Protein–Protein Interactions

DOI: 10.1002/anie.201201717

Protein Tango: The Toolbox to Capture Interacting Partners

*Anna Rutkowska and Carsten Schultz**

cross-linkers · dimerizers · protein–protein interactions · small molecules

The evaluation of protein function in the context of the whole cell is crucial for understanding of living systems. In this context, the identification and modulation of protein–protein interactions in and outside cells is of ample importance. Several methods have been developed in the past years to detect and/or actively induce protein–protein interactions in living cells. As a result, tools are now available to manipulate intracellular events by reversible or irreversible cross-linking of proteins in a specific manner. These techniques open many new doors and enable the dissection of complicated protein networks. Herein we describe which cross-linkers and inducers of dimerization are out there and how to make use of this great toolbox.

1. Introduction

In cells, protein function is usually determined by formation of multi-protein complexes as well as the dynamics of protein–protein interactions (PPIs). Techniques to enable the discovery and investigation of PPIs on a short time scale in living cells include 1) visualization of protein localization and interactions by protein fragment complementation assays or by light microscopy through Förster resonance energy transfer (FRET) measurements or fluorescence cross correlation spectroscopy (FCCS), 2) manipulation of cellular protein levels (by overexpression or knockdown), and 3) immunoprecipitation of protein complexes from cell lysates.[1] Moreover, protein interaction maps (interactomes) are currently produced with the help of biochemical methods performed at the proteomic scale (e.g. yeast-2-hybrid screens, protein arrays, and affinity purification techniques combined with mass spectrometry).[2] In all cases, the large set of detectable new PPIs is limited to fairly strong interactions, and the dataset requires validation and further characterization by complementary methods.

One possible solution to improve the analysis is based on the use of small molecules, that is, cross-linkers and chemical inducers of dimerization (CIDs; Figure 1),[3] to extend the lifetime of protein complexes and to demonstrate functionality of the PPI. Two general approaches are pursued: covalent fixation of existing PPIs through chemical cross-linkers or induction of protein interaction by CIDs. In both cases, the proteins of interest are linked in a stable, reversible or irreversible, manner. These techniques can be used to control protein activity and localization or to manipulate the oligomeric state of proteins.

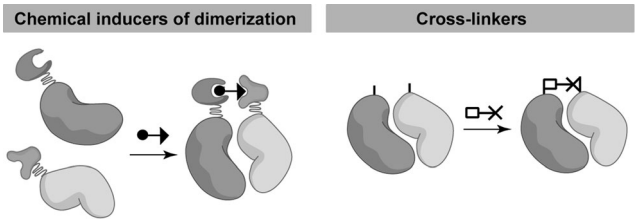

Figure 1. Concepts behind cross-linkers and dimerizers/chemical inducers of dimerization (CIDs). CIDs are applied to artificially force dimerization of any two proteins of interest by forming a stable ternary complex. In contrast, cross-linkers are designed to detect and monitor existing protein–protein interactions. They covalently bind to specific functional groups on the protein of interest.

Herein we highlight the available chemical dimerizers and cross-linkers and provide examples of their biological applications in signal transduction, gene expression, or protein secretion. Moreover, novel strategies are discussed that may lead to improved tools and broader applications in the future.

[*] Dr. A. Rutkowska, Priv.-Doz. Dr. C. Schultz
Cell Biology & Biophysics Unit
European Molecular Biology Laboratory
Meyerhofstr. 1, 69117 Heidelberg (Germany)
E-mail: schultz@embl.de

2. Application Spectrum of Chemical Dimerizers

Chemical inducers of dimerization (CIDs) have two binding surfaces that simultaneously recognize the same (homodimerizers) or two different (heterodimerizers) protein domains (Figure 2).[3] Although not covalent, the dimerization reaction is usually very specific, highly efficient, and stable. In fact, in most cases it is irreversible. Thus, virtually any two proteins of interest, which are fused to the additional domains, are rapidly brought into close proximity. When they are membrane-permeant and metabolically stable, CIDs offer potent, real-time, and spatial control of protein interactions in living cells.

