Inducible Protein Dimerization: New Tools and Applications to Understanding the Mitotic Checkpoint

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Abstract
Cellular processes such as growth, migration, signaling and cell division require choreographed interactions between dozens or hundreds of proteins carefully organized in time and space. In order to test hypotheses about complex cellular functions, it is desirable to experimentally perturb the interactions of individual proteins that perform these functions with a level of spatial and temporal control commensurate with the time and space scales over which the system is naturally organized. Inducible protein dimerization offers the ability to experimentally control protein-protein interactions. Inducible dimerization can be used to test the immediate effects of dimerizing two proteins, or it can be engineered to create or destroy a protein or change a protein's localization. Several different techniques for inducible dimerization using small molecules or light have been developed, each with its own strengths and weaknesses. Ultimately, only light-inducible dimerization offers the potential for both temporal and spatial experimental control. In this thesis, I describe the application of inducible dimerization to further our understanding of a complex signaling network, the Mitotic Checkpoint, which monitors chromosome segregation and is regulated by the localization of its constituent checkpoint proteins. I discovered that relocating a single key checkpoint protein, Mad1, to kinetochores at metaphase is sufficient to reactivate the checkpoint. I also describe the development of a novel photochemical technique which has allowed us to achieve light-induced dimerization at centromeres, a cellular compartment which has not been successfully targeted by previously reported light-inducible dimerization systems. This technology enables us to perform experimental biology on living cells with a new level of spatial and temporal control.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Biochemistry & Molecular Biophysics

First Advisor
Michael A. Lampson

Keywords
Chemical Biology, Chemical Dimerization, Mad1, Mitotic Checkpoint, Rapamycin, Spindle Assembly Checkpoint

Subject Categories
Biology | Chemistry | Molecular Biology

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ACKNOWLEDGMENTS

I would like to acknowledge Michael Lampson, who has been an excellent advisor and mentor since welcoming me into his lab in 2009. Thank you, Mike, for having faith in me and giving me the freedom to pursue my own ideas, and for arranging for me to work with Zach Feldman, Muyi Li, Michelle Riegman and Alyssa Mayo. To Zach, Muyi, Michelle and Alyssa: thanks for all your help and hard work. I really enjoyed teaching you what I could and working alongside you. It was a privilege to work with such bright, motivated and fun people, and to watch you all pursue your own bright futures. May you always remember how to clone! Thanks also to the rest of the Lampson Lab for your help and ideas, for making lab a fun group to belong to. Thanks especially to my bay-mate, Lukas Chmatal, for setting an inspiring standard of scientific enthusiasm and excellence, for always being interested in hearing about my results and sharing yours, and for countless afternoon coffee breaks.

Thanks to David Chenoweth, for enthusiastically supporting my chemistry projects and opening his lab to me, you’ve really been a second mentor. Thanks to Bob Rarig for so patiently and clearly teaching me everything I now know about organic synthesis, and for being such a fun guy to hang out with late at night with the lights turned low. A huge thanks to Jay Aonbangkhen for being such a great partner on the dimerizer synthesis. Without you, Jay, I’d only have immeasurably small quantities of poorly characterized compounds! I look forward to seeing what you dream up with trifunctional linkers, etc.

Thanks to Ben Black, Ekaterina Grischuk and their labs for our wonderful Friday morning joint group meetings, and for your valuable research advice. I learned a lot from
your insightful and critical discussions of the literature. Thanks also to Ben and Kevan Salimian for welcoming me into the Aurora B enrichment project. I’m very proud we’re linked in the literature as co-authors. Thanks also to the rest of my thesis/dissertation committee: Mark Lemmon, Phong Tran, Michael Ostap, Ravi Radhakrishnan and Silke Hauf for your support and guidance.

Thanks to Josh Wand, Kate Ferguson, Ruth Keris and the whole BMB/B&B community for making Penn a great place to be a grad student.

I thank my wonderful friends Dave Slochower, Nick Bessman, Kathleen Molnar and Liz Sweeny, Matt Sochor, Lauren Naliboff, Matt Puster and Kacy Wander for your friendship and support over the last seven years. I had a LOT of fun with you guys. Bikes, baseball, beer, barbecue: I loved every minute of it. I’m sad we can’t all do postdocs in the same city, but I look forward to visiting you wherever you go next, and hosting you all in England.

I thank my parents Ronnie and Ray, my sisters Darcy and Caity, and the rest of the Ballister family for being so understanding and supportive of my move out to the East Coast. I’ve missed you all so much, sorry I had to go so far.

Finally, and most of all, I thank Laurel MacKenzie.
ABSTRACT

INDUCIBLE PROTEIN DIMERIZATION: NEW TOOLS AND APPLICATIONS TO UNDERSTANDING THE MITOTIC CHECKPOINT

Edward Raymond Ballister
Michael A. Lampson

Cellular processes such as growth, migration, signaling and cell division require choreographed interactions between dozens or hundreds of proteins carefully organized in time and space. In order to test hypotheses about complex cellular functions, it is desirable to experimentally perturb the interactions of individual proteins that perform these functions with a level of spatial and temporal control commensurate with the time and space scales over which the system is naturally organized. Inducible protein dimerization offers the ability to experimentally control protein-protein interactions. Inducible dimerization can be used to test the immediate effects of dimerizing two proteins, or it can be engineered to create or destroy a protein or change a protein’s localization. Several different techniques for inducible dimerization using small molecules or light have been developed, each with its own strengths and weaknesses. Ultimately, only light-inducible dimerization offers the potential for both temporal and spatial experimental control. In this thesis, I describe the application of inducible dimerization to further our understanding of a complex signaling network, the Mitotic Checkpoint, which monitors chromosome segregation and is regulated by the localization of its constituent checkpoint proteins. I discovered that relocating a single key checkpoint protein, Mad1, to kinetochores at metaphase is sufficient to reactivate the
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Chapter 1: Inducible dimerization: principles and techniques

Detailed outline of Chapter 1

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4) Conclusions
1) Summary

Inducible dimerization is a powerful technique for controlling the function of proteins in living systems. Inducible dimerization refers to any system in which a pair of proteins can be forced into a high-affinity interaction by means of an experimental intervention such as chemical addition or light exposure. Dimerization can be used to activate or degrade a protein, reconstitute a “split” protein, or change the localization of a protein, among other possible effects. Inducible dimerization can function on a timescale of seconds, which is much faster than genetic manipulations, transcriptional induction or RNAi. Light-inducible dimerization can be applied in a spatially controlled manner on a subcellular scale. In the first section of this chapter, I discuss the diversity of applications of inducible dimerization. In the second section, I then describe the most commonly used dimerization techniques, and weigh their relative strengths and weaknesses, highlighting areas for further development, particularly within light inducible dimerization.

2) Dimerization schemes and applications

2a) Introduction

Although conceptually simple, dimerizing two proteins can have an almost limitless number of effects and be used to perturb and study almost any process in biology. Inducible dimerization can be achieved using any protein or pair of proteins that can be forced into a tight association by an externally controlled intervention (Figure 1.1 A), such as the addition of a chemical or light (Figure 1.1 B,C). When two copies of the same protein are dimerized, this process is called homodimerization; when different proteins are dimerized, heterodimerization (Figure 1.1 D,E). Through the use of
Figure 1.1: schematic diagrams illustrating the basic principles of inducible dimerization. 

**A:** Inducible dimerization is triggered by an experimental intervention. 

**B:** In chemically induced dimerization, the experimental intervention is the addition of a small molecule. 

**C:** In light induced dimerization, the experimental intervention is illumination with a specific wavelength of light. 

**D:** Homodimerization 

**E:** Heterodimerization 

**F:** Inducible dimerization is a general technique: arbitrarily chosen proteins can be inducibly dimerized by genetic fusion to a pair of proteins with desired dimerization properties.
recombinant DNA technology, these dimerizing proteins can be genetically fused to, theoretically, any proteins of interest, making inducible dimerization a general technique for controlling protein interactions (Figure 1.1 F). Although I will use the term “dimerization” throughout this chapter, in some cases this refers to oligomerization rather than monodisperse 1:1 dimerization. For this review, I have grouped the applications of dimerization into those intended to control a specific biochemical interaction between two proteins, and those intended to control the subcellular localization of a protein of interest. I have chosen several examples from the literature that illustrate the diversity of schemes to which inducible dimerization has been applied.

2 b) Dimerization that induces a specific biochemical interaction

2 b i) Dimerization to turn proteins on: cell signaling pathways

Controlling biochemical activity, without directly changing localization, was the first application of inducible dimerization as a general technique in seminal work from the Schreiber and Crabtree labs (Spencer et al., 1993) (Figure 1.2 A). In this study, the authors fused the intracellular domain of T-cell receptor (TCR) zeta to an engineered membrane-tethered version of FKBP12, a protein that could be homodimerized by the addition of a semisynthetic small molecule. Induced dimerization using this system was found to be sufficient to activate the TCR signaling pathway, in the absence of any native TCR ligand and without the TCRzeta transmembrane domain. Previous work, including dimerization experiments using antibodies, had established that dimerization is a key step in the signaling of a number of membrane-bound receptors (Rönnstrand et al., 1988; Yarden, 1990; Spaargaren et al., 1991), but this was the first report of a general system that could be applied to almost any protein of interest.
**Figure 1.2**: activation or inhibition of a target protein by inducible dimerization.  

**A**: Positive regulation by induced dimerization. Arrows represent subsequent steps in a biological pathway driven by the activated white triangle protein, and stars represent an observed or measured biological effect.

**B**: Negative regulation by induced dimerization. A constitutively active protein is inhibited, causing some observable biological effect.
Inducible homodimerization, heterodimerization and oligomerization have subsequently been applied to study many signaling proteins, including receptor kinases such as TGFbeta receptor and EGF receptor (Stockwell and Schreiber, 1998; Muthuswamy et al., 1999), or even unrelated pairs of receptors, such as cKit and Flt-3 (Otto et al., 2001). Once it is established that dimerization is sufficient to activate a given pathway, dimerization can be used as a tool to study the broader biology of that pathway, as in a cancer model utilizing inducible dimerization of FRGR1 (Welm et al., 2002) or even, potentially, for therapeutic applications, such as inducing proliferation of a genetically modified hematopoietic stem cell population via dimerization of the thrombopoietin receptor Mpl1 (Jin et al., 1998; Okazuka et al., 2011).

Apoptosis is another process that has been fruitfully explored using inducible dimerization. Another classic work by the Schreiber and Crabtree labs established that dimerization of the Fas protein is sufficient to trigger apoptosis in tissue culture and targeted cell populations in mice (Belshaw et al., 1996b; Spencer et al., 1996). Inducible dimerization has been applied to at least 10 different proteins in the apoptosis pathway: Fas, FADD, Asc, Apaf, Bid, Bax and caspases 1, 3, 8 and 9 (Spencer et al., 1996; Hu et al., 1998; Fan et al., 1999; Mallet et al., 2002; Pajvani et al., 2005; Srinivasula et al., 2002; Grinberg et al., 2002; Gross et al., 1998). In most cases, dimerization of an individual apoptotic protein has proven sufficient to induce apoptosis in the absence of any additional apoptotic signal. Inducible dimerization of apoptotic proteins has been developed into a widely used tool for targeted killing of selected cells in mice. Any cell
population that can be genetically marked can be engineered to express a dimerizable apoptotic protein and apoptosis can be temporally controlled by addition of dimerizer.

While most examples from the literature involve the activation of proteins by dimerization, in at least one case inducible dimerization has been used to study a signaling protein that is naturally inhibited by dimerization (Figure 1.2 B). The pro-apoptotic receptor \( p75^{NTR} \) promotes apoptotic signaling in its monomeric state, and inducible homodimerization is sufficient to suppress its signaling (Wang et al., 2000).

Dimerization of signaling kinases or caspases induces a state of “general activation”: an increase in the activity of these enzymes for their full range of normal substrates. A subtly different use of inducible dimerization is to direct the activity of a broadly active enzyme towards a specific substrate, as has been achieved with sumoylation enzymes (Zhu et al., 2006; Zimnik et al., 2009).

2 b ii) Protein surgery using inducible dimerization: split proteins, inteins and proteases

Signaling kinases and caspases are proteins that are naturally activated by dimerization and oligomerization. Inducible dimerization can be applied to achieve control over proteins that normally function as monomers through the technique of split protein reconstitution (Figure 1.3 A). In the split protein approach, the amino acid sequence of a protein is separated into two nonfunctional fragments. When these fragments are dimerized, they can reconstitute the activity of the intact protein. Sequence splitting is relatively straightforward in proteins with discrete functional domains. Glycosyltransferases, for example, can be split into a Golgi-targeting domain and a
Figure 1.3: protein creation, destruction and surgery

A: split protein reconstitution. The amino acid sequence of a protein can be split into separate, individually non-functional fragments. Fusion to dimerization domains allows inducible protein reconstitution.

B: The use of a split intein allows for release of the reconstituted protein from the intein and dimerization domains.

C: Inducible dimerization can trigger degradation of an otherwise stable protein, eliminating its function.

D: Inducible dimerization can suppress degradation of an unstable protein, restoring its function.
catalytic domain, allowing small-molecule control over protein glycosylation through inducible dimerization of these fragments (Czlapinski et al., 2008). Producing a split protein from a single globular domain is a nontrivial protein engineering challenge, but it has worked for GFP, DHFR, ubiquitin, beta-lactamase, beta-galactosidase, firefly luciferase, DnaE Intein and TEV protease among others (Kerppola, 2006).

DnaE Intein and TEV protease merit special mention, as they are protein elements that themselves can control protein ligation and cleavage, respectively. Split intein dimerization is attractive because protein splicing releases the reconstituted protein free of the genetically fused dimerization and intein domains (Figure 1.3 B), which could be valuable for target proteins that are sensitive to protein fusion tags (Mootz et al., 2003; Schwartz et al., 2007). TEV is a highly specific protease that can be expressed in human cells with negligible off-target cutting. The TEV recognition sequence can be genetically introduced into a protein of interest, rendering it susceptible to TEV cleavage. The activity of split TEV can be chemically controlled either by direct fusion to dimerization domains (Scheek et al., 2006; Gray et al., 2010) or by fusion to a dimerizable split intein (Sonntag and Mootz, 2011).

2 b iii) Controlling protein degradation through inducible dimerization

Inducible dimerization can also be used to positively or negatively regulate the degradation of a target protein (Figure 1.3 C,D). The first such system achieved degradation by directly dimerizing a target protein with a proteasomal subunit, but this system has not been successfully applied outside of budding yeast (Janse et al., 2004). In human cells, dimerization has been used to halt the degradation of proteins fused to a
constitutive degradation tag (Stankunas et al., 2003; Pratt et al., 2007). Another approach to induce degradation of a target protein is to dimerize it with a ubiquitin ligase. This has been elegantly achieved using a hormone receptor system borrowed from plants (Nishimura et al., 2009).

