probed.

Another approach involves the use of small protein domains, or “affinity reagents”, that bind selectively to a given conformation of target proteins. Affinity reagents are often derived from endogenous proteins but have also been generated by screening libraries of engineered protein scaffolds. Affinity reagents provide several distinct advantages over smFRET or in-cell NMR. In contrast to smFRET, both small and large conformational changes can be detected in live cells, and unlike NMR, affinity reagents are not limited by the size of the target protein. Affinity reagents are also compatible with endogenous levels of target protein expression and can be used for ensemble or single molecule measurements. However, their application has been limited because they remain difficult to design, requiring considerable optimization for each target. Here, we describe a general approach for developing affinity reagents based on highly-specific protein-peptide interactions. We demonstrate that this approach can be used to create affinity reagents to detect conformational changes for multiple different proteins at the ensemble and single-molecule level in live cells. We also demonstrate that the same approach can be used to control protein conformation. The ability to detect and control protein conformation will likely
have many uses in understanding the link between protein structure and function in the context of living organisms.

3.2 Results

Development of BinderTag, a System for Sensing and Controlling Protein Conformation via Insertion of a Small Peptide.

Multidomain proteins often undergo conformational changes that unmask binding sites for other proteins that regulate and/or relay information downstream of its binding partner.\textsuperscript{111,112} We therefore sought proteins that bind to small peptide motifs, which we could insert into a target multidomain protein to engineer novel conformation-dependent binding interactions. These engineered interactions could be used to either sense or control a target protein’s conformation by varying the affinity and/or relative concentration of the binding protein and inserted peptide (Figure 3.1A).

We identified stringent starvation protein B (SspB), an 18.3 kDa protein from \textit{Haemophilus influenzae} as a potential candidate.\textsuperscript{113,114} SspB binds to the seven-residue peptide SsrA with high affinity (K\textsubscript{D} = 16 nM). We used a mutant SspB (Y11K/A15E) that is monomeric to prevent artifacts due to dimerization.\textsuperscript{115,116} Due to its small size, we reasoned that SsrA could be inserted into target proteins without affecting its folding or regulation. Additionally, mammalian proteins notably lack significant sequence homology with the SsrA peptide and therefore SspB would be unlikely to exhibit off-target interactions with untagged proteins. We tested the ability of SspB to bind SsrA-tagged proteins in mammalian cells by co-expressing SspB and the transmembrane protein stargazin (Figure 3.1B). SspB co-expressed with
wild type stargazin displayed a diffuse cytoplasmic localization indicating SspB was properly folded and stable. In contrast, when SsrA was inserted in stargazin’s intracellular loop we observed robust recruitment of SspB to the plasma membrane, indicating efficient binding of SspB to the inserted peptide.

Figure 3.1 Identification of a Peptide-Protein Pair That Can Be Inserted into Proteins.

(A) Schematic of BinderTag approach.
(B) GFP-SspB was co-expressed with either stargazin-mCherry (top) or stargazin-mCherry with ssrA inserted into an intracellular loop (bottom) in HEK293 cells, and cells were imaged using epifluorescence microscopy.

Next, we tested whether we could engineer selective SspB binding to a particular conformation of a protein. We focused on Src kinase because it is an important regulator of diverse cellular processes and the ability to monitor Src conformation in live cells would provide new biological insights.\textsuperscript{117–119} Src kinase activity is regulated by its SH2 and SH3 domains (hereafter referred to as autoinhibitory domain or AID), which bind to and inactivate the kinase
domain (hereafter referred to as AID engagement). AID engagement is stimulated by phosphorylation of a C-terminal tyrosine (Y527) by C-terminal Src Kinase (CSK), whereas dephosphorylation weakens the interaction and promotes Src activation. We therefore sought to engineer selective SspB binding to Src’s active conformation by screening SsrA insertion sites in the interface between the AID and kinase domain (Figure 3.2).

Figure 3.2 Identification of a Peptide Insertion Site for Src.
Crystal structure of Src with tested insertion sites labeled as numbered and color-coded spheres, color-coding indicates whether ssrA insertion affected kinase activity and/or resulted in conformation-dependent binding of SspB. Domains are labeled for clarity; kinase (grey), SH2 (yellow), and SH3 (cyan).
Insertion sites were evaluated to determine whether SsrA insertion disrupted Src regulation and/or produced selective SspB binding. Phosphotyrosine blots of cell lysates from LinXE cells transfected with different Src constructs revealed that SsrA insertion at the C-terminal tail region maintained normal regulation (Figure 3.3A). Next, we tested for selective binding via pull-down experiments in LinXE cells co-expressing SspB and SsrA-tagged Src (Figure 3.3B). Active mutant (Y527F) Src co-immunoprecipitated with SspB to a greater degree than wild type Src, indicating SspB bound selectively to Src’s active conformation.

![Figure 3.3](image)

Validation of a Peptide Insertion Site for Src.

(A) LinXE cells were transfected with different Src constructs and whole-cell lysates were immunoblotted for indicated proteins. Non-transfected (NT), wild type (WT), and active mutant (Y527F) Src are shown as controls. Numbered lanes correspond to different SsrA-insertion sites as shown in (A). Insertion site seven (star) was selected as the optimal peptide insertion site and is hereafter referred to as nanoTag-Src. Blot representative of three independent replicates.

(B) Co-immunoprecipitation of nanoTag-Src and FLAG-SspB with anti-flag antibody for wild type and active mutant (Y527F/D388R) Src. Numbered lanes correspond to different SsrA-insertion sites as shown in (A). Blot representative of three independent replicates.
We named the new Src analog tagSrc, for SsrA-tagged Src. To test whether SspB and tagSrc interacted in live cells, we co-expressed SspB and active mutant tagSrc in HeLa cells (Figure 3.4A). In cells expressing high levels of tagSrc, we observed relocalization of SspB from the cytosol to the perinuclear region, consistent with SspB binding to tagSrc. In addition, analysis of crystal structures indicated SspB binding at this site was unlikely to affect tagSrc interactions with other regulatory or effector proteins (Figure 3.4B).

Figure 3.4 Localization of tagSrc and Analysis of Regulator Interactions.

(A) HeLa cells were co-transfected with mCherry-SspB and active mutant (Y527F) nanoTag-Src-GFP. Cells were imaged using epifluorescence microscopy. White arrow indicates low Src-expressing cells, grey arrow indicates high Src-expressing cells.

(B) Crystal structure of Src (green) showing optimized peptide insertion site (red) and interacting proteins (grey surface).

Identifying an alternative peptide-protein pair would be useful, as it would enable simultaneously detecting the conformation of two different proteins in the same cell. We replaced SsrA with the ARVCF peptide, which binds to ePDZ with high affinity (Figure
3.5A). Pulldown experiments showed that ePDZ bound selectively to active mutant ARVCF-tagged Src (Figure 3.5B).

The Src-family kinase Fyn shares significant structure and sequence homology with Src but emerging evidence suggests they regulate distinct cellular processes. We tested whether insertion of SsrA into Fyn at the same site used for tagSrc would also produce conformation-dependent binding of SspB. Similar to tagSrc, active mutant (Y527F) Fyn co-immunoprecipitated with SspB to a greater degree than wild type Fyn, indicating SspB bound selectively to Fyn’s active conformation (Figure 3.6A).
The ability to engineer conformation-selective protein-protein interactions via peptide insertion would be more valuable if it could be readily applied to proteins with different domain organization and regulatory mechanisms. We focused on the non-receptor tyrosine kinase Syk,

Figure 3.6 Development of TagFyn and TagSyk Analogs

(A) Co-immunoprecipitation of nanoTag-Fyn and FLAG-SspB with anti-flag antibody for wild type and active mutant (Y527F/K299M) Fyn (bottom). SsrA inserted after indicated residue.

(B) Schematic of BinderTag Syk.

(C) Co-immunoprecipitation of nanoTag-Syk and FLAG-SspB with anti-flag antibody for inactive (YYFF) and active (YYAA) mutant Syk. NanoTag was inserted at multiple locations as indicated (top). The insertion site with the best dynamic range (N11) was replicated, along with Syk without the nanoTag insertion as a control (bottom).