Homodimerizers are employed to induce cellular events that depend on protein self-association. The most common example is dimerizer-induced activation of cell surface receptors in the absence of extracellular ligands (Figure 2A).[4]

Heterodimerizers allow the specific and independent control of any cellular event that requires protein interactions (Figure 2B). To the most common applications belong the rapidly induced translocation of a protein of interest to the desired site of action[5] and the transcriptional activation of target genes.[6] High speed and selectivity are the largest advantages of such small-molecule systems when compared to genetic approaches, such as RNAi or tetracycline-induced expression. Accordingly, heterodimerizers give rise to the three-hybrid technology that allows studies on interactions of small molecules with target proteins in living cells (mostly in yeast, Y3H), even in a high-throughput fashion.[7] Slightly modified versions of such an approach, termed "chemical complementation", enable screens for enzymatic activity.[8]

Another interesting dimerization variant is based on a reverted system, where a protein of interest is sequestered into aggregates owing to oligomerization-prone properties of the fused domain (Figure 2C). The addition of ligand (reverse dimerizer) enables rapid disaggregation of fusion proteins and restoration of their activity. This technology can be used to study intracellular trafficking by inducing protein secretion.[9] A related approach utilizes heterodimeric compounds that upon binding to the added domains destabilize the possible PPIs by covering the interaction surface or by causing unfavorable structural clashes. This can be used to develop enzyme inhibitors,[10] engineer chemical selectivity by creating CIDs that display context-dependent cellular activity,[10,11] or to create new non-native protein interactions and thereby for example inhibit protein aggregation.[12]

Finally, further modification of the CID technique leads to tools for post-translational control of protein levels by deactivation (controlled degradation) or activation (controlled stabilization or induced protein splicing). For instance, in the absence of a CID, the protein of interest is rapidly proteolytically degraded owing to the destabilizing properties of an artificially added unstable domain or a degron sequence. This process is, however, easily modulated by addition of a specific dimerizer that leads to either stabilization[13] or removal[14] of such a degradation-prone domain. In this way, the stability and thereby also the function of the fused protein of interest is restored. Other approaches for controlling degradation are based on inducible localization of proteins to the E3 ubiquitin ligase[15] or the proteasome.[16] Finally, small molecules are used to initiate and control protein splicing, thereby leading to the formation of new protein variants with specific functions.[17]

3. Dimerizers—the Molecules

The concept of chemically induced dimerization was inspired by the naturally occurring immunosuppressive drug FK506, which simultaneously and tightly binds to the small cytoplasmic protein FKBP (FK506-binding protein 12) and the signaling phosphatase calcineurin.[18] Thus, dimerizers are either obtained from natural bifunctional products or synthetically produced by connecting two protein binding motives with a flexible linker (Figure 3, Table 1). The latter type is derived from well-characterized ligand–receptor pairs or enzyme–substrate interactions.

3.1. Homodimerizers

Introduced by the research groups of Schreiber and Crabtree, the first homodimerizer comprised the bivalent ligand of FK506 (FK1012) that was able to bind two FKBP proteins.[18] By today, a whole spectrum of FK1012 variants has been synthesized with improved conformational rigidity, higher specificity, and reduced affinity to endogenous pro-

Carsten Schultz received his PhD in chemistry from Bremen University (with Bernd Jastorff). After a postdoc with Roger Tsien at the University of California, San Diego, he finished his habilitation in organic chemistry in Bremen. He then picked up a position as group leader in the institute of Herbert Waldmann at the MPI Dormund. Since 2001, he is a group leader at the EMBL Heidelberg and since 2008 he holds a position as senior scientist. His main research interest is to improve our understanding of signal transduction networks. For this aim, the group develops tools to image and manipulate intra- and extracellular signaling events.

Anna Rutkowska completed her PhD at the University of Heidelberg with Bernd Bukau in the field of protein folding. Currently she is working at the EMBL Heidelberg as an interdisciplinary post-doc (EIPOD) in the groups of Carsten Schultz, Christian Haering, and Jan Ellenberg. Her research is focused on the development of tools to study protein–protein interactions in living cells as well as relevant application for biological questions.