2 b iv) Other applications: cohesin, clathrin, amyloid

In other cases, dimerization has also been applied to induce more specific, idiosyncratic effects. The Nasmyth lab used inducible dimerization to control the topology (closed or open) of the cohesin complex, a supramolecular protein ring (Gruber et al., 2006). Homodimerization has been used to inactivate clathrin, allowing clathrin-dependent processes to be chemically controlled (Zlatic et al., 2011). Amyloid formation has been probed using inducible dimerization of alphaSynuclein, PrP, and APP (Eggert et al., 2009; Béland et al., 2012; Roostaee et al., 2013).

2 c) Inducible translocation: dimerization to change localization

In the above examples, inducible dimerization primarily exerts a biological effect by increasing the effective concentration of two proteins, without necessarily changing their localization. By contrast, inducible dimerization can used to control the localization of a protein of interest. This is generally achieved via heterodimerization between a “recruiter” protein with strong intrinsic localization to the desired address and a “recruit,” a second protein of interest. In these experiments, the specific identity of the recruiter protein is not as important as its localization; for instance, recruiting a signaling kinase to the plasma membrane using different membrane-localized recruiters may have the same
effects. The recruit may lack any intrinsic localization, or it may naturally localize to a different compartment, but it must at least transiently encounter the recruiter. Proteins have been successfully recruited via dimerization to the plasma membrane, mitochondria, peroxisomes, the nucleus, specific genomic loci, and, recently, kinetochores. The chief advantage over genetically fusing the recruit to the recruiter is the temporal (and in some cases, spatial) control afforded by inducible dimerization.

2c i) Turning ON a process via inducible translocation

Recruiting a protein to the location in which it is normally active is a powerful way to turn on a signaling pathway (Figure 1.4 A). Many cellular pathways are controlled by regulated access of signaling proteins to the plasma membrane, and this has been a common target for inducible protein translocation. In the first example of inducible protein relocalization, it was found that recruiting Src kinase to the plasma membrane is sufficient to activate a Src signaling cascade (Spencer et al., 1995). In another early application, ZAP70 kinase signaling was studied by comparing homodimerization in the cytosol to membrane recruitment using different chemical dimerizers and different configurations of the dimerization domains (Graef et al., 1997). Homodimerization in the cytosol failed to activate ZAP70. Notably, some of the membrane recruitment schemes activated ZAP70, but others did not.

Inducible translocation has been used to control heterotrimeric G proteins (Castellano et al., 1999; Inoue et al., 2005) and the kinases and phosphatases that modify phospholipids (Fili et al., 2006; Suh et al., 2006; Varnai et al., 2006; Zoncu et al., 2009; Ueno et al., 2011; Kakumoto and Nakata, 2013). My own work exploited the temporal
Figure 1.4: Positive or negative control over a biological process through inducible translocation. **A:** Inducible dimerization drives interaction between a diffusible protein and an intrinsically localized protein, activating a pathway. **B:** A pathway driven by interactions between a soluble protein and an intrinsically localized protein can be silenced by recruiting the first protein to a distal cellular compartment, where it cannot interact with its partner.
power of inducible translocation to recruit a spindle assembly checkpoint protein, Mad1, back to kinetochores after it has been removed through the normal checkpoint silencing pathways at metaphase (Ballister et al., 2014) (also cf Chapter 2 of this thesis).

2 c ii) Turning OFF a process via inducible translocation

Inducible translocation can also be used to rapidly inhibit a protein by sequestering it in a location in which it cannot function (Figure 1.4B). Proteins with strong nuclear import or export sequences can be used as recruiters to force a target protein through inducible dimerization (Haruki et al., 2008; Xu et al., 2010; Raschbichler et al., 2012; Yang et al., 2013). Forced nuclear import or export could be activatory or inhibitory, depending on the target protein. Clathrin adaptor proteins and ADP-ribosylation proteins have been inactivated by forced translocation to mitochondria, in a technique called “knock-sideways” (Robinson et al., 2010; Hirst et al., 2012; Robinson and Hirst, 2013; Cheeseman et al., 2013).

2 c iii) Inducible translocation to a specific chromatin locus

Perhaps the most precise cellular location to which proteins can be recruited is a specific DNA sequence, which may be present at a single site in the nucleus. Chemical- and light-inducible dimerization were used to recruit various transcription and chromatin modifying factors to defined chromatin loci to study the interplay between heterochromatin establishment and maintenance and transcription (Hathaway et al., 2012; Konermann et al., 2013). Inducible recruitment of a transactivator to a target promoter has also been used as a generic gene induction tool. Chemical dimerization-induced
transcription was first demonstrated in tissue culture and in mice in 1996 (Rivera et al.,
1996; Ho et al., 1996; Belshaw et al., 1996a) and has been used in numerous applications
since.

3) Specific dimerization tools: strengths and weaknesses

3 a) Introduction to chemical- and light-induced dimerization

An inducible dimerization system is one in which protein dimerization is
experimentally controlled by an external intervention. The two types of interventions
used to control protein dimerization in living cells are the addition of a small molecule
(chemically induced dimerization) or exposure to light (light induced dimerization).
Although biological systems can respond to many more types of stimulus, such as
changes in temperature, electric field, tension, or physical waves, none of these stimuli
have been reported as a general technique to induce protein dimerization, to my
knowledge.

Chemicals offer easy application and dosage control. Chemical dimerizers are
generally stable after addition, and in some cases, chemically inducible dimerization can
be reversed by washout or competition with a non-dimerizing antagonist. Small
molecules can access internal tissues in animals that are inaccessible to light, and
chemical dimerization does not limit the wavelengths of light available for imaging. In
the best cases, such as rapamycin, the kinetics of dimerization can be quite rapid, but
some molecules suffer from slow cellular uptake or saturation effects. The primary
disadvantage of chemically induced dimerization is that it is nearly impossible to achieve spatial control on a subcellular scale.

Photoinduced dimerization offers the potential for unparalleled spatial and temporal control, at least in tissue culture or optically accessible animal models. Light can be focused and patterned with nanometer scale resolution. Illumination can be applied for very short or long durations, and intensity is easily modulated. The persistence of photoinduced dimerization varies from completely irreversible to rapidly reversible, depending on the system used. The optimal relaxation properties for a system depend on experimental context. The disadvantages of photodimerization include sensitivity to accidental light exposure and restrictions on the wavelengths available for imaging. Some photodimerization systems require large fusion proteins, or suffer other idiosyncratic limitations, detailed below. Photodimerization using light-sensitive proteins was first reported in 2009 (Levskaya et al., 2009), and so is still in the early stages of technical development. Chemical dimerization has benefited vastly from technical refinement over the last 20 years, and new chemical dimerization tools are still being reported. Photoinduced dimerization will doubtless experience similar stunning improvement over the next few years.

3 b) Tools for chemical dimerization

Any molecule that can bind two proteins, or two copies of the same protein, can function as a chemical dimerizer. Dimerizers may either be natural products that intrinsically bind two proteins, or artificially designed molecules that link two ligands
with a tether. I have divided the discussion of chemical dimerizers into subsections on homodimerizers and heterodimerizers. The two most important characteristics of a chemical dimerizer are speed and specificity. An ideal dimerizer would rapidly enter cells and selectively dimerize its target proteins. Dimerizers that function slowly, or exhibit significant cross reactivity with endogenous proteins are inherently limited in their utility. High binding affinity can be important in some contexts, but is not as important as specificity.

3 b i) Homodimerizers

Antibodies

Antibody crosslinking is an early technique for inducible dimerization. Proteins can be homodimerized by a full-length, bivalent IgG antibody. Antibody crosslinking experiments contributed to the discovery that dimerization can be sufficient for receptor tyrosine kinase activation absence of ligand (Rönnstrand et al., 1988; Yarden, 1990; Spaargaren et al., 1991). Heterodimerization (or oligomerization) could conceivably be achieved by application of two primary antibodies and a secondary antibody. The primary advantage of antibody dimerization is that it can be applied to systems without genetic modification if a specific (ideally monoclonal) antibody is available that doesn’t interfere with protein function. If genetic modification of the protein of interest is feasible, then the use of epitope tags and well-characterized anti-tag antibodies makes antibody dimerization a generalizable technique. The primary disadvantage of antibody dimerization is that antibodies themselves are very large, so their bulk can interfere with function, and they are not cell permeable. In living cells, antibody crosslinking is limited
to extra-cellular or cell-surface proteins (or intracellular proteins fused to a membrane-crossing epitope tag) or else requires microinjection.

Symmetric artificial FKBP ligands

The first rationally designed dimerization reagent was **FK1012** (**Figure 1.5 A,B**), formed by linking two copies of the natural product **FK506** (Spencer et al., 1993). FK506 is an immunosuppressive natural product that heterodimerizes the proteins FKBP12 (FK506-Binding Protein of size 12 kD, hereafter simply “FKBP”) and calcineurin, potently inhibiting calcineurin (Liu et al., 1991). FK1012 consists of two copies of FK506 joined with a flexible linker to form a symmetric, semisynthetic dimer. A number of different linkers have been used, even in the initial report, so “FK1012” is really a family of molecules (Spencer et al., 1993). The linkers in FK1012s are attached to the calcineurin-binding surface of each FK506 monomer, blocking interaction with calcineurin and rendering the molecule non-immunosuppressive.

A protein of interest can be fused to FKBP and dimerized by the addition of FK1012. A serious limitation of FK1012 is the fact that it binds endogenous FKBP, which is a conserved, highly abundant protein in yeast, animals, plants and bacteria (Siekierka et al., 1990; Trandinh et al., 1992; Sampson and Gotschlich, 1992). In an inducible dimerization experiment, the exogenous FKBP fusion protein would ideally interact only with its intended dimerization partner, namely another copy of itself in a homodimerization experiment. Abundant endogenous FKBP will readily form undesired heterodimers with an exogenous FKBP fusion protein, competing with and limiting the
Figure 1.5: Small molecules that homodimerize FKBP. A: schematic diagram of chemical homodimerization. Two copies of the same ligand are joined by a chemical linker. B: FK1012 combines two copies of FK506, the ligand for which FKBP is named. FK1012 binds wild-type FKBP. C: AP1510 exploits a fully synthetic FKBP ligand (SLF) that is structurally simpler than FK506. D: Addition of a bulky adduct to SLF confers specificity for the FKBPF36V mutant. AP1903 homodimerizes mutant FKBP, and is not prone to competition from endogenous FKBP. E: AP20187 has a modified linker which improves pharmacological properties. AP20187 is commercially available, and is currently the most widely used FKBP homodimerizer.
desired homodimerization of the exogenous FKBP fusion. Another drawback of FK1012 is the chemical complexity of the natural product FK506.

Simplified, non-cyclic, fully synthetic FKBP ligands were developed as inhibitors of FKBP peptidyl-prolyl isomerase activity (Holt et al., 1993; Armistead et al., 1995). Replacing FK506 with synthetic ligands in the context of a symmetric homodimerizer yielded dimerizers with greatly reduced chemical complexity (Figure 1.5 C) (Amara et al., 1997; Keenan et al., 1998). The monomeric synthetic ligand for FKBP was not named in either of the 1998 manuscripts, but has subsequently been referred to as **SLF**, for Synthetic Ligand of FKBP, which we adopt (Czlapinski et al., 2008). The symmetric dimer of SLF, **AP1510**, functions similarly to FK1012, albeit with weaker affinity (Figure 1.5 C). While this work did not address the fundamental problem of competition with endogenous FKBP, it did set the stage for the development of a novel ligand that is highly specific for a mutant form of FKBP (Clackson et al., 1998).

Clackson and coworkers used a classic “bump-hole” approach, in which a small panel of SLF analogs with bulky adducts were screened for binding to a pool of FKBP mutants in which a phenylalanine in the ligand binding pocket was replaced with smaller non-polar amino acids (Ala, Val, Ile or Leu). The best molecule bound mutant FKBP_{F36V} with an affinity of 1.8 nM, and wild-type FKBP with an affinity of 2.9 µM, a specificity of >1000 fold (Clackson et al., 1998). Homodimerization reagents developed from this bumped FKBP ligand, including **AP1903** and **AP20187** (Figure 1.5 D,E), are effectively bioorthogonal, allowing much more specific dimerization between exogenous, mutant
FKBP constructs (Clackson et al., 1998; Yang et al., 2000). AP1903, AP20187 and related compounds differ only in their linkers.

A further potential limitation of some chemical dimerizers, particularly artificial dimerizers consisting of two ligands joined by an inert linker, is the lack of cooperativity between the binding of the first and second proteins (Corson et al., 2008). This poses a risk of dimerizer saturation. Excess dimerizer can drive the system to a state of zero protein-protein dimerization, in which every copy of the target protein binds a unique copy of the small molecule, but none are cross-linked. Optimal dimerizer concentrations must be empirically determined for every dimerization experiment.

Other non-covalent homodimerizers

Dimeric cyclosporin-A CsA2 (Figure 1.6 A) is another artificial homodimerizer derived from an immunosuppressive natural product (Belshaw et al., 1996b). CsA2 dimerizes cyclophilinA (CypA) and was used as a complement to FK1012 in early work from the Schreiber and Crabtree labs (Stockwell and Schreiber, 1998), but has not been widely used since.

Coumermycin (Figure 1.6 B) is a natural product homodimerizer, a symmetric molecule that binds two copies of the bacterial protein DNA GyrB (Farrar et al., 1996). Coumermycin is nontoxic and bioorthogonal in animal cells, a major advantage over the original FK1012 and AP1510 dimerizers. As with other noncooperative dimerizers, “high coumermycin concentrations attenuated this activation, suggesting that binding site saturation leads to dissociation of the dimers, which was confirmed with novobiocin, a ‘monomeric’ form of coumermycin that does not dimerize Raf-GyrB” (Corson et al.,
Figure 1.6: other chemical homodimerizers

A: CsA-2 is a semisynthetic, symmetric dimer of Cyclosporine-A. CsA-2 homodimerizes Cyclophilin A. B: Coumermycin is the only widely used natural product homodimerizer. Coumermycin homodimerizes the bacterial protein GyrB, and does not have natural receptors in animal cells. C: CoDi3 (one member of the CoDi family) is a synthetic dimerizer that covalently crosslinks the SNAP-tag protein. D: X-CrASH is both a dimerizer and a fluorescent probe. X-CrASH covalently homodimerizes proteins tagged with a short tetracysteine peptide motif. This reaction turns on X-CrASH fluorescence.
2008). Coumermycin has successfully been used to study at least 12 different signaling proteins including Raf (Farrar et al., 1996), Jak2 (Mohi et al., 1998), Stat3 (O’Farrell et al., 1998), VEGFR (Knight et al., 2000), Alk (Gouzi et al., 2005) Tyk2 (Mizuguchi and Hatakeyama, 1998), PKR (White et al., 2011), FAK (Toutant et al., 2002), hPAFR (Perron et al., 2003), ERIS (Sun et al., 2009) and RIPK3 (Moujalled et al., 2014), and the bacterial toxin CagA (Nagase et al., 2011). The antagonistic combination of coumermycin and the non-dimierizing GyrB ligand novobiocin have been used to create a tuneable gene expression system (Zhao et al., 2003).