(D) Syk with and without the nanoTag insertion at N11 were expressed in LinXE cells and whole-cell lysates were immunoblotted for indicated proteins.
which plays an important role in immune cell signaling as a master regulator of classical immunoreceptors including B cell receptors (BCRs), T cell receptors (TCRs) and Fc receptors (FcRs).\textsuperscript{125,126} Syk kinase activity is regulated by its AID (consisting of tandem SH2 domains), which binds to and inactivates its kinase domain (Figure 3.6B).\textsuperscript{127} Similar to Src, we sought to engineer selective SspB binding to Syk’s active conformation by screening SsrA insertion sites in the interface between the AID and kinase domains. We found that SsrA insertion after residue N11 produced a Syk analog that bound SspB only in the active state (Figure 3.6C). Phosphotyrosine blots of cell lysates showed that the Syk analog was functional and regulated similar to wild type Syk (Figure 3.6D).

SspB’s selectivity for the active conformation of our target proteins is likely due to SspB binding and AID engagement (and protein inactivation) being mutually exclusive. Accordingly, disengagement of the AID upon protein activation would enable SspB to bind the inserted SsrA peptide. However, it is possible that SspB could also bind an inactive protein and displace the AID. Such “artificial activation” by SspB would hinder efforts to monitor protein activation by endogenous regulators. We tested whether SspB could activate tagSrc by titrating SspB expression in LinXE cells co-expressing tagSrc (Figure 3.7A). SspB expression induced tagSrc activation in a dose-dependent manner, as indicated by heightened substrate phosphorylation (paxillin pY31). SspB-induced tagSrc activation also resulted in the appearance of cell rounding, a well-documented phenotype of Src hyperactivation, which causes destabilization of cell-matrix adhesions (Figure 3.7B and C).
Figure 3.7  SspB-induced Src Activation and Development of Mass-action Model.

(A) LinXE cells were co-transfected with paxillin and either Src or nanoTag-Src. Whole-cell lysates were immunoblotted for indicated proteins.

(B) LinXE cells were transfected with indicated constructs and the number of rounded cells per image fields were counted by a blinded evaluator. Error bars are 95% confidence interval. n=4

(C) Representative images of LinXE cells expressing indicated constructs. Cells were imaged with DIC (top) and epifluorescence (bottom).

(D) Schematic of BinderTag model.

(E) Model-derived binding curves for SspB and Src with high affinity nanoTag or low affinity microTag. Shaded area indicates hypothetical SspB expression range that is below assay detection limit.
In order to better understand the factors governing whether SspB will activate tagSrc, we constructed a mass action-based model of SspB binding to ssrA-tagged proteins (Figure 3.7D). The effect of AID engagement on SspB binding is incorporated by applying the Schild model of competitive antagonism, which states that the presence of a competitive inhibitor will shift the binding curve to the right. The magnitude of the shift is determined by the concentration and affinity of the inhibitor. In our model, tagSrc’s AID acts as a competitive inhibitor of SspB binding. When tagSrc is inactive, the affinity of the AID is high (due to enhanced interaction of the SH2 domain with a phosphorylated C-terminal regulatory tyrosine) and the shift in the binding curve is large. When tagSrc is active, the affinity of the AID is low (due dephosphorylation of the C-terminal regulatory tyrosine) and the shift in the binding curve is small.

Our model indicates that selective binding of SspB to active tagSrc (and prevention of spurious tagSrc activation from SspB binding to inactive tagSrc) can be achieved by carefully controlling the expression level of tagSrc and SspB (Figure 3.7E). For some applications (such as single cell FRET imaging), working at lower expression levels may not be feasible due to insufficient signal. In these cases, selectivity at higher expression levels can be achieved by lowering affinity via use of ssrA mutants.

Visualizing Src Activation in Living Cells with BinderTag FRET.

Applying our newly developed method to sense protein conformation in living cells required the ability to detect when SspB is bound to its target protein. We therefore sought to measure SspB binding to tagSrc by fluorescence resonance energy transfer (FRET) (Figure 3.8A).
Figure 3.8 Development of BinderTag FRET Sensor

(A) Schematic of BinderTag FRET sensor.

(B) Screening of different combinations of CFP variants (CyPet, mCerulean3, and mTurquoise) and YPet circular permutants (wild type, CP173, CP157, CP229). Src and SspB were attached to CFP and YFP, respectively. Sensitized FRET emission (bleedthrough corrected) is displayed on the y-axis. Star indicates optimized FRET pair (mCerulean3 and YPet CP229).

(C) High-content live-cell imaging of LinXE cells expressing FRET sensor. SspB expression level is titrated against a constant Src expression level. Blue, wild-type Src; red, active mutant Src.

(D) Top: MEF cells expressing FRET sensor for wild-type and active mutant Src. Src and SspB are expressed at approximately equal levels as measured by CFP and YFP emission intensities. Color scale indicates sensitized FRET/CFP emission values. Bottom: Histogram of FRET/CFP ratio values for each cell displayed.

(E) Sensitized FRET/CFP emission values for MEF cells expressing wild type (n=14) or active mutant (n=12) Src FRET sensor. P value was calculated with unpaired two sample Student’s t test.
A FRET-based readout would also provide an efficient means to identify ssrA-mutants with reduced affinity, as well as enable us to test our model’s predictions by measuring SspB binding to tagSrc across a range of SspB expression levels.

We screened variants of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) attached to active mutant tagSrc and SspB, respectively (Figure 3.8B). The fluorescent proteins we tested differed in several properties important for efficient FRET including brightness and fluorophore orientation. We selected mCerulean3 and YPet (circularly permutated at residue 229) as the optimal FRET pair (indicated by star in Figure 3.8B). While CyPet gave the highest sensitized FRET emission, the superior brightness of mCerulean3 was required to achieve sufficient signal to noise during image processing required to calculate single-cell FRET.

Our initial screen measured FRET at a single fixed ratio of SspB and tagSrc expression. In order to determine the maximal FRET response, we used high-content live-cell imaging to measure FRET across a range of different SspB expression levels (Figure 3.8C). FRET increased with SspB expression in a dose-dependent manner. Importantly, we observed a shift in the binding curve for active mutant Src relative to wild type, which closely paralleled our model’s predictions. At high SspB expression levels, FRET was similar for active mutant and wild type tagSrc, likely because high SspB expression was activating tagSrc. We obtained similar results in single-cell experiments with mouse embryonic fibroblasts (MEFs), where active mutant tagSrc produced higher FRET than wild type tagSrc (Figure 3.8D and E).

As mentioned previously, lower affinity ssrA-mutants would be useful for preventing tagSrc activation when working at higher expression levels. We therefore conducted a screen to identify ssrA mutants with reduced affinity (Figure 3.9A and B). Several mutants displayed a significant reduction in affinity and were confirmed via co-immunoprecipitation (Figure 3.9C).
For clarity, we named the new Y7I mutant SsrA “microTag” and the wild type SsrA “nanoTag”.

Incorporating microTag into our FRET sensor would likely result in a significant reduction in FRET and hinder using the probe in single cell imaging experiments. We therefore sought to enhance the FRET efficiency of our sensor by introducing two point-mutations (S208F and V224L) into mCerulean3 (Figure 3.10A). In a previous study, these mutations were shown to promote a weak interaction (K_D ≈ 100 µM) between fluorescent proteins that enhances FRET efficiency. Our new mCerulean3 variant (named “sticky” mCerulean3) demonstrated enhanced FRET efficiency without compromising dynamic range (Figure 3.8C and Figure 3.10B).

**Figure 3.9** Identification of SsrA-mutants with Reduced Affinity.

(A) Initial screen to identify nanoTag mutants with reduced affinity. Normalized sensitized FRET/CFP emission values for LinXE cells expressing nanoTag-Src FRET sensor with indicated nanoTag mutants. Src and SspB are expressed at approximately equal levels as measured by CFP and YFP emission intensities. n=1

(B) Measurements of selected mutants from (A) were replicated. Error bars are 95% confidence interval. n=4

(C) Co-immunoprecipitation of nanoTag-Src and FLAG-SspB with anti-flag antibody for indicated nanoTag mutants. Reduced affinity SspB (A70Q) was used as a control. Band densities were quantified and normalized Src/SspB is displayed on y-axis. n=1

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Figure 3.10  Development of "Sticky" mCerulean3 with Enhanced FRET.