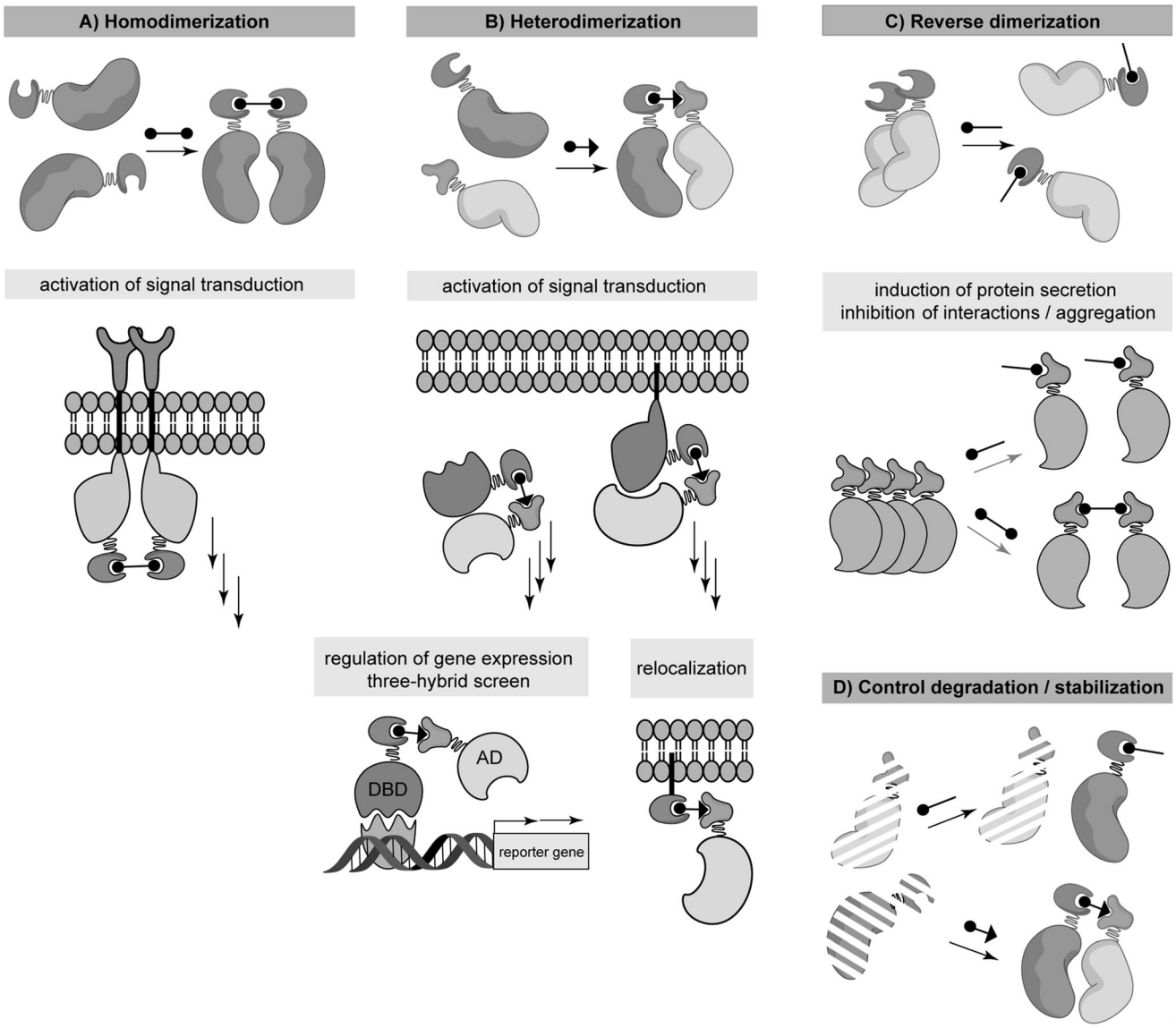

Figure 2. General principle of chemically induced dimerization. A) Symmetrical ligands (homodimerizers) link two molecules of the same protein. This approach is used to study processes that depend on self-dimerization, like initiating intracellular signaling by activation of receptors in the absence of stimulus.[20] B) Nonsymmetrical ligands (heterodimerizers) connect two different proteins. Such tools are used to study steps in signaling networks, control protein localization, or induce gene expression. They are applied in well-known three-hybrid systems to screen for small molecules or particular binding domains. Binding domains are in this case fused to a DNA binding protein (DBD) and a transcription activator (AD). C, D) Derivatives of CIDs and their binding domains, like "reverse" dimerizers or destabilizing domains are used to regulate protein stability and solubility, modulate protein secretion, and inhibit protein interactions by blocking existing or introducing new interaction surfaces.

teins (SLF, synthetic ligand for FKBP, for example, AP1510 or AP20187).[19] As an alternative, the FKBP domain has been modified to increase its affinity for SLF (e.g. FKBP F36V mutant). The optimized systems are commercially available and have been employed to activate a variety of signaling events in intact cells.[20]

Another prominent homodimerizer is based on coumermycin (Figure 3) that connects the N-terminal domains (220 amino acids) of bacterial DNA gyrase B (GyrB).[21] Importantly, the small molecule novobiocin, which interacts with only one GyrB subunit, can be used to rapidly reverse coumermycin-driven dimerization. Both coumermycin and novobiocin are commercially available and are nontoxic as GyrB has no function in higher eukaryotes. This approach was successfully used to activate signaling pathways (such as Raf/Ras or Jak/Stat[4a,22]), to study the dimerization-mediated activation of many membrane-bound receptors,[23] to investigate the role of dimerization on protein function,[24] and to control gene expression.[25] Another important application is represented by synthetic analogues of coumermycin with changed specificity.[26] They dimerize the endogenous heat shock protein 90 (Hsp90) and thereby display antiproliferative activity against breast cancer and prostate cancer cell lines.