Covalent homodimerizers

Several techniques for covalent protein labeling have been developed in the last decade, including SNAP-tag (Keppler et al., 2003), CLIP-tag (Gautier et al., 2008) and Halo-tag systems (Los et al., 2008). In the SNAP-tag system, a mutant version of the DNA repair protein O6-alkylguanine-DNA alkyltransferase (hAGT) forms a covalent bond with O-benzylguanine (BG)-conjugated compounds. A series of symmetric homobifunctional BG molecules, termed CoDis (Figure 1.6 C), were introduced in 2007 (Lemercier et al., 2007). These molecules were shown to covalently crosslink SNAP-tag fusion proteins in living cells, and were used to test for colocalization of different proteins in proof-of-principle experiments. These molecules appear to exhibit slow kinetics, as this paper only reports results of 6-hour labeling reactions (Lemercier et al., 2007).
xCrAsH (Figure 1.6 D) is another system for covalent dimerization with several interesting properties (Rutkowska et al., 2011). xCrAsH is based on the biarsenic/tetracysteine labeling system developed in the Tsien lab (Griffin et al., 1998). Biarsenic probes selectively covalently label proteins (in living cells) containing a short tetracysteine peptide CCPGCC. This labeling can be reversed by addition of membrane-permeable dithiols. xCrAsH probes are homobifunctional molecules consisting of two fluorogenic CrAsH (carboxy-fluorescein arsenic) units linked with a flexible tether (Rutkowska et al., 2011). Each CrAsH subunit can label an individual CCPGCC motif, enabling dimerization of two CCPGCC-tagged proteins. Protein labeling causes a gain of fluorescence, allowing a functional readout of dimerization. xCrAsH was used successfully in living cells to detect the presence of stable homodimeric and heterodimeric protein complexes. XCrAsH labeling kinetics are not thoroughly detailed, but all results in the paper are from incubations of 1H or longer. As with CoDis, above, one strength of covalent crosslinking is that it allows direct detection of protein-protein interactions through electrophoretic analysis of cell lysates.

3 b ii) Heterodimerizers

Rapamycin and analogs

Rapamycin (with its analogs) is the most widely used chemical heterodimerizer (Figure 1.7 A) (Putyrski and Schultz, 2012). Rapamycin was first reported as an antifungal antibiotic produced by Streptomyces hygroscopicus in soil isolates from Rapa Nui (Easter Island) (Vezina et al., 1975). It was quickly discovered that rapamycin is also a potent immunosuppressive (Martel et al., 1977). The antifungal and immunosuppressive effects of rapamycin share a common mechanism involving
Figure 1.7: Chemical heterodimerizers with obligate sequential binding mechanisms. 

A: chemical structures of FK506, Rapamycin, and AP21967. Binding surfaces indicated. 

B: Rapamycin first binds FKBP, then this complex binds FRB (or mTOR). All three molecules contact each other in this complex. This property underlies the design of rapamycin analogs specific for mutant FRB. 

C: The plant hormones Abscisic Acid (ABA) and Gibberellin 3 (Gibberellic Acid 3 or GA3). GA3-AM is a prodrug form of GA3 in which the carboxylic acid moiety is masked by an ester. This modification significantly improves cellular permeability. Inside the cell, the masking group is removed by promiscuous esterases, yielding GA3. 

D: These plant hormones induce dimerization through a sequential binding mechanism that is similar to that of rapamycin, but that differs in the structural details. Like rapamycin, ABA and GA first bind an initial receptor, and this complex binds a secondary receptor. ABA and GA do not bind the secondary receptor in their free forms. Unlike FK506 or rapamycin, ABA and GA do not directly contact the secondary receptor in the ternary complex. Instead, these hormones induce structural rearrangements in their primary receptors that allow direct binding of the primary receptor to the secondary receptor.
conserved proteins in yeast and animals: rapamycin first binds FKBP; this complex then
binds and inhibits the kinase Target of Rapamycin (TOR in yeast, mTOR in mammals)
(Heitman et al., 1991b; a; Sabatini et al., 1994; Brown et al., 1994; Chiu et al., 1994).
Rapamycin binds FKBP with 0.2 nM affinity, and the FKBP:rap complex subsequently
binds with 2 nM affinity to mTOR, or a small fragment of mTOR termed FRB (Figure 1.7 B)
(Chen et al., 1995; Banaszynski et al., 2005). Rapamycin was first used as a
general tool to dimerize FKBP and FRB fusion proteins in 1996 (Ho et al., 1996; Rivera
et al., 1996). Rapamycin is useful for its rapid cell permeability, very high affinity and its
highly cooperative, sequential binding mechanism. Because FRB will only bind a pre-
formed FRBK:rapamycin complex, there is no danger of free rapamycin binding FRB
and competing with FKBP:rapamycin (Figure 1.7 B). Rapamycin can therefore be
applied at very high concentrations relative to its kD without blocking
heterodimerization. High levels of rapamycin can be used to maximize intracellular flux
and achieve rapid dimerization (Ballister et al., 2014) (also cf Chapter 2 of this thesis).

There are three major limitations to rapamycin: 1) mTOR inhibition, 2)
competition with endogenous FKBP, and 3) irreversibility on short timescales. The
natural function of the FKBP:rapamycin complex as a potent, specific inhibitor of
mTOR has the potential to introduce confounding effects to any inducible dimerization
experiment, and has serious immunosuppressive effects in animals. This problem has
been effectively addressed by the development of rapamycin analogs with bulky adducts
that bind selectively to a mutant FRB with larger rapamycin binding pockets, but cannot
bind endogenous mTOR (Figure 1.7 A,B) (Bayle et al., 2006). The most widely used rapalog, AP21967 (Figure 1.7 A), is now commercially available.

Rapamycin also suffers from the same competition with endogenous FKBP that drove the development of bump/hole ligand/FKBP homodimerizers. Unfortunately, rapamycin analogs that are “bumped” on the FKBP binding surface have not been reported. Achieving inducible dimerization with rapamycin or any of its analogs requires high levels of expression of exogenous FKBP and FRB fusion proteins to overcome competition with endogenous FKBP. This problem has probably been underreported in the literature since it would typically manifest as a “failed” experiment. In at least one case, however, the failure of a rapamycin induced dimerization experiment has been reported (Coutinho-Budd et al., 2013), although only after the wasted investment of the substantial resources required to generate transgenic mice. This limitation can be overcome, at least in tissue culture cells, by deleting or depleting endogenous FKBP (Ballister et al., 2014) (also cf Chapter 2 of this thesis). A more elegant solution would be to develop a rapalog that is “bumped” on the FKBP binding surface to be specific for a mutant FKBP, or to generate mutually specific mutants of FKBP and FRB. Finally, the affinity of the FKBP:rap:FRB ternary complex is so high that rapamycin washout is effectively impossible within short timescales, even using competitive non-dimerizing FKBP ligands (Putyrski and Schultz, 2012).

Abscisic Acid and Gibberellin

Two highly promising new inducible dimerization tools were recently reported, both using plant hormones and their natural receptors (Figure 1.7 C) (Liang et al., 2011;
Miyamoto et al., 2012). Abscisic acid (ABA) and Gibberellin (also known as Gibberellic Acid or GA) naturally induce dimerization between their immediate receptors and a downstream target protein. “Gibberellin” describes a large family of over 100 related plant hormones; Miyamoto et al. used GA$_3$ in their work. The mechanism of action is similar to that of rapamycin, FK506 and CyclosporinA, in which the molecule forms a tight complex with an initial receptor, which then binds to and acts on (typically inhibiting) a second protein (Figure 1.7 D) (Murase et al., 2008; Miyazono et al., 2009).

ABA and its receptor Pyl bind to and inhibit a protein phosphatase PP2C, and GA and its receptor GID1 bind to proteins containing a DELLA motif and trigger their degradation. As with rapamycin and FK506, this strictly sequential binding mechanism precludes the possibility of saturation of the system by an excess of dimerizer. On a structural level, the binding mechanism of ABA and GA differs from rapamycin and FK506. Both ABA and GA bind in deep pockets in their receptors and induce a dramatic conformational shift that completely occludes the hormone. This rearranged surface of the hormone receptor forms the binding site for the target protein, which does not directly contact the buried hormone molecule (Figure 1.7 D) (Murase et al., 2008; Miyazono et al., 2009). In contrast, rapamycin and FK506 are not buried by FKBP, and there is extensive contact between rapamycin and FRB (mTOR) and FK506 and calcineurin (Figure 1.7 B) (Van Duyne et al., 1991, 1993; Choi et al., 1996).

In their initial descriptions as dimerizers, ABA and GA were both successfully applied to signal transduction and protein translocation, and ABA was also shown to be effective for inducing transcription in typical proof of principle experiments (Liang et al.,
As their names imply, Abscisic acid and Gibberellic acid both contain carboxylic acid moieties that are deprotonated and charged at physiological pH. Negatively charged groups can interfere with cell permeability, however. Recognizing the importance of cellular permeability, Miyamoto and coworkers masked the carboxylic acid moiety of GA3 with an ester protecting group (acetoxymethyl, or AM) that is readily cleaved by promiscuous intracellular esterases (Figure 1.7 C). The resulting neutral prodrug (GA3-AM) enters cells much more readily than unmodified GA3, allowing maximal dimerization within 60 seconds and greatly enhancing its usefulness as an inducible dimerizer (Miyamoto et al., 2012). ABA, on the other hand, appears to have relatively slow kinetics, requiring ~30 minutes to achieve maximal protein translocation, which limits its applicability in studying any cellular process which occurs on a faster timescale (Liang et al., 2011). It is not clear whether the difference in localization kinetics between ABA and GA3-AM is entirely due to cellular uptake or the intrinsic kinetics of the protein binding events, but the performance of ABA might also be improved by a similar charge-masking prodrug modification.

Both ABA and GA3 are non-toxic at the concentrations required for inducible dimerization, and both occur naturally in common food plants. The kinetics of GA3-AM dimerization are somewhat slower than those of rapamycin (~60 seconds for GA3-AM to reach maximum dimerization, compared to ~10 seconds for rapamycin), perhaps because of the requirement for activation by intracellular esterases. GA3-AM still acts fast enough for effective perturbation of any process that occurs on a timescale of minutes or longer. GA3-AM is not yet commercially available, but this should not present a
significant barrier to its adoption. ABA and GA₃-AM both hold great promise as chemical dimerizers due to their cooperative binding mechanism and total orthogonality in animal backgrounds, but the rapid kinetics of GA₃-AM set it apart as the superior dimerizer. GA₃-AM should be considered the chemical homodimerizer of choice for future applications.

Non-covalent artificial heterodimerizers

A great variety of artificial heterodimerizers have been reported, but few have been used for more than a handful of applications. The following series of molecules all non-covalently heterodimerize two different fusion proteins and require genetic engineering of the experimental system. The overall effectiveness of these molecules is again a function of their permeability and orthogonality. None appear to exhibit substantial cooperative binding, and so must be applied at low enough doses to prevent saturation effects.

**FK506 (Figure 1.7 A)** could theoretically be used as a chemical heterodimerizer, analogously to rapamycin, but it appears this has not been done. Instead, FK506 was fused to Cyclosporin-A to generate **FK-CsA (Figure 1.8 A)**, the first artificial heterodimerizer. FK-CsA was used to dimerize proteins fused to FKBP and CyclophilinA (Belshaw et al., 1996a). **Dex-FK506 (Figure 1.8 A)** combines FK506 with dexamethasone, a glucocorticoid hormone, allowing dimerization of wild-type FKBP and glucocorticoid receptor (GR) fusion proteins (Licitra and Liu, 1996). FK-CsA and Dex-FK506 are limited by competition with endogenous FKBP12. Virginia Cornish’s lab has developed several dimerizers mixing and matching ligands for steroid receptors and
Figure 1.8: artificial heterodimerizers are formed by chemically linking two independently functional small-molecule ligands. Artificial dimerizers dimerize proteins that do not naturally interact so these molecules are fundamentally modular. Different combinations of interchangeable parts create dimerizers with unique properties. In these diagrams, linkers are represented schematically.

A: fully non-covalent artificial heterodimerizers B: hemi-covalent and fully covalent heterodimerizers. BG-Mtx is a hemi-covalent dimerizer that covalently bonds with one of its receptors (SNAP-tag) and non-covalent binds the other (DHFR). HaXS forms covalent linkages with both of its receptors (SNAP-tag and Halotag).
dihydrofolate reductases (DHFRs). The first Cornish lab dimerizer was **Dex-Mtx** (Figure 1.8 A), which combines dexamethasone with methotrexate, a dihydrofolate reductase (DHFR) inhibitor that broadly binds DHFRs from bacteria and eukaryotes (Lin et al., 2000). Dex-Mtx dimerizes GR and DHFR fusions in yeast, but suffers from binding to endogenous yeast and human DHFRs. **Dex-TMP** (Figure 1.8 A) solves the problem of off-target DHFR inhibition by replacing Mtx with trimethoprim (TMP), a DHFR inhibitor that is highly selective for bacterial DHFR (Baccanari et al., 1982; Gallagher et al., 2007). In 2008, Carolyn Bertozzi’s lab, in collaboration with the Cornish lab, published **TMP-SLF** (Figure 1.8 A), a dimerizer combining TMP with a synthetic ligand of FKBP (SLF) (Czlapinski et al., 2008). TMP-SLF is selective for bacterial DHFR, but binds endogenous FKBP, meaning that it will suffer from competition with endogenous FKBP. In summary, the best non-covalent artificial heterodimerizer is probably Dex-TMP, but this molecule does not offer any apparent advantages over Gibberellin.

**Covalent artificial heterodimerizers**

Two heterodimerizers have been reported which exploit covalent protein labeling. The first, **BG-Mtx** (Figure 1.8 B), combines the SNAP-tag ligand BG with methotrexate (Gendreizig et al., 2003). This molecule forms a covalent attachment with the SNAP-tag protein HAGT, but a non-covalent interaction with DHFR. The most attractive feature of a semi-covalent dimerizer is that excess or unreacted dimerizer can be washed out, so that the only MTX available in the cell is that which has successfully reacted with the SNAP-tag protein. Surprisingly, semi-covalent protein dimerization has not been further
developed in the decade since this initial report, and BG-Mtx itself appears never to have been used beyond the proof-of-principle experiments in the initial manuscript.

A family of fully covalent heterodimerizers combining Halotag and SNAP-tag ligands has been reported under the name **HaXS (Figure 1.8 B)**, for Halo-X-Snap, wherein the X refers to the linker (Erhart et al., 2013). The authors carefully explored the effects of various linkers on cell permeability, dimerization kinetics and dynamic range of inducible dimerization. They found a strong correlation between cell permeability and overall performance, with the most permeable molecule giving the highest maximum levels of dimerization (65% of total protein) with the fastest kinetics (>15 minutes). Even with the optimal linker, however, saturation is observed when excess dimerizer is used. The fully covalent crosslinking offered by HaXS may be of value in some applications, but the slow kinetics are a serious limitation.

3 c) Light-induced dimerization

The ideal light-induced dimerization system would be non-toxic, bioorthogonal, rapid and efficient. Toxicity is a function of the proteins and/or molecules used, as well as the wavelength and intensity of light required to induce dimerization. Visible light is less toxic than UV light per photon, but overall toxicity must be considered in terms of wavelength and the intensity required to induce the desired effect. As with chemical dimerization, bioorthogonality principally requires that the proteins/molecules used should not interact with endogenous proteins. The basic speed limit for inducible protein dimerization in cells is diffusion of the dimerizing proteins within the cell, generally on the order of a few seconds. Many photochemical processes occur much faster than this,
but some protein-based dimerization systems respond to light much more slowly. Efficiency is the yield of the dimerization reaction which can be practically achieved.