(A) Schematic of dimerization-enhanced mCerulean3.
(B) Sensitized FRET/CFP emission values for LinXE cells expressing microTag-Src FRET sensor incorporating either wild-type or S208F/V224L (dimerization enhanced) mCerulean3. n=1

Figure 3.11  Single-cell FRET Measurements with "Sticky" mCerulean3.

(A) Histogram of sensitized FRET/CFP emission values for MEF cells expressing Src FRET sensor using either microTag or nanoTag.
(B) Sensitized FRET/CFP emission values for MEF cells expressing Src FRET sensor using microTag (n=10) or nanoTag (n=8). P value was calculated with unpaired two sample Student’s t test.
(C) MEF cells expressing microTagSrc FRET sensor. Src and SspB are expressed at approximately equal levels as measured by CFP and YFP emission intensities. Color scale indicates corrected FRET emission (left), corrected FRET to YFP (SspB) emission ratio (middle), or corrected FRET to CFP (microTagSrc) emission ratio (right).
Single-cell FRET experiments demonstrated that using sticky mCerulean3 with our microTag sensor enabled measurement of tagSrc activation in living cells similar to the nanoTag sensor (Figure 3.11)

**Generation and characterization of MEFs stably expressing Src and SspB.**

Src and SspB expression must be carefully controlled to achieve selective SspB binding to active Src. We used retroviral-mediated gene transduction to generate MEFs stably expressing Src and SspB under the control of a doxycycline-responsive promotor. Src and SspB were fused to HaloTag and SnapTag, respectively, to facilitate labeling with membrane permeable dyes. Successful transduction of both proteins was confirmed by imaging cells following dye labeling (Figure 3.12A).

Fluorescence-activated cell sorting (FACS) was then used to select homogenously expressing populations with different Src to SspB expression ratios (Figure 3.12B). Variability in expression levels after FACS was similar to variation in endogenous Src levels and likely due to stochastic fluctuations in gene expression that occur in individual cells over time (Figure 3.12C). Because expression was relatively even across cells, ensemble measurements based on biochemical techniques could be used to infer properties of individual cells.

Src overexpression can result in transformation and perturbation of many cellular functions including adhesion. We measured tagSrc expression relative to endogenous Src by blotting cell lysates and found tagSrc expression was approximately 10-fold over endogenous (Figure 3.12D). In order to control tagSrc expression we titrated doxycycline to achieve tunable tagSrc expression ranging from 0.2- to 10-fold over endogenous (Figure 3.12D). Importantly, the ratio of tagSrc to SspB expression was constant over a range of expression levels (Figure 3.13A).
Single-molecule imaging of tagSrc was possible at all expression levels, however visualization of SspB was hindered at tagSrc expression levels below endogenous Src. To ensure that higher tagSrc expression levels did not perturb Src regulation or downstream signaling we assessed Src phosphorylation (Y416 and Y527), and phosphorylation of paxillin, a Src substrate (Figure 3.13B). Src expression up to 10-fold above endogenous did not alter Src phosphorylation patterns or increase paxillin phosphorylation. However, tagSrc expression at 40-fold above
endogenous was associated with the loss of inactivating pY527, gain of activating pY416, and an increase in phospho-Paxillin.

As indicated by our model, SspB expression must be carefully controlled to prevent tagSrc activation. Therefore, we assessed whether increases in SspB expression affected Src regulatory phosphorylation. MEFs expressing high and low amounts of SspB did not display significantly different Src phosphorylation relative to MEFs lacking SspB (Figure 3.14). This

Figure 3.13 Validation of BinderTag Stable Cell Line.

(A) Whole-cell lysates of BT MEF and parental MEF cells were immunoblotted for indicated proteins. Doxycycline concentration was titrated to control level of expression. Src-HaloTag and SnapTag-SspB band density was quantified and normalized to either endogenous Src or fully-induced Src-HaloTag or SnapTag-SspB.

(B) Whole-cell lysates of BT MEF, parental MEF cells, and electroporated parental MEF cells (GFP and Src) were immunoblotted for indicated proteins. Normalized band density for Src-HaloTag, paxillin, and phospho-paxillin were quantified.
indicates that SspB concentrations are below the threshold for binding inactive tagSrc and SspB is very likely binding to tagSrc activated by endogenous regulatory mechanisms.

![Immunoblot image showing protein expression levels](image)

Figure 3.14 Validation of SspB Expression Level for BinderTag MEFs.

Whole-cell lysates of BinderTag and parental MEF cells were immunoblotted for indicated proteins. Doxycycline concentration was titrated to control level of expression.

**Single-molecule tracking of Src conformation.**

Visualizing protein conformation at the single-molecule level in live cells has proven challenging. Currently available methods rely on smFRET to detect changes in protein conformation. However, live-cell smFRET suffers from poor signal to noise and is technically challenging, which has limited adoption. Therefore, we sought to leverage the unique advantages of the BinderTag approach to develop a new method for probing the conformational changes of individual molecules in live cells. Our method relies on the use of TIRF microscopy to
selectively visualize proteins near or at the ventral plasma membrane. We hypothesized that because the Binder normally localizes to the cytosol, detection of the Binder at the plasma membrane would indicate that it is bound to an active TagSrc molecule, thereby revealing the single-molecule localization of activated tagSrc (Figure 3.15).

Figure 3.15 Schematic of BinderTag single-molecule tracking of protein conformation.

TIRF microscopy produces a thin region of illumination (yellow shaded area) that selectively visualizes molecules near or at the plasma membrane (PM). The majority of Binder (B) molecules are cytosolic and therefore are not detected (grey spheres). However, when Binder associates with a protein in the open conformation that has an inserted tag (T) it can be detected (green sphere). Binder molecules in plane of illumination not associated with a PM-bound protein exhibit fast diffusion rates and can be filtered out (fuzzy green sphere).
We tested this hypothesis by expressing active mutant TagSrc and Binder in live cells and used TIRF microscopy to track tagSrc and binder molecules (Figure 3.16). We observed many TagSrc and Binder tracks, which displayed a high degree of co-localization, indicating that binder accurately reported the localization of active TagSrc. In contrast, when we expressed Binder by itself we only observed a few tracks, likely due to Binder molecules that diffused into the illumination plane but were not bound to TagSrc.

As mentioned previously, the expression of TagSrc and Binder must be carefully controlled to achieve selectivity of the Binder for active TagSrc. Therefore, we performed SPT of our BinderTag MEFs, which have been validated to ensure Binder selectivity for active Src, in order to compare the single-molecule properties of active and inactive Src (Figure 3.17). We
observed many tracks for both TagSrc and Binder, which appeared to have different cellular localizations. Binder appeared to preferentially localize to the edge of the cell, whereas TagSrc displayed a more random localization. Analysis of tracks for TagSrc and Binder revealed several important differences in their single-molecule behavior (Figure 3.18). The mean squared displacement (MSD) for Binder was lower than TagSrc, indicating active Src has a slower diffusion rate. Binder and TagSrc also displayed different diffusive behaviors, where Binder underwent less random diffusion and more confined diffusion relative to TagSrc. Finally, Binder tracks were confined to a smaller area than TagSrc.

Figure 3.17  Single-molecule tracking of BinderTag MEFs.
Left and Middle: Single-molecule trajectories of TagSrc and Binder were collected from BinderTag MEFs. Tracks are color coded to indicate diffusion rate. Right: Track density maps reveal that Binder preferentially localizes to cell edge, but TagSrc does not. Warmer colors reflect relative higher track density.
3.3 Discussion

Advantages of BinderTag System:

The BinderTag system represents one of the most versatile and non-perturbing systems for generating conformation-selective binding proteins and has several major advantages over existing methods. First, the small size of the peptide tag (seven residues) enables insertion into target proteins without disrupting their regulation or interaction with other proteins. This enables generation of fully functional protein analogs that can be used to replace the endogenous version using gene editing techniques such as CRISPR-Cas9. Second, the high specificity of the SspB-SsrA interaction minimizes artifacts due to off-target interactions with other cellular proteins and ensures accurate reporting of protein conformation. Third, the ability to easily tune the affinity of the SspB-SsrA interaction enables broad application to proteins with different interdomain
interaction strengths. Finally, the ability to use other peptide-protein systems such as ARVCFP-ePDZ can enable multiplexed detection of two different protein’s conformations in the same cell. This could be used to explore signaling relationships between two proteins to better understand complex cell signaling networks.