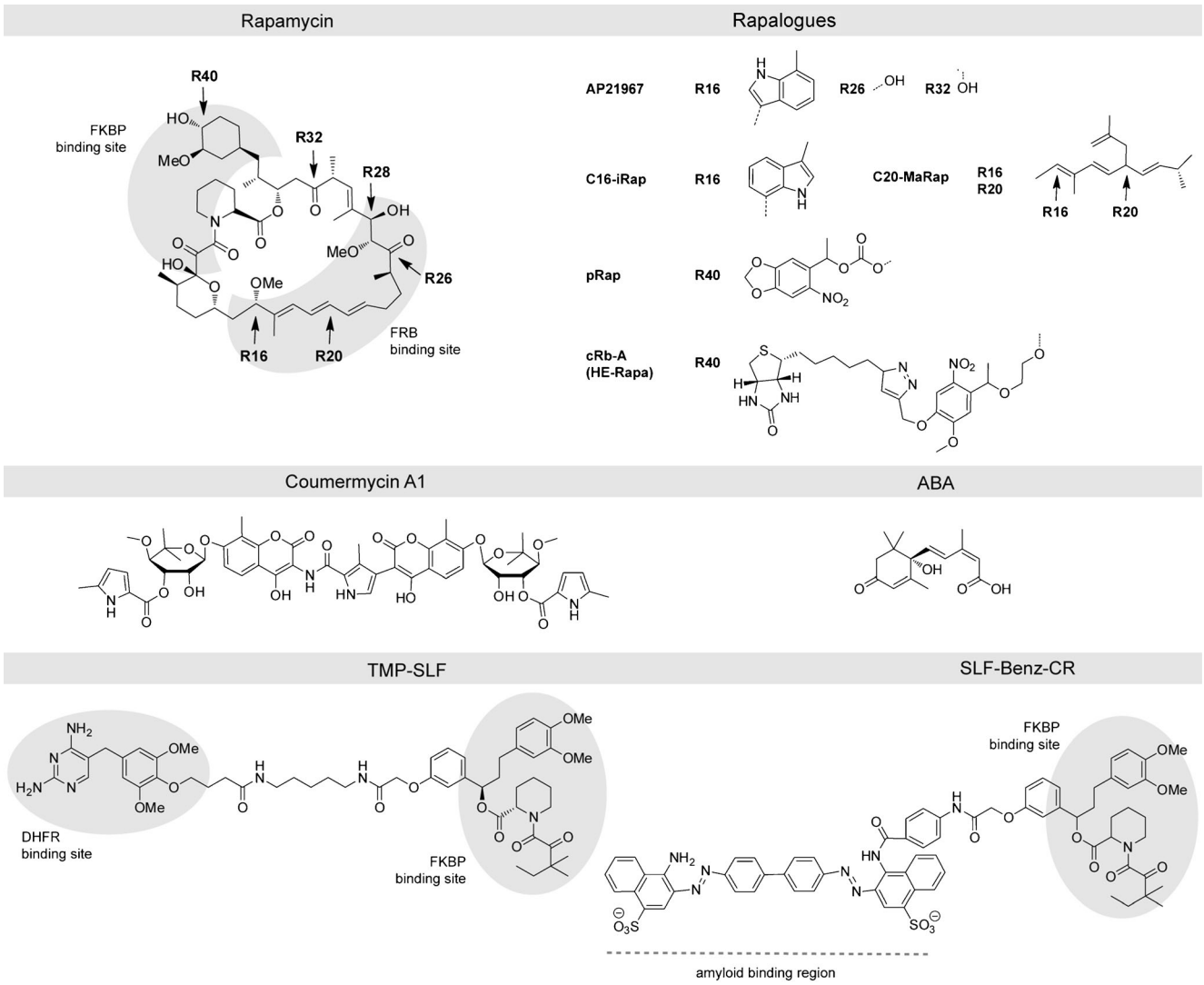

Figure 3. Examples of dimerizers/chemical inducers of dimerization: the most commonly applied natural heterodimerizer rapamycin and its synthetic analogues (rapalogues); at the indicated positions, the functional groups in the natural product are replaced by the substituents listed on the right to give the different rapalogues (except for rapalogue C20-MaRap). The rapalogues pRap and cRb-A are photocaged derivatives; the natural homodimerizer coumermycin interacts with bacterial DNA gyrase B; the plant hormone abscisic acid (ABA) induces as a heterodimerizer interaction between two proteins (PYL1, ABI1) involved in the ABA stress signaling pathway in plants; the heterodimerizer TMP-SLF contains synthetic substrates for dihydrofolate reductase (DHFR) and FKBP binding domains; the heterodimerizer SLF-Benz-CR conjugates FKBP to Aβ (amyloid β) dimers or small oligomers (through the congo red dye) and thereby inhibits amyloid aggregation.