Unlike small molecules, light can be applied in a spatially defined manner, so an ideal light-induced technology should exploit this potential. Two types of systems have been reported for light-controlled dimerization: photoreactive small molecules and photosensitive proteins.

3 c i) Photoreactive chemical dimerizers

Four photoreactive chemical dimerizers have been reported: two photoactivatable rapamycin derivatives (Figure 1.9 A) (Karginov et al., 2010; Umeda et al., 2011) and two photo-cleavable dimerizers (Figure 1.9 B) (Ahmed et al., 2014; Erhart et al., 2013). \textbf{pRap} (Figure 1.9 A) is rapamycin with a bulky photocleavable group added at the C40 position, which was intended to prevent binding to FKBP. Surprisingly, this modification does not block binding to wild-type FKBP or FKBP:FRB dimerization. The authors were required to introduce mutations to FKBP to prevent binding to pRap, yielding iFKBP, and were able to achieve light-induced dimerization between iFKBP and FRB. Umeda et al took a different approach, designing a system to sequester rapamycin outside the cell until it is locally released with light (Figure 1.9 A). Specifically, they attached rapamycin to biotin with a photocleavable linker, then added avidin to generate a photocleavable rapamycin-biotin-avidin complex termed \textbf{cRb-A} that does not enter cells. Local illumination near the plasma membrane releases free rapamycin, which can diffuse into the cell and induce dimerization.
Figure 1.9: Photosensitive dimerization systems

A: Two photocaged versions of rapamycin. pRap enters cells but does not bind the mutant iFKBP until uncaged by UV light. cRb-A is tethered to biotin-Avidin by a photocleavable linker. This rapamycin-protein complex cannot enter cells. Illuminating a region near the cell membrane liberates a small quantity of rapamycin, which is free to diffuse into the cell and induce dimerization. These molecules release functionally equivalent analogs of rapamycin, as indicated.

B: PhAP and MeNV-HaXS are photocleavable chemical dimerizers. PhAP is a member of the AP1903 family of homodimerizers specific for the FKBPF36V mutant. MeNV-HaXS covalently heterodimerizes SNAP-tag and Halotag fusion proteins. Both molecules induce dimerization when added to cells. This dimerization can be reversed in a spatiotemporally controlled manner by light.

C: schematic diagram of light induced dimerization using photosensitive proteins. The photosensitive protein, represented as a gray circle, contains a photosensitive cofactor. Illumination induces a conformational shift that allows dimerization with the binding partner. In the case of the Phy/Pif system, reversal can be induced with far-red light.
The most significant fundamental limitation of both caged rapamycins is that the effective dimerizer, free rapamycin (Figure 1.9 A), can diffuse throughout the cell after uncaging. While both have been successfully used to induce localized cellular responses at the membrane, the actual confinement of dimerization achieved is on the micrometer scale, and not sharply defined. This limits the usefulness of these caged rapamycins for localized dimerization. cRb-A is not applicable for regulating processes in compartments other than the plasma membrane, and the spatial confinement of pRap would be, at best, limited.

An alternative approach is to add a photocage to the FRB-binding face of rapamycin, by analogy with the bumped rapalog s. This face of rapamycin is chemically accessible, and modifications there have proven to block binding of wild-type FRB. A photocleavable “bump” could be added to rapamycin, or, even better, the photocage could be added to a rapalog’s bulky adduct. These molecules would presumably be able to bind to FKBP prior to uncaging, allowing the FKBP fusion protein to spatially confine the uncaged rapamycin. Rapamycin has been photocaged on its FRB binding face (Sadovski et al., 2010), but, curiously, this promising compound has not been tested for localized inducible dimerization.

The recently reported photocleavable dimerizers are valuable additions to the dimerization armamentarium. PhAP (Figure 1.9 B) is an analog of AP20187 in which a photocleavable group has been inserted into the linker, allowing photo-induced release of homodimerized FKBP fusion proteins. Although the authors do not demonstrate photocleavage on a subcellular region, there is no reason this should not work. MeNV-
HaXS (Figure 1.9 B) is a photocleavable version of the HaXS Halotag/SNAPtag covalent heterodimerizer (Erhart et al., 2013). In this report, the authors elegantly demonstrate release of a heterodimerized protein on a subcellular scale, specifically, from individual vesicles. Many inducible dimerization experiments could be enhanced by the ability to reverse the effects of dimerization with spatiotemporal control.

3 c ii) Photosensitive protein dimerizers

Four distinct systems for light-inducible dimerization based on naturally photosensitive proteins have been developed since 2009: Phy/Pif (Levskaya et al., 2009), FKF1/GI (Yazawa et al., 2009), Cry2/CIB1 (Kennedy et al., 2010) and TULIPs (Strickland et al., 2012). All four systems work in a conceptually similar way: a photosensitive protein (which contains a photosensitive cofactor) undergoes a conformational change upon exposure to light which allows dimerization with a second (non-photosensitive) protein (Figure 1.9 C).

Of these systems, Phy/Pif has the most outstanding photophysical properties (Levskaya et al., 2009). Phy is a photosensitive protein and Pif is its non-photosensitive binding partner. Phy adopts a Pif-binding conformation when exposed to red light (650 nm) and rapidly returns to a non-binding conformation when exposed to far-red light (750 nm). The system is shown to be stable over many cycles of dimerization and release. The ability to suppress dimerization globally using 750 nm illumination and induce dimerization locally using focused 650 nm illumination means that localized dimerization can be achieved even with proteins that are not themselves intrinsically stably localized. The non-photosensitive half of the dimerization pair, Pif, is quite small, so it should be
minimally disruptive as a fusion protein. One minor obstacle is that Phy/Pif requires the purification and addition of an exogenous photoactive cofactor, phycocyanobilin, that is not commercially available and is prone to photodegradation. A more important limitation is that Phy is a “difficult” fusion partner. Phy is very large protein (908 amino acids), so it may be simply too bulky to fuse to many proteins of interest. In the initial report, Phy/Pif was only used to recruit freely diffusible Pif to membrane-bound Phy. Phy/Pif dimerization was recently tested in budding yeast by fusing Phy to 20 different proteins that naturally localize to several distinct compartments (Yang et al., 2013). In this study, greater than half (11/20) of the Phy fusion proteins failed to localize properly or proved toxic to the cells. Notably, 7/8 spindle-pole body fusions failed, and all 3 kinetochore fusions failed. Phy/Pif holds great promise, but these technical difficulties are likely preventing the system from enjoying the widespread application its outstanding photophysical properties merit.

In the **FKF1/GIGANTEA** system, FKF1 is the light sensing protein and GIGANTEA is its binding partner (Yazawa et al., 2009). FKF1 shifts to a GIGANTEA-binding conformation when exposed to blue light, and very slowly relaxes to a non-binding state in the dark. Both proteins are large: FKF1 is 619 AAs and the appropriately named GIGANTEA is 1,173 AAs. The most significant drawback of this system is that dimerization is very slow: dimerization continues to increase for over 5 minutes after illumination, and 30 minutes of continuous illumination is required to achieve maximum dimerization. Relaxation in the dark takes several hours.
**Cryp/Cib1** (Kennedy et al., 2010) also uses blue light to induce dimerization, and does not offer direct reversal of dimerization. A minor disadvantage of this system is that its sensitivity to blue light precludes the use of GFP or YFP for imaging. The primary advantages of Cryp/Cib1 are that it does not require exogenous cofactors and it uses modestly sized dimerization domains (~400 AAs). This system has been more widely adopted than others, appearing in original research articles from four other labs, in which it was successfully used to induce transcription in fish (Liu et al., 2012), to regulate phosphoinositides in tissue culture (Idevall-hagren et al., 2012; Kakumoto and Nakata, 2013), and to control the activity of chromatin modifying factors in neurons in mice (Konermann et al., 2013).

Unlike other systems, **TULIPs** do not consist of a pair of interacting proteins borrowed from a naturally light sensitive system (Strickland et al., 2012). Instead, TULIPs utilize a synthetic interaction between a photosensitive LOV domain protein and an engineered PDZ domain protein. The LOV domain normally undergoes a conformational switch upon blue-light illumination that disrupts its C-terminal alpha-helix and exposes those residues as an unstructured peptide. This feature was engineered for dimerization by appending a short peptide sequence on the C-terminal helix that can be bound by the PDZ domain when unfolded, but not when folded into an alpha-helix as in the dark state. Various kinetic parameters of this interaction can be “tuned” by mutations in either the LOV protein or the PDZ receptor. This system holds great promise, but it has failed to function properly in our hands when the photosensitive LOV domain is fused to histone H2B or centromere protein CENP-B.
4) Conclusions

In conclusion, the choice of technique for inducible dimerization depends on the particular biological system being investigated. For rapid, global perturbations, chemical dimerization is a good option, as it is the simplest and most robust technique to implement. **Of all the chemical dimerization techniques, Gibberellin (in its prodrug form) offers the best combination of rapid cell permeability, bio-orthogonality and cooperative binding mechanism.** This technique is highly promising and merits widespread adoption as the front-line dimerization tool of choice. Development of more rapidly permeable forms of Absciscic Acid also hold great promise. Probably the only persistent limitation of Gibberellin is that its full effects may not be able to be pushed faster than ~30 seconds. Rapamycin still has value for its rapid permeability, high affinity and cooperative binding. The drawbacks of rapamycin, namely off-target binding to endogenous FKBP and undesired mTOR inhibition, must be taken into consideration, and, if necessary, addressed through the use of bumped rapalogs and/or depletion of endogenous FKBP (cf Chapter 2). One lesson from the history of chemical dimerization is that a tool such as rapamycin need not be perfect to be widely used. The further development and refinement of several mutually orthogonal chemical dimerization tools will allow for combinatorial control of protein-protein interactions.

**Light-induced dimerization offers a powerful level of spatial control that can enables precise perturbations in living cells.** Light-induced dimerization is generally more technically difficult to implement than chemically-induced dimerization, but the experimental advantages of spatial and temporal control well justify the investment. As
with chemical dimerization, there is probably no single light-induced dimerization system that is optimal for all experiments. Chemical dimerization has benefitted from 20 years of technological improvement since it was introduced in 1993. Continued refinement of existing light-induced dimerization systems, and the discovery of entirely new systems has the potential to enable a new paradigm in experimental cell biology.
Chapter 2: Recruitment of Mad1 to metaphase kinetochores is sufficient to reactivate the mitotic checkpoint

Summary

The mitotic checkpoint monitors kinetochore-microtubule attachment and prevents anaphase until all kinetochores are stably attached. Checkpoint regulation hinges on the dynamic localization of checkpoint proteins to kinetochores. Unattached, checkpoint-active kinetochores accumulate multiple checkpoint proteins, which are depleted from kinetochores upon stable attachment, allowing checkpoint silencing. Because multiple proteins are recruited simultaneously to unattached kinetochores, it is not known what changes at kinetochores are essential for APC/C inhibition. Using chemically induced dimerization to manipulate protein localization with temporal control, we show that recruiting the checkpoint protein Mad1 to metaphase kinetochores is sufficient to reactivate the checkpoint without a concomitant increase in kinetochore levels of Mps1 or BubR1. Furthermore, Mad2 binding is necessary but not sufficient for Mad1 to activate the checkpoint; a conserved C-terminal motif is also required. The results of our checkpoint reactivation assay suggest that Mad1, in addition to converting Mad2 to its active conformation, scaffolds formation of a higher-order mitotic checkpoint complex at kinetochores.

Introduction

The mitotic checkpoint inhibits the Anaphase Promoting Complex/Cyclosome (APC/C) in the presence of unattached kinetochores and silences this inhibition once all kinetochores are stably attached to spindle microtubules. Checkpoint activity (i.e.,
APC/C inhibition) and silencing are correlated with changes in the kinetochore localization of checkpoint proteins, including Mad1, Mad2, Bub1, BubR1, Bub3, Mps1 and Cdc20 (Kops and Shah, 2012). These proteins are enriched at kinetochores until stable MT attachment and are required for checkpoint function. A central challenge in understanding the mitotic checkpoint is to dissect how local changes in checkpoint protein occupancy at kinetochores drive global changes in checkpoint activity.

Preventing the removal of Mad1 or Mps1 from kinetochores via genetic fusion to the stable kinetochore component Mis12 blocks anaphase, demonstrating that the removal of these proteins is required for checkpoint silencing (Jelluma et al., 2010; Maldonado and Kapoor, 2011). To probe checkpoint activation (i.e., switching the checkpoint from an “off” state to an “on” state), experimental intervention when the checkpoint is silenced, such as at metaphase, is an attractive approach. The checkpoint can be reactivated at metaphase by disrupting K-MT attachments, using either spindle poisons or laser microsurgery (Clute and Pines, 1999; Dick and Gerlich, 2013). It is unknown if the checkpoint can be reactivated after metaphase without compromising K-MT attachment. Metaphase kinetochores are stably attached and depleted of checkpoint proteins, so they provide a context in which to test the effect of increasing the kinetochore concentration of an individual protein in the absence of the full set of signals associated with the unattached state.

We tested whether increasing kinetochore localization of Mad1 at metaphase is sufficient to reactivate the checkpoint. Mad1 and its partner Mad2 are essential checkpoint proteins (Li and Murray, 1991). Mad1 constitutively binds a single copy of Mad2 in the closed conformation, and this bound population of Mad2 serves as the
kinetochore receptor for cytosolic, open-conformation Mad2 (Luo et al., 2004; De Antoni et al., 2005; Lara-Gonzalez et al., 2012). Continual recruitment of open-Mad2 and its conversion to the closed conformation, concomitant with binding to Cdc20, constitutes the catalytic engine of the SAC at kinetochores (Han et al., 2013). We used an improved technique for rapamycin-induced protein dimerization to achieve temporal control over Mad1 kinetochore localization.

**Results and Discussion**

*Depleting endogenous FKBP improves efficiency of rapamycin dimerization*

Rapamycin-induced dimerization is a well-established technique to experimentally control the association of two proteins in living cells (Rivera et al., 1996; Putyrski and Schultz, 2012). Rapamycin is a small molecule which induces the dimerization of the proteins FKBP12 (hereafter, FKBP) and mTOR, or mTOR’s minimal rapamycin binding fragment FRB (Chen et al., 1995). To test the feasibility and kinetics of recruiting an unlocalized protein to kinetochores during mitosis, we generated a stable HeLa cell line constitutively expressing Mis12-GFP fused to a tandem trimer of FKBP (Mis12-GFP-FKBP) and inducibly expressing mCherry-FRB (mCh-FRB) (**Figure 2.1 A,B**). The expression level of mCh-FRB varied between cells, and rapamycin induced recruitment of mCh-FRB to kinetochores was only detectable in cells with high mCh-FRB expression (**Figure 2.2 B,C**). Highly overexpressing Mad1 can compromise the checkpoint (Ryan et al., 2012; Heinrich et al., 2013), so we sought to improve the efficiency of rapamycin mediated dimerization.
Figure 2.1: Endogenous FKBP depletion improves efficiency of rapamycin-mediated recruitment.