**Identification of Tag Insertion Sites:**

Our application of BinderTag to Src and Syk provides a blueprint for application of the technique to other proteins. Successful application of BinderTag to other proteins will depend on several factors that were important in the present study. First, our application to Src and Syk were greatly aided by the wealth of information from previous studies that provided detailed information about the activation mechanism and associated conformational changes. Selective binding of SspB to the inserted tag likely depends findings a SsrA-insertion site that undergoes a change in solvent-accessible surface area (SASA) during the conformational change that is to be measured, which ensures that SspB can only bind the inserted peptide in one of the conformations. In particular, high-resolution crystal structures of different conformations of the target protein are useful in identifying such regions, as well as data from hydrogen deuterium exchange (HDX) experiments. Second, the availability of mutants or experimental conditions that induce different conformations of the target protein are essential for testing putative insertion sites for conformation-dependent binding. Third, information about the binding of regulators and effectors is important for identifying insertion sites that are unlikely to perturb normal function. This information can come from multiple sources, including crystal structures of the interacting proteins, as well as mutagenesis data that can localize potential binding sites. Finally, the recent advent of computational biology has the potential to enable application of BinderTag to proteins for which this information is not available. Several studies have
demonstrated the ability to determine both protein structure and predict regulatory interactions from amino-acid sequence data alone, which should be available for any protein of interest.

**Application of BinderTag to New Proteins:**

As mentioned previously, BinderTag excels at detecting the change in SASA of specific regions in target proteins due to conformational changes. Therefore, proteins with high potential for successful application of the BinderTag approach are multidomain proteins that undergo large conformational changes. As an example, many proteins that are either regulators of, or regulated by, SFKs are likely to be amenable to application of BinderTag. Src is activated by a variety of phosphatases that dephosphorylate its C-terminal regulatory tyrosine, which promote the formation of the open and catalytically-active conformation. The phosphatase Shp2, contains a phosphatase domain and two SH2 domains, which bind to and inactive the phosphatase domain in the inactive conformation. The N- and C-terminus regions of the protein appear to undergo significant changes in SASA upon activation in a manner similar to Src, and thus would be a promising application for BinderTag. Src is also known to regulate a number of Rho guanine nucleotide exchange factors (RhoGEFs), which also undergo large conformational changes upon activation. In most RhoGEFs, an autoinhibitory domain (AID) binds to and inactivates that catalytic Dbl homology (DH) domain responsible for activating downstream GTPases. Displacement of the AID is associated with a large change in SASA, which provides many potential insertion sites for the SsrA peptide. While these are only two examples, they illustrate how the conformational changes of many proteins are associated with changes in SASA, and therefore BinderTag is likely to be successfully applied to many other proteins to generate conformation-selective binders. Finally, the generation of new BinderTag analogs combined with
the potential to multiplex multiple BinderTag analogs in the same cell may provide a powerful window into the complex regulatory interactions at the heart of cell signaling networks.

**Versatility of BinderTag:**

An important advantage of the BinderTag system is that it greatly simplifies protein engineering by enabling one analog to serve several purposes. Once a peptide insertion site has been identified, the BinderTag system can be used to detect protein conformation both at the population level using a FRET-based sensor and at the single molecule level. As demonstrated above, high FRET efficiency was achieved via the use of a mCerulean3/YPet FRET pair with mCerulean3 fused at the N- or C-terminal of the inserted peptide for Syk or Src, respectively. Given that the binding orientation of SspB relative to the inserted peptide is likely to be the same regardless of target protein, achieving efficient FRET for a new BinderTag analog will require minimal optimization. The BinderTag system also provides an important advantage over current approaches to developing FRET sensors based on affinity reagents derived from effector proteins, because the peptide insertion site can be chosen to prevent competition with endogenous regulators and downstream effectors, which preserves normal signaling and prevents dominant-negative effects. However, it is important to note that a minimum expression level of the target protein is required to provide sufficient signal for FRET image processing, which may be considerably higher than the target protein’s endogenous expression level. This must be assessed for each new target protein to determine whether higher expression levels will perturb regulation of the target protein or cell behavior. In contrast, single particle tracking with BinderTag should be compatible will almost all target proteins because single particle tracking can be accomplished with extremely low expression levels. Due to the high specificity of the SspB-SsrA interaction, BinderTag can be used to localize target protein activation on the plasma
membrane at a single-molecule level. This is because the cytosolic localization of SspB prevents detection unless it is bound to an activated target protein molecule, which localizes it to the membrane and renders it detectable with TIRF illumination. Current methods for generating affinity reagents generally do not provide complete selectivity for the protein of interest, and therefore membrane localization would not a reliable indicator of binding to the target protein with these approaches. BinderTag also provides advantages over smFRET, which can also detect protein conformation at the single molecule level. While smFRET as traditionally been limited to in vitro use, recent studies have demonstrated smFRET in live cells. However, smFRET in live cells is limited by poor signal to noise, which imposes several limitations on the range of target proteins and conformational changes that can be probed (reviewed in more detail in the introduction). Further, smFRET requires the use of two detection channels, which limits the opportunity to correlate protein conformation with other cellular structures. In contrast, BinderTag requires only one channel, and thus can be multiplexed with multiple fluorescent markers. Finally, BinderTag may also provide the ability to activate target proteins. As mentioned earlier, SspB can either activate or sense the conformation of a target protein depending on its relative concentration or affinity. Future efforts to control SspB binding with light or small molecules may enable optogenetic or chemogenetic control of protein activity. Control of protein activity with BinderTag would also provide important advantages over current methods of protein control. Whereas current methods for controlling protein activity render the analogs inactive in the basal state, BinderTag analogs retain their native regulation and activity. This could enable replacement of the endogenous version for more physiological control of protein signaling.
3.4 Materials and Methods

Epifluorescence Microscopy

All imaging was performed on an Olympus IX-81 microscope equipped with an UPlanFLN 40x objective (Pil, N.A 1.30). Metamorph software (Molecular Devices) was used to control the microscope and acquire images. Light source was a 100-Watt mercury arc lamp. All dichroic beamsplitters and bandpass filters were purchased from Semrock unless otherwise indicated. For assessing the localization of Src and SspB in LinXE and HeLa cells, a FF444/521/608 dichroic beamsplitter and either Chroma HQ470/40X (GFP) or Zeiss BP-585/35 (mCherry) bandpass filter was used to select excitation wavelengths. Emission selection was accomplished with either a Chroma HQ525/50m (GFP) or FF-647/57 (mCherry). Images were collected with a Photometrics Cool Snap ES2 CCD camera. For FRET imaging in MEF cells, a FF462/523 dichroic beamsplitter and either FF-434/17 (CFP) or FF-510/10 (YFP) bandpass filter was used to select excitation wavelengths. Simultaneous collection of CFP and YFP emission was accomplished with an Andor TuCam using a FF509-FDi01 dichroic beamsplitter, FF-550/49 and FF-482/35 bandpass filters. Images were collected with two Hamamatsu Flash 4 V2 sCMOS cameras. The two cameras were manually aligned prior to image analysis and images were further aligned after collection using a custom MatLab script described previously. Image processing including flat-field correction, background subtraction, bleed-through subtraction, photobleaching calculations, and ratio corrections were accomplished with a custom MatLab script described previously.129
Single-molecule microscopy

Single molecule tracking was performed on a home-built total internal reflection microscopy based on IX81 (Olympus), equipped with four solid-state lasers (Coherent OBIS 405 nm, 488 nm, 561 nm and 647 nm). A four-band dichroic (Di01-R405/488/561/635, Semrock) mirror was used for multi-color imaging. Fluorescence images were collected by a 150 X TIRF objective (UAPON 150XOTIRF, NA 1.45, Olympus), and projected to an electron-magnified charge-coupled device (EMCCD) camera (Evolve® 512 Delta, Photometrics). A 10-position emission filter wheel (FW) (Sutter Instrument) and an imaging splitting optics (W-View GEMINI, Hamamatsu) was mounted in front of the camera to enable flexibly switching between different imaging modes.