A third important system utilizes methotrexate (MTX), which is a potent inhibitor of dihydrofolate reductase (DHFR).[27] The bisMTX dimerizer, containing two MTX molecules connected by a flexible linker has so far mostly been used in cell-free systems to create nanoassemblies and nano ring structures.

3.2. Heterodimerizers

The most popular heterodimerizer is based on the bifunctional natural product rapamycin, which simultaneously binds to the otherwise not interacting FKBP protein and the FRB domain (FKBP-rapamycin binding domain) of the protein kinase FRAP/mTOR (Figure 3).[28] Rapamycin and its synthetic derivatives (rapalogues) with reduced effects on endogenous cell biology have recently been extensively reviewed and are therefore not detailed herein again (Table 1).[29] Some of the most prominent applications are the intracellular modification of phosphoinositide levels,[30] GTPase signaling pathways,[5a] G protein-coupled receptor signaling,[31] secretion,[32] or glycosylation.[33] Among the most exciting current developments was the introduction of caged rapamycin analogues that permitted light-induced activation of PPI (Figure 3, Table 1).[34]

In 2011, another naturally occurring heterodimerizer was introduced: the plant phytohormone *S*-(+)-abscisic acid (ABA; Figure 3).[35] This very small and easily obtainable

Table 1: The current toolbox to induce protein–protein interactions: dimerizers.

Chemical inducers of dimerization/dimerizers							
Dimerizer	Description	Additional domains/tags		Size [kDa]	Comments	In vivo	Ref.
Homodimerizers							
FK1012	FK506 dimer	FKBP		12		+	[18]
SLF e.g.: AP1510	synthetic FKBP ligands	FKBP		12	more potent, decreased immunosuppressive activity	+	[29a]
AP1903 AP20187	bumped analogues of AP1510	FKBP F36V		12	no interaction with endogenous FKBP	+	[19a, 29a]
(CsA)2	cyclosporin A dimer	cyclophilin (CyP)		21	no interaction with T-cell signaling	+	[63]
BisMTX	methotrexate dimer	eDHFR/mDHFR		18	nanoassemblies	?	[27]
coumermycin	natural product	gyrase B (GyrB)		24	2–220 N-terminal aa	+	[21]
Reverse dimerizers							
AP21998	bumped ligand analogous to one half of AP1903	FKBP F36M		12	ligand disrupts self-dimerization of FKBP mutants	+	[9]
Heterodimerizers							
rapamycin	natural product	FKBP	FRB	12/11		+	[28]
Rapalogues:							
AP21967	synthetic, bumped analogues of rapamycin	FKBP	FRB T2098L	12/11	reduced interaction with endogenous FRB	+	[29, 13b]
C20-MaRap		FKBP	FRB PLF (KLF, KTF)	12/11	good pharmacological properties, destabilizing FRB	+	[13a]
C16-iRap		FKBP	FRB PLF (KLF, PLW)	12/11	weak pharmacological properties, destabilizing FRB	+	[13a]
pRap	photoactivatable analogue	iFKBP	FRB	12/11	shorter version of FKBP	+	[34b]
cRb-A	photoactivatable analogue	FKBP	FRB	12/11	conjugated to biotin, after cleavage HE-Rapa released	+	[34a]
FK506-DEX	FK506 and dexamethasone	FKBP	GR	12/32	Y3H system	+	[64]
FKCsA	FK506–CsA dimer	FKBP	CyP	12/21		+	[65]
MTX-DEX TMP-DEX	methotrexate or trimethoprim and dexamethasone	DHFR	GR	18/32	Y3H system	+	[36]
MTX-SLF	methotrexate and a synthetic FKBP ligand	DHFR	FKBP	18/12	bacterial and Y3H system	+	[10, 11, 37a]
TMP-SLF	trimethoprim and SLF	eDHFR	FKBP	18/12	no endogenous protein targets	+	[37b]
MTX-BG	methotrexate and a benzylguanine derivative	DHFR	SNAP-tag	18/20	one-side covalent binding Y3H system	+	[40]
DEX-biotin		GR	streptavidin	18/52	one-side covalent binding	+	[38b]
estradiol-biotin		streptavidin	estrogen receptor	52/29	one-side covalent binding Y3H system	+	[38a]
ABA	*S*-(+)-abscisic acid	PYL_{CS}	ABI_{CS}	20/33	plant-derived, stable, nontoxic, good pharmacokinetics	+	[35]
SLF-Benz-CR	synthetic FKBP ligand and congo red	FKBP	Aβ dimers/oligomers	12/x	inhibits amyloid aggregation	+	[12]