(A) Diagram of a DNA cassette used to constitutively express Mis12-GFP-FKBP and miRNA, and inducibly express mCherry-FRB. The cassette is integrated between Lox acceptor sites downstream of the EF1a promoter (Khandelia et al., 2011), see Chapter 4 for details. (B) Schematic representation of rapamycin-mediated recruitment of mCherry-FRB to kinetochore-localized Mis12-GFP-FKBP. (C) HeLa cells expressing Mis12-GFP-FKBP, mCherry-FRB and either an empty miRNA backbone or miRNA against the 3’UTR of endogenous FKBP were imaged before and ~1 min after the addition of 500 nM rapamycin (rap). Images are representative of three independent experiments (quantified in Figure 2.2 C). Scale bars 5 µm.
Figure 2.2: Effects of endogenous FKBP knockdown on rapamycin-mediated recruitment to kinetochores. HeLa cells were expressing Mis12-GFP-FKBP, mCherry-FRB and either empty miRNA backbone or miRNA against the 3’UTR of endogenous FKBP. (A) Cells were analyzed by immunoblot for FKBP (top), GFP (middle) and tubulin (bottom), as a loading control. Arrows indicate expected bands for endogenous FKBP and Mis12-GFP-FKBP. Note that our Mis12-FKBP constructs include a tandem trimer of FKBP, resulting in a total MW of 88 kDa for Mis12-GFP-FKBP. miRNA expression in this system effectively depletes endogenous FKBP. (B, C) Cells were imaged before and ~1 min after treatment with 500 nM rapamycin. Images (B) show mCherry at different expression levels of mCherry-FRB. In the presence of endogenous FKBP, mCherry-FRB recruitment is only observed at higher expression levels, but not at low expression levels (Figure 2.1 C). (C) mCherry-FRB expression levels were quantified based on cytoplasmic fluorescence. Recruitment was quantified by measuring the increase in mCherry intensity at kinetochores (identified by GFP signal, not shown) after rap treatment. Each data point represents a single cell, n ≥ 138 cells for each condition, pooled from 3 independent replicates. Knockdown of endogenous FKBP improves recruitment at all expression levels. (D) Cells expressing Mis12-GFP-FKBP, mCherry-FRB and FKBP miRNA were imaged before and after treatment with 50 nM or 500 nM rapamycin, as indicated. Scale bars 5 μm.
We reasoned that a fundamental limitation of this system is competition for FRB-binding between an exogenous, targeted FKBP construct and endogenous FKBP, a highly abundant cytosolic protein. Depletion of FKBP, which is not essential (Hoeffer et al., 2008; Weiward et al., 2006; De Angelis et al., 2009; Gerard et al., 2010), should remove this limitation. We added an artificial miRNA targeting the 3’UTR of endogenous FKBP to the constitutively expressed Mis12-GFP-FKBP transcript. This miRNA effectively depleted endogenous FKBP, but not exogenous Mis12-GFP-FKBP (Figure 2.2 A), without any overt effect on cellular morphology or growth. Endogenous FKBP depletion dramatically improved the efficiency of rapamycin-induced dimerization (Figure 2.1 C; Figure 2.2 B,C). We also found that the kinetics of FRB recruitment to kinetochores are sensitive to applied rapamycin concentration, with maximal recruitment in ~10 seconds using 500 nM rapamycin (Figure 2.2 D).

Mad1 recruitment to kinetochores at metaphase reactivates the checkpoint

We constructed a cell line that combined constitutive expression of FKBP miRNA and Mis12-GFP-FKBP with doxycycline-inducible expression of FRB-mCherry-Mad1. FRB-mCherry-Mad1 localized robustly to misaligned kinetochores and was removed as kinetochores aligned at the metaphase plate (Figure 2.3 A; Figure 2.6 A). In the absence of rapamycin, cells expressing FRB-mCherry-Mad1 proceeded through mitosis normally and rarely entered anaphase with misaligned chromosomes. After addition of rapamycin, both FRB-mCherry-Mad1 and endogenous Mad2 were recruited to kinetochores (Figure 2.3 B), which did not compromise metaphase plate organization or chromosome alignment, consistent with the results of constitutive tethering (Maldonado and Kapoor,
Figure 2.3: Mad1 recruitment to metaphase kinetochores activates the mitotic checkpoint. Cells were expressing FRB-mCherry-Mad1, FKBP miRNA and either Mis12-GFP-FKBP (A,C,D) or Mis12-FKBP (B). (A) Timelapse shows FRB-mCherry-Mad1 removal from the last mCherry-positive kinetochore pair before anaphase (at 22 min). (B) Cells were treated with rapamycin or vehicle (DMSO) control for 10 min, fixed and stained for Mad2. Control cells, which have not recruited mCherry, were also stained for Hec1. Images shown are representative of three independent replicates. (C,D) Cells were treated with either rapamycin or vehicle at metaphase (t = 0), then monitored for anaphase onset for 30 min. Images show Mad1 recruitment and metaphase arrest in rapamycin-treated but not control cells (C). Graph shows cumulative % of cells entering anaphase over time (D, n ≥ 24 cells for each condition, pooled from 6 independent replicates). Scale bars 5 µm.
These results indicate that FRB-mCherry-Mad1 is functional and does not exert dominant negative effects at the expression levels in our cells.

To test the effect of recruiting Mad1 to kinetochores after metaphase, we identified metaphase cells in an asynchronous population, added either rapamycin or a vehicle (DMSO) control, then monitored for 30 minutes to determine progress to anaphase (Figure 2.3 C,D). We only considered cells with a clearly defined metaphase plate, without misaligned chromosomes identifiable by Mis12-GFP signal, and without mCherry-Mad1 positive kinetochores. 100% of control cells proceeded to anaphase within 15 minutes of mock treatment. In contrast, after recruiting Mad1 to metaphase kinetochores, only 34% of cells entered anaphase within 30 minutes (Figure 2.3 D). In a related experiment, rapamycin was added at a fixed time (3 min) after removal of Mad1 from the last unattached kinetochore. In this case, very few (1/11) cells entered anaphase, consistent with recent results demonstrating that checkpoint reactivation depends on time in metaphase (Dick and Gerlich, 2013). We conclude that increasing Mad1 levels at bioriented, stably attached kinetochores is sufficient to switch them from a checkpoint-silenced to a checkpoint-active state.

Mad1 recruitment does not increase BubR1 and Mps1 levels at metaphase kinetochores

We next asked whether recruiting Mad1 to metaphase kinetochores activated the checkpoint through a concomitant increase in two other key checkpoint proteins, Mps1 and BubR1, that are enriched at unattached kinetochores. Mps1 kinase activity is required upstream of Mad1, to recruit Mad1 to unattached kinetochores, and downstream of Mad1, to recruit cytosolic Mad2 to kinetochore-bound Mad1:Mad2 (Hewitt et al.,
2010). BubR1 contains a conserved Mad3-homology domain that is the key inhibitor of Cdc20 and a pseudokinase domain that is an important signaling scaffold (Han et al., 2013; Suijkerbuijk et al., 2012). Levels of other checkpoint proteins, including Bub1, CENP-E, P150, ROD, and ZW-10, at metaphase kinetochores are not affected by including constitutive tethering of Mad1 to kinetochores (Maldonado and Kapoor, 2011).

We measured levels of endogenous BubR1 and Mps1 at kinetochores using immunofluorescence. As expected, Mps1 and BubR1 were significantly higher at misaligned prometaphase kinetochores than at aligned metaphase kinetochores in vehicle-treated controls. Recruiting Mad1 to metaphase kinetochores produced no detectable increase in levels of kinetochore-bound BubR1 or Mps1 (Figure 2.4 A,B). These data indicate that an increase in Mad1 levels at kinetochores is sufficient to activate the checkpoint without triggering an increase in kinetochore levels of Mps1 or BubR1.

*The Mad1 N-terminal localization domain is not required for checkpoint activation*

To determine what functional domains of Mad1 are necessary for checkpoint activation, we first tested the role of the N-terminal domain, which is required for kinetochore localization in *Xenopus* (Chung and Chen, 2002) and mammalian cells (Kim et al., 2012). It is not known whether this domain contributes to checkpoint activation beyond its role in localization. We removed the first 484 residues to generate Mad1\(^{ΔN}\), which matches a fragment used for *in vitro* studies of Mad2 conformational templating (De Antoni et al., 2005). FRB-mCherry-Mad1\(^{ΔN}\) fails to localize to unattached kinetochores in the absence of rapamycin (Figure 2.6 A), as expected, but recruits Mad2
Figure 2.4: Kinetochore levels of Mps1 and BubR1 are not affected by Mad1 recruitment at metaphase. Cells expressing Mis12-FKBP, mCherry-FRB and FKBPI miRNA were treated with rapamycin or vehicle for 10 minutes, then fixed. (A) Cells were stained for BubR1, together with Hec1 to label kinetochores in control cells, which have not recruited mCherry, and BubR1 levels at kinetochores were quantified. (B) Cells were stained for Mps1, together with CENP-C to label kinetochores in control cells, and Mps1 levels at kinetochores were quantified. Scale bars 5 µm. Error bars represent SEM from 3 independent experiments.
to metaphase kinetochores upon treatment with rapamycin (Figure 2.5 B; Figure 2.7 C), and induces a metaphase arrest (Figure 2.5 C,D). These results show that the N-terminal domain of Mad1 is not required for checkpoint activation beyond localizing Mad1 to kinetochores.

**Mad1 C-terminus is required for checkpoint activation; Mad2-binding is not sufficient**

Because Mad2 binding is the best-characterized activity of Mad1, we tested whether Mad2-binding is sufficient for Mad1 to activate the checkpoint. We truncated Mad1\(^{\Delta N}\) at the C-terminus to construct Mad1\(^{\text{MID}}\), comprising residues 485-584, including the Mad2 interaction loop flanked by alpha-helical regions, as shown in the crystal structure of the tetrameric Mad1:Mad2 complex (Sironi et al., 2002). FRB-mCherry-Mad1\(^{\text{MID}}\) recruits Mad2 to metaphase kinetochores upon treatment with rapamycin to similar levels as Mad1 and Mad1\(^{\Delta N}\) (Figure 2.5 B; Figure 2.7 C) but does not induce a metaphase arrest (Figure 2.5 C,D). In contrast, deletion of the Mad2-binding region prevents checkpoint reactivation (Figure 2.7 D,E), consistent with previous results (Maldonado and Kapoor, 2011). These results indicate that localizing Mad2 to kinetochores is necessary but not sufficient to activate the checkpoint, and that the C-terminal domain of Mad1 is also required.

**A conserved RLK motif in the Mad1 C-terminus is required for checkpoint activity**

In *S. cerevisiae*, mitotic checkpoint activity can be abolished by mutating three conserved amino acids in the C-terminus of *S. cerevisiae* Mad1 to alanine: R653, L654 and K655, which correspond to residues 617-618 in human Mad1 (Brady and Hardwick,
Figure 2.5: A conserved RLK motif in the C-terminal domain of Mad1 is required to activate the checkpoint. (A) Schematic representation of Mad1 constructs. Black outline indicates Mad2-binding region and black bar indicates RLK motif mutated to AAA. (B-D) Cells were expressing Mis12-GFP-FKBP, FKBP miRNA and FRB-mCherry-Mad1 fragments as indicated. (B) Cells were treated with rapamycin or vehicle for 10 min, then fixed and stained for DNA and Mad2. Images are representative of three independent experiments (quantified in Figure 2.7 C). (C) Cells were treated with rapamycin at metaphase (t = 0), then monitored for anaphase onset for 30 min. Vehicle controls shown in Figure 2.6 B. (D) Graph shows cumulative % of cells entering anaphase over time (n ≥ 26 cells for each condition, pooled from ≥ 4 independent replicates). Scale bars 5 µm.
Figure 2.6: Characterization of Mad1 truncation mutants. Cells were expressing Mis12-GFP-FKBP, FKBP miRNA and FRB-mCherry-Mad1 fragments as indicated. (A) In early prometaphase cells, full length Mad1, but not N-terminal truncation mutants, localizes to unaligned kinetochores in the absence of rapamycin. Note: Mad1$^{ΔN-AAA}$ cell was imaged separately, so localization can be compared to the other images, but not overall brightness. (B) Cells were imaged live before and after addition of DMSO (t = 0), as a vehicle control to the rapamycin experiment in Figure 2.5 C. Images are representative of ≥3 independent replicates. Scale bars 5 μm.
Figure 2.7: Mad2-binding is required for checkpoint activation by Mad1. (A) Schematic representation of truncation mutant Mad1ΔN549 lacking the Mad2-binding region, compared to full-length Mad1. (B) Cells expressing Mis12-GFP-FKBP, FKBP miRNA and FRB-mCherry-Mad1ΔN549 were treated with rapamycin or vehicle for 10 min, then fixed and stained for DNA, Mad2 and Hec1. Mad1ΔN549 does not recruit Mad2 to kinetochores after rapamycin treatment. (C) Parental HeLa cells and cells expressing Mis12-GFP-FKBP, FKBP miRNA and indicated FRB-mCherry-Mad1 constructs were treated with rapamycin for 10 min, then fixed and stained for DNA, Mad2 and Hec1. Mad2 levels at metaphase kinetochores were quantified in >8 cells per condition in each of three independent replicates. Error bars represent SEM. (D, E) Cells were expressing Mis12-GFP-FKBP, FKBP miRNA and FRB-mCherry-Mad1ΔN549. (D) Cells were treated with rapamycin or vehicle at metaphase (t = 0), then monitored for anaphase onset for 38 min. (E) Graph shows cumulative % of cells entering anaphase over time (n ≥ 31 cells, pooled from 3 independent replicates). Scale bars 5 µm.
2000). Mutating either R617 or K619 in human cells impairs kinetochore targeting, presumably by disrupting interactions with other kinetochore proteins (Kim et al., 2012), but the role of the RLK motif in checkpoint activation independent of targeting has not been tested. To determine whether checkpoint activation depends on the C-terminal RLK motif we mutated these three residues to alanines in the context of Mad1ΔN. Mutating the RLK motif does not reduce Mad2 recruitment (Figure 2.5 B; Figure 2.7 C), but almost completely abolishes the ability of Mad1 to activate the checkpoint when recruited to metaphase kinetochores (Figure 2.5 C, D). This result demonstrates that a conserved protein interaction motif in the C-terminus of Mad1 is required for full checkpoint activity.

Conclusions

In conclusion, our results show that the spindle assembly checkpoint can be reactivated after silencing by relocalizing Mad1 to metaphase kinetochores. Checkpoint activation does not require the increased levels of Mps1 and BubR1 associated with unattached kinetochores: the basal levels of these proteins present at metaphase kinetochores are sufficient to sustain APC/C inhibition when combined with increased Mad1:Mad2. Both Mps1 and BubR1 exhibit rapid turnover at metaphase kinetochores (Howell et al., 2004), so a large fraction of Mps1 and BubR1 may transiently encounter kinetochore-localized Mad1:Mad2 even though steady-state levels of Mps1 and BubR1 at kinetochores remain low. Additionally, Mps1 and BubR1 play other roles at the kinetochore independent of their checkpoint functions, for example regulating microtubule interactions (Lampson and Kapoor, 2005; Maure et al., 2007; Jelluma et al.,
2008; Meyer et al., 2013). It is possible that the increased levels of some checkpoint proteins at unattached kinetochores contribute to functions other than APC/C inhibition.