Single-molecule tracking and analysis

Single B/T MEF cell was imaging for 60 seconds at 50 Hz, generating a 3000 frames video. We first applied an àtrous-wavelet decomposition method to extract individual single molecules. Each identified molecule was then fitted with a 2D-gaussian function to obtain its precise centroid location. We then adopted a well-established trajectory linking algorithms find the corresponding single molecules in successive frames.

Mean square displacement (MSD) is one of the most common approaches to describe the diffusion property of single molecules. The MSD at different time lag \( \tau = n \Delta t \) was calculated as below:

\[
MSD(\tau) = \langle (x_{\tau+k} - x_\tau)^2 + (y_{\tau+k} - y_\tau)^2 \rangle,
\]
Where \( x_{i+k} \) and \( y_{i+k} \) describe the position following a time interval, \( k \), after starting at positions \( x_{\tau} \) and \( y_{\tau} \).

To get the diffraction coefficient of each trajectory, the MSD curve was fitted to a nonlinear anomalous diffusion model:

\[
MSD(\tau) = 4D\tau^\alpha + 4\sigma^2.
\]

Where \( D \) is the diffusion coefficient, \( \alpha \) is the anomalous exponent, and \( \sigma \) is the localization precision. To get a reliable estimation of the diffusion coefficient, the minimum track length was set to 12 frames and only the first 4 points were used when fit to the diffusion model.

We use moment scaling spectrum (MSS) to categorize each trajectory to different modes, such as pure Brownian, confined or directional movement. The moments of displacement of order \( \nu \) is defined as:

\[
MSS(\tau, \nu) = \langle (x_{\tau+k} - x_{\tau})^\nu + (y_{\tau+k} - y_{\tau})^\nu \rangle,
\]

Where \( \tau \) is the time shift. The mean square displacement is a special case when \( \nu = 2 \). To quantify the motion types, these moments were calculated for \( \nu = 0 \sim 6 \).

**In-cell kinase activity assay**

HEK293T cells were seeded into 6-well plates with 0.8 M cells per well. The next day cells were transfected with indicated constructs using Fugene6 (Promega) according to manufacturer’s protocol. At 24 h post-transfection the cells were collected by removing media and washing 1X with 1 mL ice-cold DPBS. To each well, 250 \( \mu \)L of IP lysis buffer (Pierce) was added and cells were removed with a cell lifter (Corning), followed by pipetting the entire volume over the surface of the well several times to remove remaining cells and mix lysate.
Lysis buffer contained protease inhibitors (cOmplete EDTA-free; Millipore Sigma) and phosphatase inhibitors (PhosSTOP; Roche). Lysate was then transferred to prechilled Eppendorf tube on ice and placed on rotating mixer for 20 m at 4 C. Following incubation lysates were centrifuged at 6000 rcf for 10 m at 4 C. Supernatant was removed and combined with 4X Laemmli sample buffer (10% 2-mercaptoethanol) and boiled for 5 m. Samples were Western blotted for total phosphotyrosine and kinase expression. Initial optimization of transfection conditions was required to identify kinase expression level that produced detectable changes in total cell phosphotyrosine between controls.

**Co-immunoprecipitation assay**

HEK293T cells were transfected and lysed as described for the in-cell kinase activity assay. Following centrifugation of lysates, 20 µL was removed from each sample to assess lysate protein expression. To the remaining lysate, 2 µL Anti Flag M2 (1 mg/mL) was added to each sample and placed on rotating mixer at 4 C for 3 h. Following incubation, 30 µL of PureProteome™ Protein G magnetic beads (Millipore Sigma) were added to each sample and incubated on rotating mixer at room temperature for 15 m. Following incubation, samples were washed 3X with IP lysis buffer and resuspended in sample buffer, boiled for 5 m, and stored at 4 C. Samples were Western blotted for indicated proteins.

**Identification of effector binding sites**

Analysis of Src effector binding sites was accomplished by searching the Research Collaboratory for Structural Bioinformatics Protein Data Dank (RCSB PDB) for x-ray crystal structures of Src bound to other proteins. We identified the following structures: active Src
(1Y57), Csk (1K9A), Cbl-c (3VRO), PTPN-22 (3BRH), and p130cas (1X27). The structures were loaded in PyMol and aligned using Src as a common reference point.

**Cell Rounding**

HEK293T cells were plated on fibronectin coated coverslips and allowed to spread overnight. The next day, cells were transfected with indicated constructs using Fugene6 (Promega) according to manufacturer’s protocol. At 24 h post-transfection cells were imaged in Ham’s F-12 (Kaighn’s Modification) (Caisson Laboratories, Inc) supplemented with 1 mM HEPES and 5% FBS. Images were acquired using differential interference contrast (DIC) microscopy. Image fields were scored for the number of rounded cells by a blinded evaluator.

**Modeling of binding curves**

We used the law off mass action to model the binding equilibrium formed between Src and SspB. We describe the reversible binding of SspB to Src in terms of their equilibrium dissociation constant, $K_D$.

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[SspB_{free}][Src_{free}]}{[Complex]}$$

Substitution of the equation to describe $K_D$ solely in terms of total concentrations of SspB, Src, and the SspB-Src complex gives:

$$K_D = \frac{([SspB_{total}] - [Complex])([Src_{total}] - [Complex])}{[Complex]}$$

Rearrangement of the equation and application of the quadratic formula yields:

$$[PA] = \frac{([SspB_{total}] + [Src_{total}] + [K_D]) \pm \sqrt{([SspB_{total}] + [Src_{total}] + [K_D])^2 - 4[SspB_{total}][Src_{total}][K_D]}}{2}$$

$$\% \text{ Src Bound} = \frac{[PA]}{[Src_{total}]} \times 100$$
We input the final formula into MatLab to plot binding curves for a range of SspB concentrations given the concentration of Src and affinity of their interaction.

**High content assay**

High-content live cell FRET imaging was performed as described previously. Briefly, HEK293T cells were seeded onto 96-well plate and cells were transfected with the indicated constructs with Lipofectamine and Plus reagent (Invitrogen) according to manufacturer’s protocol. Plates were imaged with an automated microscope (Olympus IX-81 with automated stage, filter wheels and X/Y/Z stage with autofocus) and Metamorph software was used to image each well for CFP, YFP, and FRET emission. A custom-written MatLab script was used to calculate the sum intensity of each well and perform background subtraction, bleed-through corrections, and normalization of FRET/Donor ratios for each well.

**Fluorometer assay**

Fluorometer assays were performed as described previously. Briefly, HEK293T cells were seeded into 6-well plates and transfected with indicated constructs with Lipofectamine and Plus reagent (Invitrogen) according to manufacturer’s protocol. At 24 h post transfection cells were harvested by trypsinization, resuspended in DPBS (10% FBS). Cells were then centrifuged at 1000 rcf, supernatant was removed, and cells were resuspended in 700 µL DPBS (10% FBS), and placed in Eppendorf tubes on ice, protected from light. To collect CFP and FRET emission, samples were excited at 433 nm and emission was collected from 450 to 600 nm. To collect YFP emission, samples were excited at 505 nm and its emission at 525 nm was measured. Cells transfected with empty cDNA (pBABE-puro) was used to measure autofluorescence and light.
scatter, which was subtracted from the data. FRET emission was corrected for bleed-through and FRET/Donor ratio was calculated by dividing corrected FRET emission at 525 nm by CFP emission at 474 nm.