Light-activated dimerizers						
Light source	Additional domains/tags		Size [kDa]	Comments	In vivo	Ref.
Red (650 nm)	PhyB	PIF6	99/11	very fast, dissociation induced by far red (750 nm), requires cofactor PCB	+	[41]
Blue (450 nm)	FKF1	GIGANTEA (GI)	70/125	slow (0.5 h), very slow, irreversible	+	[42]
Blue (488 nm)	CIBN	CRY2	20/70	very fast, spontaneous dissociation (min)	+	[43]

molecule induces dimerization of proteins involved in stress response and developmental decisions in plants, namely the PYL1 domain (PYL_{CS}) of the regulatory component of the ABA receptor (PYR/PYL/RCAR) and the ABI1 domain (ABI_{CS}) of the ABI1 protein serine/threonine phosphatase (PP2C). ABA is nontoxic, does not interfere with endogenous signaling pathways in mammalian systems, and has favorable pharmacokinetics in mice. The comparison with rapalogues revealed its high stability, a linear dose response, good responsiveness, and easy reversibility in mouse embryonic stem cells.[35] To definitely avoid toxic effects, a mutant of ABI_{CS} domain was engineered with diminished phosphatase activity (ABI_{D143A}).[35] Although very promising, the biological applications of this tool are yet to come.

Other types of heterodimerizers were generated through fusion of two ligands or enzyme substrates. The Cornish research group developed heterobifunctional molecules containing the DHFR ligand MTX or less toxic trimethoprim (TMP) and dexamethasone (DEX).[36] Such molecules allowed rapid dimerization of DHFR and a glucocorticoid receptor (GR) fusion protein and have been mostly used in bacterial and yeast three-hybrid systems.[36] Similar heterodimers between MTX or TMP and synthetic FKBP ligand SLF (Figure 3) were applied to modulate post-translational modifications of proteins[37] or to induce context-dependent activity, when simultaneous binding to DHFR and FKBP was impossible owing to the short linker used.[10–11] Another interesting "specialized" dimerizer derivative utilized the synthetic FKBP ligand SLF fused to the dye congo red (SLF-Benz-CR, Figure 3). The molecule induced fusion between FKBP molecules and dimers or small oligomers of the Aβ peptide.[12] Formation of these new complexes physically abolished further Aβ interactions and thereby blocked amyloid aggregation.

Finally, a group of CIDs was developed that rely on quasi-covalent irreversible interactions. Thereby, the specificity of the reaction and stability of the dimerized complex is increased in a way that also allows detection of targeted PPIs by using standard molecular biology techniques (e.g. immunobloting). Initially, the natural ligand biotin was used with proteins fused to streptavidin. Two different heterodimerizers were synthesized: DEX-biotin (for GR and streptavidin fusion proteins, very potent in mammalian systems) and estrogen-biotin (for estrogen receptor and streptavidin fusion proteins, very efficient in yeast three-hybrid screens).[38] In the presence of a BirA biotin ligase, biotin itself functions as a heterodimerizer of streptavidin and AviTag fusion proteins.[39] Another dimerizer system was introduced by Johnsson and co-workers,[40] making use of O^6-alkylguanine-DNA alkyltransferase (AGT), termed the SNAP-tag, which covalently binds to O^6-benzylguanine (BG) derivatives through a cysteine residue in its active site (Figure 4). For a heterodimerizer, a BG derivative was linked to MTX, thereby providing one covalent and one high-affinity binding connection.[40] Follow-up work resulted in several molecules that owing to their truly covalent mode of action belong rather to the cross-linker category and will be discussed below.

4. Light-Activated Induction of Protein Interactions

Although the above described CIDs are widely used in cell biology and have been successfully applied for addressing many biological questions, the necessity to add exogenous dimerizer molecules may lead to delivery problems and off-site effects. Moreover, such methods lack temporal and more importantly spatial resolution at the single-cell level and in tissue. To avoid such limitations, techniques to control PPIs in living cells by light were recently developed. These genetically encoded dimerizers are based on plant photoreceptors from *Arabidopsis thaliana*, and can specifically and reversibly induce PPIs on a very short time scale (Table 1).