The results of our Mad1 mutation experiments indicate that the role of Mad1 in APC/C inhibition is not limited to localizing Mad2 to kinetochores. The potent effect of mutating the Mad1 RLK motif strongly suggests that the C-terminus of Mad1 mediates interactions with other kinetochore proteins that are essential for checkpoint activity in addition to contributing to Mad1 localization. A growing body of evidence argues that APC/C inhibition is a multistep process in which Cdc20 first binds closed Mad2 (cMad2), which catalyzes the subsequent binding of BubR1 to Cdc20, and that BubR1:Cdc20 is the primary inhibitor of the APC/C (Nilsson et al., 2008; Kulukian et al., 2009; Westhorpe et al., 2011; Han et al., 2013). The C-terminus of Mad1 may act as a scaffold to promote either the binding of Cdc20 to cMad2 or the transfer of Cdc20 from Mad2 to BubR1. An important future goal is to determine which proteins interact with the C-terminus of Mad1, and what role these interactions play in checkpoint activation.

Finally, our work demonstrates the potential for inducible protein dimerization as a tool to manipulate protein localization at kinetochores on a rapid timescale. Dynamic localization is a hallmark of many mitotic proteins, and much has been learned from disrupting localization by mutating targeting domains or conferring new localization through constitutive tethering, but these techniques are fundamentally limited by a lack of temporal control. Chemically induced dimerization overcomes this limitation, and allows us to study the effects of altering protein localization in real time.
Chapter 3: TMP-Halo and photocaged NVOC-TMP-Halo

Summary

In this chapter I describe a new technique to induce protein-protein heterodimerization with spatial and temporal control in living cells, using a photocaged bifunctional linker, which I term NVOC-TMP-Halo. This molecule combines covalent protein labeling via the Halo-tag ligand with light-induced, noncovalent capture of a second protein via photocaged trimethoprim. Using this system, I successfully achieve photo-induced recruitment of a cytosolic protein to centromeres, which has not been reported with any other light-induced dimerization system to date. This new method sets the stage for exciting future studies.

Introduction

Biological processes such as cell division, signaling and migration require the participation of tens or hundreds of components that must function at precisely the right time and place. The locations and functions of individual protein components in a biological system are often determined by protein-protein interactions, so inducing specific protein-protein interactions with spatial and temporal control is a powerful technique to interrogate biological systems. Light-induced protein dimerization offers the ability to experimentally perturb the localization and associations of proteins with a level of spatial and temporal control comparable to the time and space scales on which biological processes are naturally organized in living cells.
To this end, a number of techniques have been developed in the past 20 years to control protein-protein interactions, with various degrees of spatial and temporal precision. The first such technique was chemically induced dimerization, which can drive a protein interaction, or change the localization of a protein, with the temporal control afforded by small-molecule treatment. Chemically induced dimerization can be quite fast, but is difficult to spatially restrict on a subcellular scale.

In contrast, light-inducible protein dimerization does allow spatial and temporal control. Light can be focused on a scale of less than 1 micrometer, and exposure can be controlled on essentially any time scale. The first system for light-inducible dimerization, Phy/Pif, was introduced in 2009, in a groundbreaking paper (Levskaya et al., 2009). To date, at least 7 different systems for photo-controlled dimerization have been reported, using either photosensitive proteins or photoreactive small molecules, but none has emerged as universally optimal for all applications (Levskaya et al., 2009; Yazawa et al., 2009; Kennedy et al., 2010; Karginov et al., 2010; Umeda et al., 2011; Strickland et al., 2012; Ahmed et al., 2014). Notably, no light-induced dimerization system has been successfully used for kinetochores or centromeres, and one system (Phy/Pif) has been reported to be difficult to apply at kinetochores in budding yeast (Yang et al., 2013).

I describe here a new technique for light-induced protein dimerization using a photocaged trimethoprim-Halotag bifunctional linker. The advantage of the caged-TMP-Halo system is that it works robustly in cellular compartments that have not been successfully targeted by other light-induced dimerization systems. Photochemical
uncaging is irreversible, which may be disadvantageous for some applications but highly advantageous for others, such as studying the long-term effects of spatially localized perturbations. Our system is made entirely of “off the shelf” parts: proteins and chemicals that are very well-studied and have proven functional in a wide range of applications. The completely modular structure of this compound should aid future optimization and the introduction of novel functions.

**Molecular design rationale**

Some natural product small molecules intrinsically function as protein homodimerizers (coumermycin) or heterodimerizers (rapamycin, FK506, gibberellin and others). In principle, however, a dimerizer can be artificially constructed by linking any two small-molecule ligands, or two copies of the same ligand (*Figure 3.1 A,B*). The linker must be 1) attached at sites on the ligands that don’t interfere with binding to their protein receptors 2) long enough to allow the two protein receptors to bind to the dimerizer simultaneously, and 3) resistant to degradation in cells. This simple design principle has been well borne out, with over 15 different artificial dimerizers (pairs of ligands joined by an inert linker) reported since the first, FK1012, was introduced in 1993 (Spencer et al., 1993; Belshaw et al., 1996b; Gallagher et al., 2007; Czlapinski et al., 2008; Erhart et al., 2013; Corson et al., 2008; Putyrski and Schultz, 2012). I reasoned that adding a photocage to one half of an artificial heterodimerizer would yield a useful photo-inducible dimerization reagent (*Figure 3.1 C,D*).

For the underlying dimerizer I chose to chemically link Trimethoprim (TMP) and and the Halotag ligand (*Haloligand, Figure 3.2 A*), generating a novel heterodimerizer
Figure 3.1: Schematic diagrams of non-caged and photocaged artificial heterodimerizers. (A) Two generic ligand-receptor interactions are represented. The black circle and square represent any two small molecules that are tightly and specifically bound by a cognate protein receptor. (B) Linking the circle and square molecules produces a bifunctional molecule that can simultaneously bind both the circle receptor and square receptor proteins. (C) A photocage is represented as a 9-pointed star which can be conjugated to the circle molecule, and which, when exposed to light, is cleaved. (D) A heterodimerizer in which one ligand (circle) is photocaged, and the second ligand (square) is non-caged. This molecule is able to bind the square receptor, but does not bind the circle receptor until the cage is removed by illumination.
**TMP-Halo (Figure 3.2 B).** TMP is a dihydrofolate reductase (DHFR) inhibitor that is selective for bacterial DHFR enzymes, including that from *E. coli* (Baccanari et al., 1982). The Halotag ligand is a protein labeling tool that selectively and covalently labels an engineered mutant bacterial alkyldehalogenase enzyme (Los et al., 2008). I chose to photocage TMP (Figure 3.2 C–E), leaving the Halotag ligand as the constitutively active or “non-caged” end of the molecule (Figure 3.2 E). The term “uncaged” is ambiguous, since it can refer either to a molecule that has never been caged, or to the liberated form of a formerly caged molecule. To avoid this ambiguity, I will use the term “non-caged” to mean “never-caged” or “constitutively active.”

I reasoned that using a covalent labeling tag as the non-caged half of the dimerizer would be advantageous, as it would allow us to saturate one of the protein receptors (the Halotag fusion protein) and then remove any excess reagent by washout prior to experiment. The Halotag system is based on the alkyldehalogenase enzyme Dhha from the bacterium *Rhodococcus rhodochrous* (Kulakova et al., 1997) which has been mutated so that it cannot complete its catalytic cycle and forms an irreversible, covalent linkage to chloroalkane-containing substrates (Los et al., 2008). This system was commercially developed by Promega Corp. under the brand name “Halotag.” In product materials and scientific literature, “Halotag” is used ambiguously to refer to both the protein and its ligands. To avoid this ambiguity, I will refer to the protein as “Haloenzyme” and the chloroalkane ligand as “Haloligand.”

TMP was chosen because it is small, chemically simple and highly selective (~3000 fold in the NADPH-bound state) for bacterial DHFR over mammalian DHFR, so
Figure 3.2: Chemical structures of the molecules described in this study. (A) Trimethoprim and the Haloligand are schematically represented as a small black circle and square, respectively. (B) TMP-Halo, a non-caged dimerizer. (C) NVOC is shown with a carbamate linkage to a generic R group. (D) The ortho- and para- regioisomers (relative to the central benzylic carbon) of NVOC-TMP obtained by linking a single copy of NVOC to either of the exocyclic amines of TMP. (E) Chemical structure of NVOC-TMP-Halo, here drawn with NVOC on the para-position.
it is effectively bio-orthogonal in a human cell background (Baccanari et al., 1982; Miller et al., 2005). Virginia Cornish’s lab has used TMP for a variety of protein labeling applications, including protein dimerization (Gallagher et al., 2007; Chen et al., 2012). Linkers can be added to the \textit{para}- position of the trimethoxyphenyl ring without interfering with TMP binding to DHFR (\textbf{Figure 3.2 B,E}) (Miller et al., 2005). The aniline ring, however, is buried deeply and tightly in the DHFR substrate binding pocket. We predicted that adding a bulky photocage to one of the two exocyclic amines of TMP would effectively block DHFR binding (\textbf{Figure 3.2 D,E}) (Heaslet et al., 2009).

We chose 6-nitroveratryloxy carbonyl, (NVOC) as the photocage (\textbf{Figure 3.2 C}). NVOC is an \textit{ortho}-nitrobenzyl compound, a widely used family of photocages (Klán et al., 2013). A common strategy for photocaging amines is to attach the cage through a carbamate linkage (\textbf{Figure 3.2 C,D}), which rapidly decarboxylates upon photocleavage, releasing the native amine (Cummings and Krafft, 1988; Klán et al., 2013). Exocyclic amines analogous to those of TMP have been photocaged using carbamate linkages (Furuta et al., 2007). NVOC photocleavage is typically induced with 365 nm light, but uncaging at longer wavelengths—up to 420 nm—has been reported, even though the absorbance of NVOC at wavelengths longer than 400 nm is very low (Klán et al., 2013). We reasoned that the low absorbance of NVOC at visible wavelengths would be an advantage as it would minimize the risk of fluorescent imaging causing undesired photocleavage.
Results

Synthesis of the non-caged dimerizer TMP-Halo

We first prepared TMP-Halo to test whether this molecule was capable of entering cells, reacting with the Haloenzyme and inducing dimerization with bacterial DHFR. Amine-functionalized TMP and carboxylic acid-functionalized Haloligand were prepared following literature precedent and coupled to generate TMP-Halo, a novel bifunctional molecule. For synthetic chemistry details, see Chapter 4. TMP-Halo has a 20-atom linker between the para-oxygen of TMP and the terminal carbon of the Haloligand, for a maximum length of ~25 nm between these atoms. Simple rigid-body modeling based on crystal structures of Haloenzyme and of bacterial DHFR in complex with TMP suggest that this is sufficient to allow TMP-Halo to span these proteins without significant steric clash (PDB IDs 4KAA and 2W9H).

Generation of protein fusions and stable cell line to test TMP-Halo dimerization

I sought to test whether TMP-Halo could induce dimerization between a freely diffusible *E. coli* DHFR fusion protein and the Haloenzyme fused to a protein that localizes to a specific cellular location. I chose the DNA-binding domain of CENPB (centromere protein B) as a localization anchor, which binds a specific DNA sequence present in repetitive centromeric chromatin. CENPB has a robust, predictable localization pattern, and is tolerant of fusion proteins. DHFR was fused to mCherry to monitor its localization. I generated a stable HeLa cell line that expresses CENPB-Haloenzyme and mCherry-DHFR. These proteins are expressed from the constitutive
CAG promoter with an internal ribosome entry sequence (IRES) for bicistronic expression (Figure 3.3 A,B). We predicted CENPB-Haloenzyme would localize to centromeres but that mCherry-DHFR would localize diffusely throughout the cell. Addition of TMP-Halo would induce dimerization between DHFR and Haloenzyme, causing mCherry-DHFR to accumulate at centromeres. By contrast, photocaged NVOC-TMP-Halo would not induce dimerization until uncaged with light (Figure 3.3 C).

As expected, mCherry-DHFR diffused uniformly through the cytosol and nucleus, but appeared to be excluded from the nuclear envelope, mitochondria and dense chromatin structures such as nucleoli and mitotic chromosomes (Figure 3.4 B). CENPB-Haloenzyme expression and localization, and Halotag functionality were tested by treating cells with a commercially available Haloligand-dye conjugate, Halotag-Oregon Green (Halo-OG, Promega Corp.) (Figure 3.4 A). As expected, this dye specifically labeled punctate structures in interphase nuclei and paired puncta in metaphase chromosomes (Figure 3.4 B).

**TMP-Halo proof-of-principle testing (non-caged dimerization)**

For NVOC-TMP-Halo to function as a photo-inducible dimerizer, the underlying TMP-Halo backbone must be able to induce dimerization by simultaneously binding Haloenzyme and DHFR. To test this, cells expressing CENPB-Haloenzyme and mCherry-DHFR were treated with TMP-Halo. Encouragingly, TMP-Halo treatment caused mCherry-DHFR to localize to centromeres in interphase and mitosis (Figure 3.5). This basic success confirmed that TMP-Halo could enter cells, react with the
Figure 3.3: Schematic diagrams of transgene cassette, recombinant proteins, direct chemical dimerization and photoinduced chemical dimerization.

(A) Bicistronic expression construct used to express CENPB-Haloenzyme and mCherry-DHFR, as integrated into the genome via Cre/Lox recombinase mediated cassette exchange. (B) Schematic diagrams of CENPB-Haloenzyme and mCherry-DHFR used in this chapter. (C) Diagram of the expected behavior of a cell expressing CENPB-Haloenzyme and mCherry-DHFR on the scale of a whole cell or on a molecular scale. In the absence of any drug, there is no association between CENPB-Haloenzyme and mCherry-DHFR. Addition of non-caged TMP-Halo directly induces recruitment of mCherry-DHFR to centromeres. When NVOC-TMP-Halo is added, it initially reacts with CENPB-Haloenzyme, at centromeres, but does not recruit mCherry-DHFR. Illumination causes cleavage of the NVOC cage, unmasking TMP and triggering centromere recruitment of mCherry-DHFR.
Figure 3.4: mCherry-DHFR and CENPB-Haloenzyme behave as expected. (A) Schematic diagram illustrating Halo-Oregon Green (Halo-OG) labeling of CENPB-Haloenzyme. (B) Cells expressing CENPB-Haloenzyme and mCherry-DHFR were treated with 100 nM Halo-OG for 20 minutes, washed, and imaged live. mCherry-DHFR localizes diffusely throughout interphase and mitotic cells but is excluded from the nuclear envelope, nucleoli and mitotic chromosomes. Small bright puncta of mCherry-DHFR are sometimes observed. Halo-OG labels large amorphous puncta in interphase nuclei and paired puncta on mitotic chromosomes consistent with centromere localization of CENPB. Scale bar, 5 μm.
**Figure 3.5:** TMP-Halo functions as a chemical dimerizer. (A) Schematic diagram illustrating TMP-Halo dimerization of CENPB-Haloenzyme and mCherry-DHFR. (B) Cells expressing CENPB-Haloenzyme and mCherry-DHFR were treated with 10 nM Halo-TMP for 12 hours, or untreated as control, then briefly washed in untreated media and imaged live. Interphase and prometaphase cells are shown. Cells treated with TMP-Halo (but not control cells) exhibit strong localization of mCherry-DHFR to nuclear and chromosomal puncta typical of CENPB, indicating that TMP-Halo dimerizes CENPB-Haloenzyme and mCherry-DHFR. Scale bar, 5 μm. Note: control cells and treated cells were not imaged using the same imaging parameters in this experiment. The brightness and contrast of the mCherry images set for optimal display of the full dynamic range of the image. Interpretation of the localization pattern of mCherry-DHFR is not affected by this technical difference.
Haloenzyme and induce dimerization between Haloenzyme and DHFR, validating our overall design. The ability of TMP-Halo to enter cells was a positive indicator that NVOC-TMP-Halo might be cell permeable.