**Immunofluorescence**

For measurement of endogenous Src expression level and variability. MEF cells were processed as follows: Cells were fixed with 3.7% paraformaldehyde for 20 min at room temperature and washed 3X with DPBS. Cells were permeabilized with triton-X-100 0.1% solution in DPBS for 5 min and washed 3X with DPBS. Cells were blocked with 1% BSA and 1% FBS in DPBS for 1 h at room temperature. Cells were stained with primary and secondary antibodies at indicated dilutions in blocking buffer for 1 hour and washed 3X with DPBS after each incubation.
CHAPTER 4  CONTROLLING TUMOR CELL INVASION WITH A LIGHT-ACTIVATABLE COFILIN ANALOG

4.1 Introduction

Light-sensitive protein analogs have provided powerful tools for probing biological phenomena because they provide precise control in space and time. Many of the approaches for generating these analogs have focused on engineering light-switchable steric inhibition of binding or active sites. In a previous study, we used the photosensitive LOV2 domain from *Avena sativa* phototropin 1 (AsLOV2) to control the small GTPase Rac1. In response to blue light, the LOV2 domain undergoes a conformational change that causes LOV2’s C-terminal helix Jα to unwind. Fusing Rac1 to Jα sterically blocked Rac1 effector binding in the dark, but upon irradiation the Jα helix unwound, freeing Rac1 to interact with its effectors. Later studies have suggested that efficient inhibition of Rac1 by LOV2 was dependent on weak interactions between the two domains, which may not be present in other target proteins. We recently developed Zdark (Zdk), a protein A fragment that binds selectively to the dark state of LOV2. We hypothesized that Zdk could be used to enhance steric inhibition by controlling LOV2 orientation in the dark (Figure 4.1A). In this approach, Zdk and LOV2 are attached to the N- and C-termini of a protein of interest, respectively. In the dark, Zdk and LOV associate to sterically block a protein’s binding or active site. Upon irradiation, they diffuse away to relieve steric inhibition.
We named this approach Zlock, for Zdk-mediated locking of LOV2. We focused on the actin-binding protein cofilin because of evidence that precise regulation of its activity in space

Figure 4.1 Design of Zlock Cofilin

A. Design scheme for Zlock cofilin. Cofilin (grey) is fused to Zdk (green) at its N-terminus and LOV (blue) at its C-terminus. In the dark, Zdk binding to LOV blocks cofilin binding to F-actin (purple)

B. Actin co-sedimentation assay to measure binding of cofilin to F-actin. Wild-type cofilin and constitutively inactive cofilin S3E mutant are shown as controls. Light-dependent binding to F-actin was tested using LOV2 mutants that mimic the dark state and lit state.

We named this approach Zlock, for Zdk-mediated locking of LOV2. We focused on the actin-binding protein cofilin because of evidence that precise regulation of its activity in space
Cofilin severs F-actin filaments to drive remodeling of the actin cytoskeleton and is essential for certain types of directed cell migration. Misregulation of the cofilin pathway is a key driver of several disease states including cancer metastasis and inflammatory disorders.

4.2 Results and Discussion

Cryo-EM structures of cofilin bound to F-actin show that while the C-terminus is free and solvent exposed, the N-terminus makes several contacts with F-actin. Therefore, we first tested whether fusion of Zdk to cofilin’s N-terminus would affect F-actin binding (Figure 4.2). To do so, we relied on an in vitro actin co-sedimentation assay that measures the ability of a protein to co-sediment with actin following ultracentrifugation (Figure 4.3).

Figure 4.2 Diagram of Zdk-Cofilin Fusion Protein
Zdk fusion with a GGGSG linker (G = glycine, S = serine) did not appear to affect either F-actin binding (Figure 4.4A) or cofilin’s ability to convert F-actin to G-actin (Figure 4.4B). Previous studies have demonstrated that GFP fusion to cofilin’s C-terminus does not interfere with cofilin function and therefore LOV fusion was also unlikely to affect cofilin function.  

Figure 4.3  Validation of Actin Co-sedimentation Assay

*Upper:* SDS-PAGE gel of supernatant (S) and pellet (P) fractions from actin co-sedimentation assay.  
*Lower:* Western blot of supernatant and pellet fractions from actin co-sedimentation assay. Western blot was used to assess Zlock cofilin designs due to the similar molecular weight of Zlock cofilin and actin. Wild-type cofilin, constitutively active mutant cofilin S3A, or constitutively inactive mutant cofilin S3E were tested to validate ability of assay to measure cofilin binding to F-actin. Samples were run with and without actin to test dependence of cofilin pelleting on interaction with F-actin. F-actin and purified cofilin were incubated for 30 minutes at room temperature prior to ultracentrifugation. Reactions were run at pH 7.5 to prevent cofilin severing and generation of G-actin.
To engineer light-switchable steric inhibition of cofilin, we needed to attach LOV2 and Zdk to cofilin with linkers of appropriate length and composition that would correctly position the Zdk-LOV complex to block cofilin F-actin binding in the dark state but not the lit state. To design linkers, we performed Rosetta structure prediction simulations and assessed orientation of the Zdk-LOV complex. We were able to achieve precise positioning of the complex over the actin binding interface only if linkers of intermediate length were used (Figure 4.5A).

Figure 4.4 Validation of Zdk-Cofilin Fusion Protein

A. Actin co-sedimentation assay for Zdk2-cofilin fusion proteins with different linkers.
B. Actin co-sedimentation assay for Zdk2-cofilin fusion proteins with different linkers. F-actin and purified cofilin were incubated for 2 hours at room temperature. Samples were tested at pH 6.8 and 7.5 to measure ability of cofilin to convert F-actin to G-actin.
If linkers were too long, the complex failed to reliably orientate over cofilin’s actin binding site. Conversely, if linkers were too short, the Rosetta algorithm was unable to connect the complex to cofilin, indicating Zdk and LOV would be unable to bind each other in the dark. Next, we tested whether attaching Zdk and LOV to cofilin with our optimized linkers would block F-actin binding. We tested two variants of Zdk (Zdk1 and Zdk2) in our initial designs that differed in affinity and LOV2 binding orientation. Using Zdk1 achieved a modest ($\approx 50\%$) reduction in F-actin binding in the dark state that fully recovered in the lit state (Figure 4.5B). Using Zdk2 achieved robust inhibition ($\approx 95\%$) in the dark state, but surprisingly did not recover in the lit state (Figure 4.1B).

Next, we attempted to improve the dark state inhibition of the Zdk1 design and lit state binding of the Zdk2 design. Comparing structural models of Zdk1 and Zdk2 designs revealed a
potentially strained linker conformation connecting Zdk1 to cofilin, which we hypothesized was hindering Zdk1 binding to dark state LOV2 (Figure 4.5A). To improve the Zdk1 design, we focused on optimizing linkers connecting Zdk1 to cofilin. We tested whether removing a proline and/or lysine from the C-terminus of Zdk1 would enhance linker flexibility and improve binding to dark state LOV2 (Figure 4.5B). The best linker resulted in a 3-fold reduction in dark state binding, however lit state binding was also reduced.

To improve the Zdk2 design, we focused on optimizing Zdk2 affinity. We hypothesized that Zdk2 was binding to LOV2 in the lit state and blocking F-actin binding. This hypothesis is supported by Zdk2’s significant affinity for the LOV2 lit state (761 ± 78 nM) and the high local concentration of LOV2 relative to Zdk2 that arises because the two proteins are physically linked together (Figure 4.6).

We reasoned that lowering Zdk2 affinity would facilitate dissociation from LOV2 in the lit state. Therefore, we performed Rosetta mutational analysis to identify point mutants that
would modestly decrease Zdk2 affinity. Positions to mutate were chosen based on two criteria (Figure 4.7A). First, we avoided mutating residues contacting the Jα helix, which could reduce selectivity for dark state LOV2. Second, we only considered mutation of non-polar residues to other non-polar residues, which are more accurately modeled by Rosetta. To assess each potential mutation, we used Rosetta to calculate the change in Gibbs free energy ($\Delta \Delta G$) for Zdk2 in isolation and the Zdk2-LOV2 complex, which yielded $\Delta \Delta G$ of binding (Figure 4.7B). We selected several Zdk2 mutants based on their modest reduction in binding affinity and neutral effect on Zdk2 stability.

Figure 4.7    Selection of Zdk2 Mutants and Computational Approach.  
A. Four residues (shown in red) along the first and second helixes of Zdk2 were mutated to generate a reduced affinity variant of Zdk2.  
B. The change in Gibbs free energy ($\Delta \Delta G$) for Zdk2 mutants was calculated for the Zdk2-LOV2 complex ($\Delta \Delta G$ complex) and Zdk2 in isolation ($\Delta \Delta G$ Zdk2). Subtracting the two values yielded the change in binding energy of the complex ($\Delta \Delta G$ binding).
All Zdk2 mutants tested displayed increased F-actin binding relative to wild type Zdk2 (Figure 4.8). Our best performing mutant, Zdk2 I32F, exhibited a roughly five-fold increase in F-actin binding for the lit relative to dark state, which we named Zlock coflin (Figure 4.1B).