One of such systems utilized phytochrom B (PhyB) and a transcription factor, phytochrome interaction factor 6 (PIF6).[17b,41] The association and dissociation kinetics of the PhyB–PIF6 complex can be triggered by red (650 nm) and far-red light (750 nm), respectively. This interaction requires, however, an externally supplied bilin cofactor (phycocyanobilin, PCB) and is therefore not entirely independent of small-molecule additions. In an alternative technique (light-activated dimerization, LAD) developed by the Dolmetsch research group, protein tags were generated from the protein FKF1 and its interacting partner GIGANTEA (GI).[42] This approach required no exogenous ligand, but the blue-light controlled dimerization was much slower and long-lasting, thus rather irreversible. Finally, the most promising, recently developed blue-light inducible dimerizer consists of an N-terminal domain of CIB1 (CIBN) and cryptochrome 2 (CRY2).[43] This system overcomes all previously mentioned limitations: no requirement of an exogenous chromophore, very fast kinetics, and almost spontaneous reversibility.

Although all three approaches are applicable in vivo and promise great spatial and temporal precision, biological applications are still missing. Moreover, all probes share the same disadvantage that is the significant size of at least one additional domain (around 100 kDa). This large tag may have a negative influence on the activity and biological properties of the fused protein of interest. Thus, these systems are currently mostly suitable for controlling protein localization.

5. Cross-Linkers to Sense and Trap Protein Interactions

While dimerizers are used to artificially manipulate PPIs, cross-linking approaches are the powerful toolbox for detection and monitoring of naturally existing PPIs in vitro and in cells. Especially useful is this technology to identify weak and transient interactions, which cannot be analyzed easily with other methods. Similarly to CIDs, cross-linkers are small molecules that contain two different (heterobifunctional) or the same (homobifunctional) reactive groups capable of binding to specific functional groups or engineered tags on the protein of interest (Figure 4, Table 2). This covalent interaction may be irreversible or reversible and has a large range of specificities ranging from unspecific chemical cross-linkers to highly specific dimerizer-based methods.