One limitation of a dimerizer such as TMP-Halo is that the presence of excess reagent can monovalently saturate its two receptors (Haloenzyme and DHFR) and prevent dimerization (Figure 3.6 A). In overnight incubation experiments, 100 nM TMP-Halo was found to be more effective than a higher dose (1 µM) (Figure 3.6 B). However, when these cells were washed and incubated for 12 hours in untreated media, dimerization was observed even in the high-dose cells that had previously shown very weak dimerization (Figure 3.6 B). This indicates that the presence of excess TMP-Halo can indeed interfere with DHFR/Haloenzyme dimerization. It may also be feasible to use excess free TMP to reverse dimerization.

**Synthesis and photocleavage of photocaged TMP test compound**

**NVOC-TMP** was prepared in a single reaction from TMP and chloroformate-functionalized NVOC, which are both commercially available. Photocaged trimethoprim is a novel compound, but there is literature precedent for analogous reactions of NVOC with exocyclic amines, and of TMP with other chloroformates. Reaction of TMP with NVOC-chloroformate produced a ~1:1 mixture of products which were separable by TLC and silica chromatography, which we interpreted as the ortho- and para-regioisomers of TMP-NVOC (Figure 3.2 D). High-resolution mass-spectrometry
**Figure 3.6:** Excess TMP-Halo inhibits mCherry-DHFR/CENPB-Haloenzyme dimerization, but is ameliorated by washout. (A) Schematic diagram illustrating how excess dimerizer can prevent dimerization by monomerically saturating the Haloenzyme and DHFR binding sites. Only the TMP-Halo which is covalently bound to CENPB-Haloenzyme is retained after washout, driving efficient dimerization. (B) Cells expressing CENPB-Haloenzyme and mCherry-DHFR were treated with 100 nM or 1 μM TMP-Halo for 12 hours, then washed briefly and imaged live. These cells were then incubated for 12 hours in untreated media as an extended washout period, and imaged again (the before and after images do not represent the same field of cells). Prior to washout, less mCherry-DHFR recruitment to centromeres is observed in with 1 μM TMP-Halo than 100 nM. Washout increases the level of dimerization observed in both populations of cells. This indicates that TMP-Halo induced dimerization persists in cells for at least 12 hours, and that excess free dimerizer inhibits dimerization. Scale bar, 5 μm.
confirmed that the products are isobaric and match the predicted mass of TMP-NVOC (529.180 AMU). Efforts are underway to unambiguously identify the isomers.

NVOC-TMP in methanol was exposed to 254 nm UV light using a handheld lamp for 2 hours or left on a bench with the room lights on overnight, and assayed by TLC. Light exposure caused the disappearance of the NVOC-TMP and the appearance of a low-mobility product that comigrated with TMP. The photoproduct was isolated and analyzed by high-resolution mass spectrometry, which revealed that it has the exact mass of TMP (290.138 AMU), confirming that NVOC-TMP can be photolyzed to yield unadulterated TMP.

*Synthesis of photocaged dimerizer NVOC-TMP-Halo*

The NVOC photocage was installed on linker-functionalized TMP using the same reaction conditions as for unmodified TMP, see Chapter 4. As before, this reaction produces a ~1:1 mixture of isobaric regioisomers (672.276 AMU) that are separable by silica chromatography. The early-eluting isomer was carried forward to synthesize NVOC-TMP-Halo.

*Development of an assay for TMP-Halo cellular uptake and Haloenzyme reactivity*

One important parameter in our system is the extent to which TMP-Halo and NVOC-TMP-Halo enter cells and react with the Haloenzyme. Neither TMP-Halo nor NVOC-TMP-Halo are fluorescent, so we cannot directly image these molecules. I developed a simple assay to measure the availability of Haloenzyme sites by exploiting the rapid kinetics of labeling using the Halo-OG dye. Staining intensity of Halo-OG
should be inversely proportional to the extent to which a competing Haloligand such as TMP-Halo or NVOC-TMP-Halo has occupied the Haloenzyme during prior treatment (Figure 3.7).

Cells were incubated with 10 nM or 1 µM TMP-Halo or 1 µM NVOC-TMP-Halo for 12 hours, then, alongside untreated control cells, were washed and stained with 100 nM Halo-OG for 20 minutes. Treatment with 1 µM TMP-Halo almost completely blocked Halo-OG staining, whereas low-dose (10 nM) TMP-Halo blocked Halo-OG to a markedly lesser extent (Figure 3.7 C). 1 µM NVOC-TMP-Halo also blocked Halo-OG, but only to a level comparable to 10 nM TMP-Halo. The blocking effect of 1 µM NVOC-TMP-Halo was much less than that of an equal concentration of the non-caged compound (Figure 3.7 C). This positive result indicated that NVOC-TMP-Halo can enter cells, but suggested that higher concentrations may be necessary to achieve complete Haloenzyme occupancy.

1-hour treatment with 20 µM NVOC-TMP-Halo fully labels CENPB-Haloenzyme

Cells expressing CENPB-Haloenzyme and mCherry-DHFR were treated with 20 µM NVOC-TMP-Halo for 1 hour (or left untreated as control), then subjected to the Halo-OG staining assay (Figure 3.8 A). This high-dose/short-time treatment effectively abolished Halo-OG staining, indicating that it is possible to achieve effective loading of NVOC-TMP-Halo on a reasonably short timescale (Figure 3.8 B). Crucially, even these high levels of Haloenzyme occupancy by TMP-NVOC-Halo did not detectably recruit
**Figure 3.7**

(A) Diagram of full Halo-OG staining in untreated cells. (B) Diagram of Halo-OG blocking by prior treatment with Halo-TMP. (C) Cells expressing CENPB-Haloenzyme and mCherry-DHFR were treated TMP-Halo or NVOC-TMP-Halo at indicated concentrations for 12 hours, then washed and stained with 100 nM Halo-OG for 20 min, washed, then imaged live. Oregon Green images displayed at two brightness levels to illustrate low signal in cells treated with 1 μM TMP-Halo. 1 μM NVOC-TMP-Halo blocks Halo-OG staining to a similar extent as 10 nM Halo-TMP. Scale bar, 5 μm.
Figure 3.8: Short treatment with a high concentration of NVOC-TMP-Halo achieves full labeling of CENPB-Haloenzyme. (A) Diagram of Halo-OG blocking by NVOC-TMP-Halo. The presence of NVOC-TMP-Halo at CENPB-Haloenzyme can be inferred by diminished Halo-OG staining and the absence of mCherry-DHFR recruitment. (B) Cells expressing CENPB-Haloenzyme and mCherry-DHFR were treated with 20 μM NVOC-TMP-Halo for 1 hour, quickly washed and stained 100 nM Halo-OG for 20 min then washed and imaged live. Halo-OG staining is almost completely blocked by this NVOC-TMP-Halo treatment. No mCherry-DHFR centromere recruitment is detectable, indicating effective blocking of DHFR-TMP binding by the NVOC photocage. Oregon Green images displayed at two brightness levels to illustrate very low signal in treated cells. Scale bar, 5 μm.
mCherry-DHFR to centromeres, suggesting that the NVOC adduct successfully blocks binding of TMP to DHFR (Figure 3.8 B).

**NVOC-TMP-Halo functions as a photocaged dimerizer**

As a final test of the overall functionality of NVOC-TMP-Halo, cells treated as above for 1 hour with 20 µM NVOC-TMP-Halo were exposed to UV illumination using a halogen lamp and a 387/11 nm bandpass filter (Figure 3.9 A). After a 2 second illumination, mCherry-DHFR rapidly translocated to centromeres in both mitotic and interphase cells (Figure 3.9 B). Recruitment was detectable after 10 seconds and steadily increased, reaching a maximum within 60 seconds. Subsequent UV exposure or longer imaging did not induce any further mCherry-DHFR recruitment (data not shown). This promising positive result indicates that NVOC-TMP-Halo successfully functions as a photocaged dimerizer and sets the stage for further testing and the application to biological experiments, which are already ongoing.

**Future directions**

*Testing recruitment to individual centromeres, kinetochores and other targets*

The next step in the development of light-inducible dimerization using NVOC-TMP-Halo is to test whether we can achieve localized protein recruitment on a subcellular scale. I have refrained from testing this using the dimerization constructs described in this chapter because the DNA binding domain of CENPB rapidly exchanges
Figure 3.9: UV illumination uncages NVOC-TMP-Halo and induces dimerization. (A) Diagram of light-induced dimerization between CENPB-Haloenzyme and mCherry-DHFR mediated by NVOC-TMP-Halo. (B) Cells expressing CENPB-Haloenzyme and mCherry-DHFR were treated with 20 μM NVOC-TMP-Halo for 1 hour, quickly washed and imaged live. Cells were imaged in the mCherry channel before (pre-UV), and immediately after a 2 second-exposure to near-UV light (1-second post-UV). Cells were imaged every 10 seconds subsequently with no further UV exposure. No mCherry signal is detectable at centromeres prior to UV illumination, but mCherry-DHFR rapidly translocates to centromeres in response to UV light. Scale bar, 5 μm.
between centromere-bound and soluble pools, rendering it unsuitable for localized protein recruitment. In FRAP experiments, the CENPB DNA binding domain exhibits >60% fluorescence recovery within 60 seconds (Hemmerich et al., 2008). By contrast, full-length CENPB binds mitotic centromeres very stably, exhibiting no turnover in FRAP experiments (Hemmerich et al., 2008). I have generated cell lines analogous to the one described in this chapter expressing full-length CENPB-Haloenzyme or full-length CENPB-GFP-Haloenzyme plus mCherry-DHFR, which will be ready for use soon.

I am also in the process of constructing cell lines for dimerization of mCherry-DHFR in combination with Haloenzyme fusions to:

1: the microtubule associated protein MAP4
2: a myristoylation domain for plasma membrane targeting
3: a centrosome-targeting PACT domain
4: a mitochondrial outer envelope targeting domain (from *L. monocytogenes* ActA)
5: the telomere protein TRF1
6: the kinetochore proteins Mis12 and Nuf2

The MT, plasma membrane, centrosome, mitochondrial and telomere targeting constructs will allow us to test the applicability of our technique in diverse cellular compartments. Kinetochore localization is of particular interest to our lab. Both Nuf2 and Mis12 exhibit slow turnover at kinetochores when measured by FRAP (Hori et al., 2003; Hemmerich et al., 2008) and so should be excellent kinetochore anchors for inducible dimerization. Spatially defined kinetochore targeting will allow us to pursue promising lines of research that are already ongoing involving the Mitotic Checkpoint and Aurora B regulation of kinetochore-microtubule dynamics.
Mitotic Checkpoint experiments

As described in Chapter 2, protein localization is central to the Mitotic Checkpoint. One of the hallmarks of Mitotic Checkpoint function is that a single unattached kinetochore is able to generate sufficient inhibitory signal to prevent anaphase (Lara-Gonzalez et al., 2012). We were able to gain insight into the requirements for checkpoint activation by recruiting Mad1 to all kinetochores at metaphase using rapamycin-induced dimerization, but a much more stringent test is whether Mad1 recruitment to a single metaphase kinetochore is sufficient for checkpoint reactivation. If Mad1 enrichment at a single metaphase kinetochore is not sufficient for checkpoint reactivation, this would imply that other factors normally present at an unattached kinetochore (such as high levels of other checkpoint proteins) are also required for checkpoint activation. If so, we could test the effects of recruiting combinations of checkpoint proteins, including Mps1 and BubR1, to a single metaphase kinetochore to define the minimal set of inputs required for full checkpoint activity.

Another question we could address is whether the kinetochore itself is strictly required for generating spindle checkpoint signaling, or whether a high concentration of checkpoint proteins at another cellular location might be sufficient. Using light-inducible dimerization, we could recruit one or more checkpoint proteins to alternative chromosomal locations or even to a non-chromosomal compartment such as a mitochondrion, and test whether this is sufficient to activate the checkpoint. Global recruitment to all chromatin, or all mitochondria might dilute the checkpoint proteins, but
light induced dimerization could produce focused puncta enriched for checkpoint proteins.

**Probing the mechanism of Aurora B regulation of kinetochore-microtubule dynamics**

A fundamental question in the field of kinetochore dynamics is the nature of the effect of Aurora B activity on the kinetochore-microtubule interface. It is well known that Aurora B phosphorylation of MT-binding kinetochore proteins decreases their affinity for MTs (Carmena et al., 2012), so an intuitive expectation might be that Aurora B activity causes a kinetochore to release its bound microtubules. Paradoxically, however, increased Aurora B activity triggers a syntelically attached pair of kinetochores to move poleward through MT depolymerization in human cells (Lampson et al., 2004). Recent biophysical studies using purified (budding) yeast kinetochores with or without Aurora B phosphomimetic mutations show that Aurora B phosphorylation both increases the rate of switching from the MT-growing to MT-depolymerizing state and increases the probability that the kinetochore will disengage from the MT (Sarangapani et al., 2013). Budding yeast kinetochores only bind a single microtubule, whereas mammalian kinetochores bind bundles of 20-30 MTs, so extrapolating from these simplified systems to predict the behavior of an intact pair of mammalian kinetochores in a living cell is not trivial.

To address these questions, I initiated a project to test the effects of increasing Aurora B activity at kinetochores with temporal control using rapamycin-mediated dimerization. I have found that global recruitment of Aurora B to well-ordered metaphase kinetochores causes a gradual but ultimately severe chromosome
misalignment phenotype, in which kinetochore pairs migrate towards the poles (Figure 3.10). In these experiments, Aurora B activity is increased at all kinetochores, so changes in the behavior of a kinetochore pair must be interpreted in light of the fact that both have been simultaneously perturbed.