![Figure 4.8 Light-dependent Change in Actin Binding for Zdk2 Mutants.](image)

Actin co-sedimentation assay to measure binding of coflin to F-actin. Wild type and inactive S3E mutant coflin are shown as controls. Zdk2 mutants are shown on the x-axis. Dark and lit state mutants were used to assess light-dependent changes in coflin F-actin binding.

The coflin pathway has been implicated in tumor cell migration during the early stages of metastasis. A previous study utilized a photoactivatable coflin analog to examine how coflin contributed to this process. Localized coflin activation was found to stimulate actin polymerization, cell protrusion, and directed cell migration. However, coflin activation with this probe was irreversible and led to accumulation of active coflin, which could potentially alter cell behavior and therefore made interpretation of these findings difficult. To address these concerns,
we assessed the effects of Zlock cofilin photoactivation at the cell edge, as activation of Zlock cofilin is reversible and does not result in accumulation of active cofilin.

Zlock cofilin or light-insensitive Zlock cofilin (LOV2 constitutively locked in the dark state) was expressed in a breast cancer cell line (MTLn3) and we assessed the effect of cofilin photoactivation via time lapse fluorescent microscopy. Analysis of cell perimeters before and after photoactivation revealed localized protrusion at the site of photoactivation (indicated by yellow dot) for Zlock cofilin but not the light-insensitive mutant (Figure 4.9A). We also assessed the directionality of cell movement before and after photoactivation by measuring the cosine of the angle between the site of photoactivation and the vector direction of cell movement (Figure 4.9B). Photoactivation resulted in reorientation of the vector toward the spot of photoactivation and consequently a significant increase in cosine values. No change in cosine values was observed for the light-insensitive control. We next investigated the effect of global photoactivation on F-actin levels in MTLn3 cells (Figure 4.9C). Photoactivation resulted in a significant increase in F-actin at 3 minutes post photoactivation, closely paralleling the increase in F-actin following stimulation with EGF.
Metastasis requires dissemination of primary tumor cells to distant organs where they
form secondary tumors.\textsuperscript{101,145} A key step in this process is tumor cell invasion into blood vessels,
which is enabled by invasive, matrix-degrading protrusions termed ‘invadopodia’.\textsuperscript{146} Previous
experiments relying on genetic perturbations have shown that cofilin is involved in the

Figure 4.9  Control of Cell Migration and Actin Polymerization

A. Changes in cell perimeter before (left) and after (right) photoactivation. Retraction = red;
protrusion = green; no change = grey. Site of photoactivation is indicated by yellow circle with
blue outline. Zlock cofilin with a light-insensitive mutant is shown as a control.

B. Analysis of directional migration in response to photoactivation of either Zlock cofilin or light-
insensitive control. The cosine of the angle between the site of photoactivation and the vector of
cell movement were calculated for two minutes before (Pre-PA) and after photoactivation (Post-
PA). Photoactivation led to increase in cosine value for Zlock cofilin (p-value = 0.03; n = 8 cells,
paired two-tailed t-test) but not the light insensitive control (p-value = 0.58; n = 9 cells, paired two-
tailed t-test). Cosine value for Pre-PA Zlock cofilin and light insensitive control were not
significantly different (p-value = 0.34, unpaired two-tailed t-test). Error bars represent mean ±
SEM.

C. F-actin content of MTLn3 cells expressing Zlock cofilin that were either unstimulated (n = 34
cells), stimulated with 5 nM EGF (n = 56 cells), or photoactivated (PA) (n = 51 cells). F-actin
content was assessed following fixation and phalloidin staining. PA cells were irradiated for one
minute and fixed three minutes after start of photoactivation. Phalloidin intensity was significantly
different for both EGF (p < 0.001) and PA (p < 0.001) relative to unstimulated cells. EGF and PA
phalloidin intensity were not significantly different (p = 0.208). P-value was calculated with
unpaired two-tailed t-test. Error bars represent mean ± SEM.
stabilization of invadopodium precursors in tumor cells following EGF stimulation.\textsuperscript{147,148} However, these studies lacked sufficient temporal resolution to determine whether the transient activation of cofilin that occurs after EGF stimulation directly contributes to precursor stabilization. We therefore assessed the effect of cofilin photoactivation on invadopodium precursors (Figure 4.10A and B). MTLn3 cells expressing either Zlock cofilin or the light-insensitive control were globally irradiated to activate cofilin for one minute, which mimics the kinetics of cofilin activation following EGF stimulation. The number of invadopodium precursors at different time points were measured quantifying the number of cortactin- and Tks5-positive puncta per cell, which were identified by immunostaining. Photoactivation led to a significant increase in invadopodium precursors five minutes after photoactivation (Figure 4.10C). No significant change was observed for the light-insensitive control at any time point. Notably, our results closely match previous findings that precursor formation peaks at five minutes following EGF stimulation.
In conclusion, we have developed a new approach to fully-reversible optogenetic control via light-switchable steric inhibition with Zdk and LOV2. Application of this approach to cofilin generated a new analog that could control actin dynamics in live cells and revealed a key role for cofilin in regulating tumor cell invasion. In the future, Zlock cofilin can potentially be used in live animals to assess how cofilin activation contributes to tumor cell migration \textit{in vivo}.

Figure 4.10  Cofilin Activation Drives Tumor Cell Invasion

A. Effect of photoactivation on number of invadopodium precursors in MTLn3 cells expressing Zlock cofilin or a light-insensitive control. Error bars represent mean ± SEM. Zlock cofilin (0 min: n = 41 cells; 1 min: n = 57 cells; 3 min: n = 51 cells; 5 min: n = 46 cells; 30 min: n = 54). Control (0 min: n = 48; 1 min: n = 48; 3 min: n = 51; 5 min: n = 45; 30 min: n = 43).

B. Representative image of MTLn3 cell stained for Tks5 and cortactin to identify invadopodium precursor.

C. Change in number of invadopodium precursors per cell following photoactivation based on data in Figure 4.10A. Photoactivation resulted in a significant increase of invadopodium precursors for Zlock cofilin (p = 0.03, unpaired two-tailed t-test) but not the light insensitive control (p = 0.55, unpaired two-tailed t-test).

4.3 Conclusion

In conclusion, we have developed a new approach to fully-reversible optogenetic control via light-switchable steric inhibition with Zdk and LOV2. Application of this approach to cofilin generated a new analog that could control actin dynamics in live cells and revealed a key role for cofilin in regulating tumor cell invasion. In the future, Zlock cofilin can potentially be used in live animals to assess how cofilin activation contributes to tumor cell migration \textit{in vivo}.
4.4 Materials and Methods

Antibodies, DNA Constructs, and Transfection

Antibodies were from the following sources: Cofilin (D3F9) XP® Rabbit mAb (Cell Signaling #5175), β-Actin (8H10D10) Mouse mAb (Cell Signaling #3700), Tks5 (Santa Cruz Biotechnology; sc-30122), Cortactin (Abcam; ab33333). The cDNA of the LOV2 domain from Avena sativa (oat) Phototropin1 (L404-L547) was used to generate photo-sensitive constructs. Three variants of LOV2 were used: wild-type, dark mutant (C450A, L514K, G528A, L531E, and N538E), and lit mutant (I510E/I539E). The cDNA of full-length rat cofilin was used for all constructs. The Z affibodies the selectively bind dark state LOV2 have been described elsewhere. For transient expression in mammalian cells, constructs were cloned into pmCherry-C1. Cells were transfected with Lipofectamine 2000 (Life Technologies) using manufacturer’s protocol and left to express the plasmid(s) for 24 h before imaging. For single cell live imaging, cells were co-transfected with mCherry Zlock cofilin and a membrane-anchored YPet (KRas C-terminus) to visualize the cell edge.

Cell Culture

Rat mammary adenocarcinoma, MTLn3 cells were cultured in MEM-alpha media (Gibco; cat # 12561-056), supplemented with 5% FBS (Gemini Bio-Products; cat # 100106). Cells were maintained in incubator set at 37 C and 5% CO2.