Using light-inducible dimerization, we can test the effects of acutely increasing Aurora B activity at a single kinetochore within a bioriented pair. If the effect of Aurora B activity is to primarily promote MT release, we predict that the treated kinetochore will detach from its MT fiber and the pair will rapidly move towards the opposite pole. Alternatively, if Aurora B activity predominantly drives microtubule depolymerization instead of kinetochore release, we expect the treated kinetochore to move towards its proximal pole, pulling the sister kinetochore behind it in a polymerizing-MT state. Intriguingly, recruitment of Mps1 kinase to metaphase kinetochores also induces a dramatic misalignment phenotype (Figure 3.11). Inducible dimerization might be useful in unraveling the role of Mps1 kinase in regulating kinetochore-microtubule dynamics, in addition to its role in the Spindle Assembly Checkpoint (Maure et al., 2007; Meyer et al., 2013).

*Spatially-resolved light-induced protein degradation*

Another appealing application of light-inducible dimerization is targeted protein degradation. Currently the most rapid and potent inducible degradation system available is Auxin-Induced Degradation, which is essentially an inducible dimerization system borrowed from plants. The plant hormone Auxin induces dimerization between its receptor, which is a ubiquitin ligase subunit, and a degron targeted by that ubiquitin
Figure 3.10: Recruitment of active Aurora B to metaphase kinetochores induces chromosome misalignment. **A:** Schematic diagram of transgene cassette used to express: miRNA against endogenous FKBP, Mis12-GFP-FKBP, mCherry-INbox-FRB. INbox is the C-terminal 100 amino acids of INCENP (residues 818-918), which binds and activates Aurora B kinase. **B:** schematic diagram of recombinant proteins in this experiment. The INbox domain is shown bound to Aurora B. **C:** cells expressing Mis12-GFP-FKBP, FRB-INbox-mCherry and a miRNA against endogenous FKBP were imaged before and after treatment with 50 nM rapamycin. Images shown are maximum-intensity Z-projections of 5 confocal sections with 1 μm spacing. Scale bar, 5 μm.
Figure 3.11: Recruitment of Mps1 kinase to metaphase kinetochores induces chromosomes misalignment.

A: Schematic diagram of transgene cassette used to express: miRNA against endogenous FKBP, Mis12-GFP-FKBP, FRB-mCherry-Mps1.  
B: schematic diagram of recombinant proteins in this experiment.  
C: cells expressing Mis12-GFP-FKBP, FRB-mCherry-Mps1 and a miRNA against endogenous FKBP were imaged before and after treatment with 500 nM rapamycin. Images shown are maximum-intensity Z-projections of 5 confocal sections with 1 μm spacing. Scale bar, 5 μm.
ligase (Nishimura et al., 2009; Holland et al., 2012). This system does not allow for spatially targeted degradation, however. A light-inducible protein degradation system was recently reported that utilizes a photosensitive LOV domain that has been engineered to expose a small peptide degron in the illuminated state (Bonger et al., 2014). Relaxation of the LOV domain to its dark state after a pulse of illumination would allow any protein that is not degraded after the illumination pulse to escape. As a result, this system requires continuous illumination with 465 nm light for 2 hours to drive complete degradation of a target protein. This would not be practical for degradation targeting on a subcellular scale, since the target locus would likely move during this time. I propose that we could exploit the irreversibility of photochemical dimerization to overcome this difficulty, by recruiting a ubiquitin ligase subunit to a target protein using NVOC-TMP-Halo dimerization. A ubiquitin ligase (CHIP) has already been successfully re-engineered for rapamycin-mediated ubiquitination by fusion to FKBP (Qian et al., 2009). We could mimic this approach by fusing DHFR to a ubiquitin ligase, following the example of Qian and coworkers, and fusing the Haloenzyme to a target protein. Since photochemical uncaging is irreversible, the engineered ubiquitin ligase would be stably dimerized with the target protein until the target is degraded.
Chapter 4: Materials and Methods

Cell culture, creation of stable cell lines, and dimerizer treatment

All experiments were performed with stable HeLa cell lines generated by recombinase-mediated cassette exchange (RMCE) using the HILO RMCE system (obtained from E. V. Makeyev, Nanyang Technological University, Singapore, reported in Khandelia et al., 2011). This system allowed us to reproducibly insert transgene cassettes at a single genomic locus. Briefly: a monoclonal acceptor cell line with LoxP and Lox2272 recombination sites at a single chromosomal locus was cotransfected with a donor plasmid containing a transgenic cassette flanked by LoxP and Lox2272 sites and a second plasmid expressing Cre recombinase, followed by selection for a marker in the donor cassette. Cells were cultured in growth medium (DME with 10% FBS and penicillin-streptomycin) at 37 °C in a humidified atmosphere with 5% CO2. Cells at ~60% confluency in a single well of a 6-well plate were transfected with 1 µg of donor plasmid + 10 ng of Cre plasmid pEM784 (see below for plasmid details) using Fugene 6 (Promega). Two days after transfection, 1 µg/mL puromycin was added to the growth medium for selection of stable cell lines. For experiments involving doxycycline-inducible protein expression (Chapter 2 only), 125 ng/mL doxycycline was added to the growth medium 2 days prior to the experiment to induce expression of protein from the TRE Tet-responsive promoter. Although dox-induced protein expression was variable within each population of cells, the variation was reproducible and similar between different cell lines produced with this technique. All rapamycin experiments were done at a final working concentration of 500 nM rapamycin, except as noted in Figure 2.2 D and Figure 3.10. Rapamycin was stored as a 500 mM stock in DMSO, then diluted in
medium to a 0.75 or 1 mM intermediate dilution in medium and prewarmed before addition to cells. Halo-Oregon Green, TMP-Halo and NVOC-TMP-Halo were similarly dissolved in DMSO and then diluted to final working concentration in media, as indicated for each figure. All work with NVOC-TMP-Halo was done under white light, turned down as low as possible.

**Plasmids**

Three plasmids were obtained from E.V. Makeyev: pEM784, expressing nuclear-localized Cre recombinase; pEM705, a donor plasmid for constitutive transgene expression; and pEM791, a donor plasmid for inducible expression of a miRNA and a transgene (Khandelia et al., 2011).

pEM705 was the backbone for the constitutive expression cassettes used in all experiments in **Chapter 3** (except **Figure 3.10**, as noted). All experiments involving dox-inducible expression used cassettes derived from pEM791 (all figures in **Chapter 2** and **Figure 3.10**). pEM705 is a relatively simple plasmid containing a Puro resistance gene (Pur) positioned for constitutive transcription from the EF1α promoter upstream of the LoxP site in the acceptor locus, a CAG promoter for strong constitutive transcription, and an Internal Ribosome Entry Sequence (IRES) for bicistronic cDNA expression (Khandelia et al., 2011). Genes of interest were added on either end of the IRES to generate the plasmids used in my work. *E. coli* DHFR was cloned from a plasmid created by Carolyn Bertozzi’s lab (Addgene #20124). cDNA for the Haloenzyme was a gift of Yale Goldman, University of Pennsylvania. Mps1 was cloned from pDONR 223-TTK (Addgene # 23857, deposited by William Hahn).
pEM791 is designed for inducible expression of miRNA-based shRNA and a reporter gene. pEM791 contains: a Puro resistance gene (Pur) positioned for constitutive transcription from the EF1α promoter upstream of the LoxP site in the acceptor locus, the gene for reverse-tetracycline transactivator 3 (rtTA3) constitutively expressed from a UBC promoter, and a tetracycline-responsive element (TRE) promoter for inducible transcription of an artificial miRNA-based shRNA nested in an intron upstream of a GFP reporter gene (Du et al., 2006; Khandelia et al., 2011).

To create the cassettes used in experiments in Chapter 2 and Figure 3.10, I modified pEM791 for constitutive expression of an additional miRNA and protein sequence. Between the LoxP site and Pur, I added: (1) a miRNA-based shRNA against the 3’UTR of FKBP12, nested within an intron; (2) Mis12-GFP-FKBP or Mis12-FKBP (tandem trimers of FKBP in both cases); and (3) an internal ribosome entry sequence (IRES). These modifications allow constitutive polycistronic coexpression of FKBP miRNA, Mis12-targeted FKBP and the Puro resistance gene from the EF1α promoter in the acceptor locus. FKBP miRNA was designed using the miR RNAi function within the Block-iT RNAi Designer (http://rnaidesigner.lifetechnologies.com/rnaiexpress/). The following oligos were used for FKBP miRNA:

5’-TGCTGATATGGATCATGTCATGGTTTTGGCCACTGACTGACCATGTGCATGAATCCATAT-3’,
5’-CCTGATATGGATCATGCATGGTCAGTCAGTGGCCAAAACCATGTGCACATGAATCCATATC-3’. mCherry-FRB-Mad1 constructs were cloned in place of GFP downstream of TRE. No shRNA sequences were added to the empty miRNA backbone
in the inducible transcript for this study. The genes for FRB and FKBP were cloned from plasmids pC4EN-F1, pC4M-F2E and pC4-RHE (obtained from Ariad Pharmaceuticals Inc, Cambridge, MA).

**Immunofluorescence**

All cell fixation procedures were done at room temperature (~22 °C). For Mad2 staining in Figures 2.3, 2.5 and 2.7, cells were fixed in PBS + 3.7% formaldehyde with 0.5% Triton X-100 for 10 min. For BubR1 staining in Figure 2.4 A, cells were pre-extracted in PBS + 0.5% Triton X-100 for 1 min, then fixed in PBS + 3.7% formaldehyde, 0.5% Triton X-100 for 10 min. For Mps1 staining in Figure 2.4 B, cells were pre-fixed for 5 s in PBS + 3.7% formaldehyde, extracted in PBS + 0.5% Triton X-100 for 1 min, then fixed in PBS + 3.7% formaldehyde with 0.5% Triton X-100 for 10 min. The following primary antibodies were used: rabbit polyclonal anti-Mad2 (1:500; PRB-452C; Covance Inc.), mouse monoclonal anti-Hec1 9G3 (1:1,000; ab3613; Abcam), rabbit anti-BubR1 polyclonal (1:1,000; a gift from W. Dai, New York Medical College, Valhalla, NY), rabbit anti-CENP-C polyclonal (1:1,000; a gift from B.E. Black, University of Pennsylvania, Philadelphia, PA), mouse anti-Mps1 monoclonal NT (1:100; 05-682; Millipore).

**Immunoblotting**

Whole cell lysates were prepared from asynchronous populations of cells. Western blot analysis was performed using the following antibodies: rabbit anti-FKBP12 polyclonal (1:2,000; ab2918; Abcam), rabbit anti-GFP polyclonal (1:10,000; a gift from
B.E. Black, University of Pennsylvania), mouse anti-tubulin monoclonal DM1α (1:10,000; Sigma-Aldrich).

**Image acquisition and processing**

For live imaging, cells were plated on 22 x 22 mm glass coverslips (no. 1.5; Fisher Scientific) coated with poly-D-lysine (Sigma-Aldrich). Coverslips were mounted in magnetic chambers (Chamlide CM-S22-1, LCI) using L-15 medium without phenol red (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin. Temperature was maintained at ~35 °C using an environmental chamber (Incubator BL; PeCon GmbH).

Except for Figures 2.5 B and 2.7 B, all images were acquired with a spinning disk confocal microscope (DM4000; Leica) with a 100x 1.4 NA objective, an XY Piezo-Z stage (Applied Scientific Instrumentation), a spinning disk (Yokogawa), an electron multiplier charge-coupled device camera (ImageEM; Hamamatsu Photonics), and a laser merge module equipped with 488- and 593-nm lasers (LMM5; Spectral Applied Research) controlled by MetaMorph software (Molecular Devices). Images in Figures 2.5 B and 2.7 B were acquired on a microscope (DM6000; Leica) with a 63x 1.4 NA objective and a charge-coupled device camera (ORCA-AG; Hamamatsu Photonics) controlled by Micro-Manager (Edelstein et al., 2010).

All image processing and analysis was performed using ImageJ (Schneider et al., 2012). For BubR1 and Mps1 quantification (Figure 2.4), kinetochores in control cells were identified by Hec1 or CenpC costaining. In rapamycin-treated cells, kinetochores were identified by recruited FRB-mCherry-Mad1 fluorescence. Each kinetochore was
defined as a circular region 6 pixels (0.72 µm) in diameter, and BubR1 or Mps1 intensity was measured in these regions and background corrected. 20 individual kinetochores were measured in each of 5 cells for each condition, and the 100 individual kinetochore measurements were averaged to yield a value for each of three independent replicates. The values shown in Figure 2.4 are averages of the three independent replicates, normalized to the metaphase control condition. Each bar, therefore, represents an average of three averages of 100 kinetochores (20 each from 5 cells). Error bars represent the SEM of the three independent replicates.

For mCherry-FRB recruitment quantification in Figure 2.2 C, total mCherry-FRB expression was measured for each cell in the pre-rap image as the average mCherry intensity within a 135 pixel (16.2 µm) diameter circle centered on the cell, then background corrected. Images were cropped to a 200x200 pixel region centered on each cell, then kinetochore regions were identified in pre-rap and post-rap GFP images by thresholding using the MaxEntropy algorithm in ImageJ. mCherry intensity within the corresponding kinetochore regions was measured, and the average pre-rap intensity was subtracted from the post-rap intensity to yield a raw mCherry-FRB recruitment value. Both mCherry-FRB expression and recruitment were thus measured on the same scale of mCherry pixel intensity. These measurements were made on 138 empty-miRNA control cells and 151 FKBP miRNA cells pooled from 6 independent replicates. Expression and recruitment values were normalized to the cell with the highest mCherry-FRB expression level.
For Mad2 quantification in Figure 2.7 C, kinetochore regions in metaphase cells were first defined in Hec1 costaining images by manually determined intensity threshold. Average Mad2 intensity within the kinetochore region was measured and background subtracted. Mad2 levels from ≥8 cells were averaged for each cell type in three independent replicates. The values from the three replicates were averaged and normalized to full-length Mad1.

**Synthetic Chemistry**

The synthesis of TMP-Halo, NVOC-TMP and NVOC-TMP-Halo is outlined in Figure 4.1. Compounds 1, 3, 13 have been previously reported in the literature (Chen et al., 2012; Erhart et al., 2013). TMP and compounds 2, 4, 7, 9 and 12 are commercially available. All compounds were purified by silica chromatography and characterized by 1H-NMR and high-resolution mass-spectrometry.
Figure 4.1: Synthesis of TMP-Halo and NVOC-TMP-Halo. The synthesis followed a convergent scheme in which TMP and the Haloligand were independently derivatized with linker and/or a photocage subunits, then coupled to form the final products. A: The TMP branch of the synthesis. A four-atom linker with Boc-protected amine functionality was added to the para-position of the trimethoxyphenyl ring to yield compound 3. Addition of the NVOC photocage produced compound 6. B: The Haloligand branch of the synthesis. The chlorohexane core Haloligand was derivatized with an 11-atom linker with a terminal carboxylic acid functional group to yield compound 13. C: TMP-Halo and NVOC-TMP-Halo were prepared by standard peptide coupling between compound 13 and 3 or 6, respectively.


Varnai, P., B. Thyagarajan, T. Rohacs, and T. Balla. 2006. Rapidly inducible changes in phosphatidylinositol 4,5-bisphosphate levels influence multiple regulatory functions