Protein expression

Proteins were cloned into the bacterial expression vector pET-14b (Novagen) containing a N-terminal His6-tag. The proteins were expressed in Escherichia coli strain BL21(DE3) (New
England BioLabs). At OD$_{600} = 0.8 – 1.0$, cultures were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma). Constructs utilizing lit mutant LOV2 (I510E/I539E) were induced for two hours at 37 C and all other constructs were induced for 5 hours at 37 C. Cultures were pelleted at 5000 RCF and stored at -80 C until purification.

**Protein purification**

Bacterial pellets were thawed and resuspended in phosphate buffer (His Buffer Kit, GE Healthcare) and lysed with BugBuster (EMD Millipore) for 20 minutes at room temperature. Lysate was cleared via centrifugation at 10,000 RCF and imidazole was added to a final concentration of 40 mM. Proteins were purified using a His GraviTrap (GE Healthcare). Column was equilibrated with phosphate buffer containing 40 mM imidazole. Lysate was applied to column and column was washed with phosphate buffer containing 40 mM imidazole. Proteins were eluted with phosphate buffer containing 500 mM imidazole. Purified proteins were concentrated with Amicon® Ultra-4 (Millipore Sigma) and buffer exchanged (10 mM Tris, 50 mM NaCl, pH 7.4) with Zeba™ Spin Desalting Columns (Fisher Scientific).

**Actin Co-sedimentation Assay**

Assay was performed using Actin Binding Protein Spin-Down Assay Biochem Kit (Cytoskeleton, Inc.) according to manufactures protocol with the following modifications. Reaction mixture contained test protein at a final concentration of 8 µM and F-actin at a final concentration of 12 µM. Reactions were incubated at room temperature for 30 minutes and centrifuged for 1 hour at 164,000 RCF. Supernatant was removed and pellets were resuspended in 50 uL Milli-Q water. Samples were combined with Laemmlı sample buffer and boiled for 5 minutes and stored at 4 C.
Whole-cell photoactivation

Cells were plated on glass-bottom dishes (MatTek Corporation) and allowed to spread overnight protected from light. For F-actin staining cells were plated on acid washed dishes. For invadopodium precursor staining cells were plated on gelatin coated dishes. All work was performed under red light to prevent unintentional photoactivation. Cells were serum-starved 4 hours prior to photoactivation in Leibovitz's L-15 media (Gibco) containing 0.35% BSA. Photoactivation was accomplished with a 470 nm LED array (Mouser Electronics, Inc. part # 828-OVQ12S30B7). During photoactivation protocol, cells were maintained in a cell culture incubator set to 37 C and 5% CO2.

Immunofluorescence

For F-actin and invadopodia analysis MTLn3 cells were processed as follows: Cells were fixed with 3.7% paraformaldehyde for 20 min at room temperature and washed 3X with PBS. Cells were permeabilized with triton-X-100 0.1% solution in PBS for 5 min and washed 3X with PBS. Cells were blocked with 1% BSA and 1% FBS in PBS for 1 h at room temperature. For invadopodia analysis: Cells were stained with primary and secondary antibodies at indicated dilutions in blocking buffer for 1 hour and washed 3X with PBS after each incubation. For F-actin analysis: DyLight™ 488 Phalloidin (Cell Signaling #12935) was incubated with cells at 1X concentration for 20 min and washed 3X with PBS.

Gelatin Coating

Glass bottom MatTek dishes were acid-washed (1N HCl for 10 min) and coated with poly-l-lysine (50 µg/mL for 20 min) followed by gelatin coating (0.2% gelatin for 10 min). Gelatin matrix was then crosslinked (0.1% glutaraldehyde for 15 min) and inactivated (5 mg/ml
NaBH4 for 15 min). After each step dishes were washed (3 x 5 min PBS). Dishes were stored at 4C in 10X Pen-Strep. All solutions were prepared fresh immediately before use.

**Single cell live imaging**

Cells were plated on acid-washed glass bottom MatTek dishes and allowed to spread overnight protected from light. Prior to imaging cells were serum starved in Leibovitz's L-15 media (Gibco) containing 0.35% BSA. A closed heated chamber was used during live cell imaging, which was performed using a Zeiss LSM 880 confocal microscope using a Plan-Apochromat 63x oil objective (N.A. 1.40). ZEN software (Zeiss) was used to control the microscope and acquire images at each time point. A GaAsP detector with tunable emission collection windows was used for detection. YFP images were acquired using a 514 nm Argon laser (25% power) with a collection window of 525 – 580 nm. mCherry images were acquired using a 561 nm DPSS laser (20% laser power) with a collection window of 580 – 650 nm. LOV2 photoactivation was accomplished with a 488 nm Argon laser (1% power) that irradiated a preselected region every 10 seconds. Images were acquired every 2.5 seconds.

**Directionality Analysis**

Changes in cell directionality was using the directionality index, which is defined as the cosine of the angle between the site of photoactivation and the vector direction of cell movement. The vector direction of cell movement was determined by measuring the cell centroid at two different time points using ImageJ. The site of photoactivation was determined by measuring the centroid of the photoactivation ROI using ImageJ. The directionality index was assessed for two intervals. First, two minutes prior to photoactivation until the time of photoactivation, which measured cell movement before photoactivation. Second, from the time of photoactivation until
two minutes after, which assessed potential changes in directionality in response to photoactivation.

**Measurement of F-actin content**

Cells fixed and stained with phalloidin were imaged on an Olympus IX-81 microscope equipped with an UPlanFLN 40x objective (Pil, N.A 1.30). Metamorph software (Molecular Devices) was used to control the microscope and acquire images. Dylight 488 and mCherry images were acquired using a 100 Watt mercury arc lamp with a 1% ND filter and a 500-550 nm or 565–595 nm band-pass filter respectively, with 1 second exposure for each channel. Flat field correction was applied to each image using a custom MatLab script. Corrected images were thresholded with Otsu's method using ImageJ to generate masks for individual cells. For each image, a region without cells was used to determine background intensity for background subtraction. Mean phalloidin intensity (shade corrected and background subtracted) was measured for individual cells using previously generated masks. Experimental replicates were imaged on the same day to enable comparison based on signal intensity. Cell intensities for each condition were averaged for sake of comparison.

**Invadopodium Precursor Analysis**

Cells were fixed and stained with appropriate antibodies and imaged on a Delta Vision epi- fluorescence microscope (Applied Precision Inc.), equipped with a CoolSNAP HQ2 camera and a 60x, NA 1.42 objective lens. Invadopodium precursors were identified as Tks5 and cortactin positive puncta. The number of invadopodium precursors per cell were counted and averaged for each condition.
Modeling of linkers connecting Zdk and LOV2 to cofilin

Structural models were generated with the RosettaRemodel package with the Rosetta3.5 series of software. This package was designed to provide a framework for flexible protein design utilizing the loop modeling tools in Rosetta. In this case, we used the domain insertion protocol to model the orientation of the Zdk-LOV complex relative to cofilin with Zdk and LOV attached to the N- and C-terminus of cofilin (PDB 4BEX), respectively. For both Zdk1 (PDB 5EFW) and Zdk2 (PDB 5DJT), we modeled linkers of different length and composition and assessed whether the Zdk-LOV complex reliably orientated over the actin binding interface of cofilin.

Computational identification of Zdk2 point mutants

The change in binding energy for Zdk2 point mutants was calculated with the ddG monomer package with the Rosetta3.5 series of software. The package was designed to predict the change in stability (the ddG) of a protein induced by a point mutation. In this case, we calculated the ddG induced by several point mutants for both Zdk2 and the Zdk2-LOV complex, using the PDB structure 5DJT as a starting point. Subtracting the ddG for Zdk2 from the ddG for the Zdk2-LOV complex yielded the change in binding energy. The shift in binding curve was calculated using the following equation:

\[ \Delta\Delta G = -RT\ln \frac{K_{d2}}{K_{d1}} \]

where \(\Delta\Delta G\) is the change in binding energy, \(R\) is the gas constant, \(T\) is the temperature, and \(K_{d2}/K_{d1}\) is the shift in the binding curve.
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