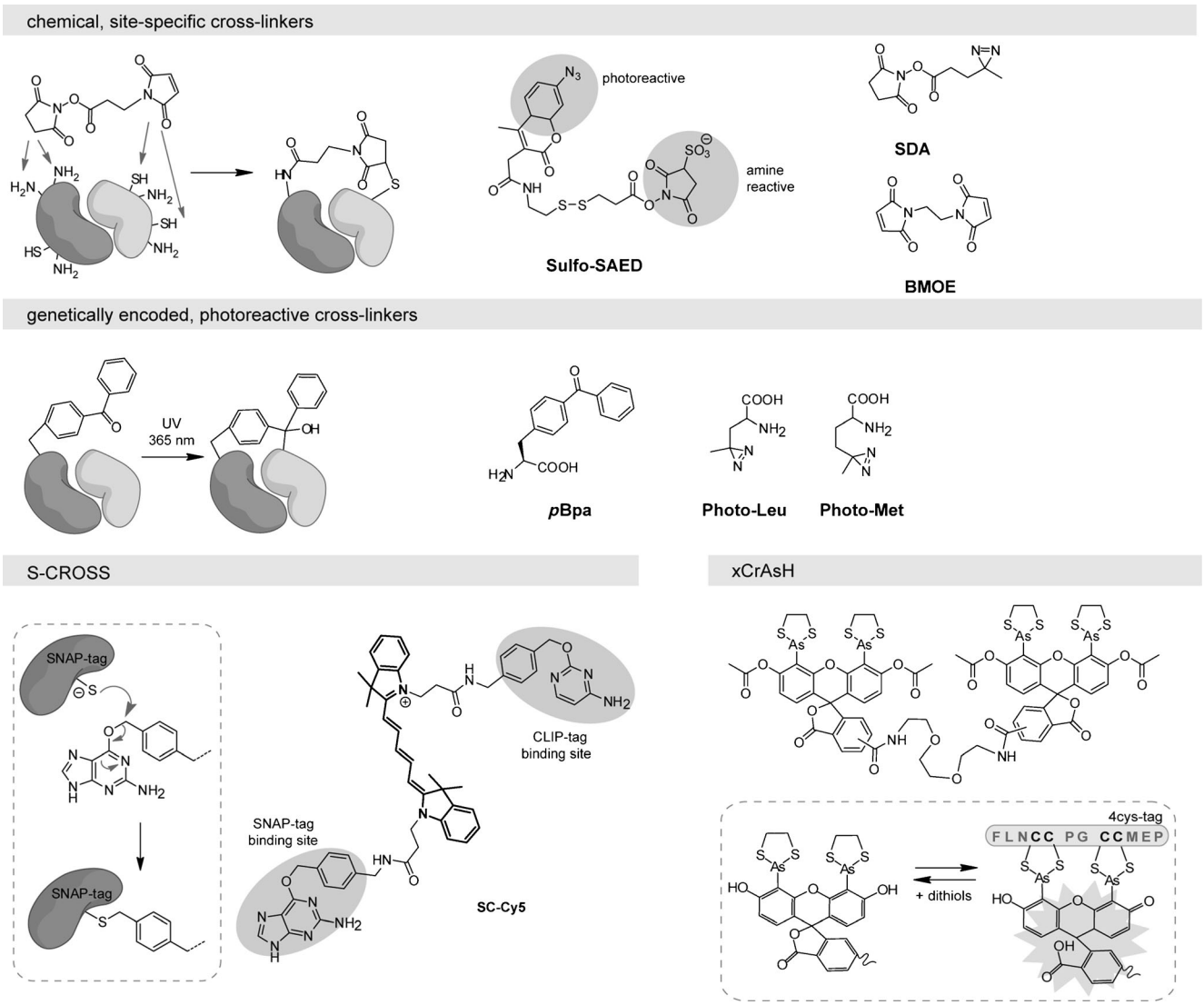

Figure 4. Examples of cross-linkers including those having characteristics of dimerizers with mechanistic details. Commercially available site-specific homo- and heterobifunctional cross-linkers that can interact with primary amines (SDA), sulfhydryl groups (BMOE), or other protein functional groups; some cross-linkers contain photoreactive nonselective groups that couple to the nearest available C–H or N–H bond (Sulfo-SAED, SDA); genetically encoded, photoreactive cross-linkers in form of unnatural amino acids, which can be introduced into one specific (*p*Bpa) or many (Photo-Leu, Photo-Met) positions in the protein of interest; the S-CROSS system based on covalent complex formation between SNAP- and/or CLIP-tagged proteins, which can be visualized through a fluorophore in the linker region (red), currently only applicable in vitro; and the reversible cross-linker xCrAsH that detects interactions in living cells between proteins equipped with a short amino acid peptide (the 4cys-tag).

The commonly used and commercially available chemical protein-cross-linking methods employ homo- or heterobifunctional chemical reagents that interact with functional groups on the protein surface, such as primary amine, sulfhydryl, and carboxyl groups (Figure 4, Table 2).[44] Although very powerful and widely used in vitro, these reagents lack suitable reactivity and specificity for applications in living cells. The best results are currently obtained with site-specific heterobifunctional photoreactive cross-linkers such as Sulfo-SAED (Figure 4), using a two-step directional reaction (first reaction with one functional group, then after light-activation a second nonselective reaction with the nearest available C–H or N–H bond).[45]

To increase specificity, several alternative methods have been developed, such as 1) site-specific photo-cross-linkers incorporated at defined positions into proteins, or 2) special protein tags that can be labeled upon PPIs with photo-cross-linkers or other chemical labels such as biotin (label transfer techniques; Figure 4, Table 2). For the first method a genetically encoded photo-cross-linker containing an amino acid (such as *p*-benzoyl-L-phenylalanine, *p*Bpa) is incorporated at a defined position through the use of an expanded genetic code (amber stop codon suppression).[46] Alternatives are photoactivatable amino acids (photo-leucine and photo-methionine, Figure 4), which are incorporated into proteins instead of natural amino acids by the unmodified mammalian translation machinery.[47] When compared with traditional

Table 2: The current toolbox to detect protein–protein interactions: cross-linkers.

Cross-linker type	Reactive groups/tags		Size [kDa]	Comments	In vivo	Ref.
chemical, e.g.:						
BMOE, DTEM	sulfhydryl-reactive			homobifunctional, maleimides	+/−	[44]
DSG, DSP, EGS	amine-reactive			homobifunctional, NHS ester	+/−	[44]
SDA, Sulfo-SAED	amine-reactive/nonselective, light-activated			heterobifunctional, NHS ester/diazirine or aryl-azide	+/−	[44]
genetically encoded:						
*p*Bpa	nonselective			covalent, not reversible	+	[46]
Photo-Leu, Photo-Met	nonselective			covalent, not reversible	+	[47]
CoDi1 (2,3)	SNAP-tag	SNAP-tag	20	covalent, not reversible, inducing dimerization	+	[50a,b]
S-CROSS:						
SC-Cy5	SNAP-tag	CLIP-tag	20/21	covalent, not reversible, fluorescence detection	−	
SS-Cy5, SS-Cy3	SNAP-tag	SNAP-tag	20			
xCrAsH	4cys-tag	4cys-tag	1	covalent, reversible, small tag	+	[51]
FlAsH, ReAsH	split 4cys-tag		>1	covalent, reversible, special design necessary	+	[54]
DOPA-biotin-FlAsH	nucleophiles	4cys-tag	1	covalent, reversible, biotin transfer	−	[55]
TRAP	nonselective	4cys-tag	1	covalent, reversible, fluorophore transfer	+	[56]

chemical cross-linkers, these novel approaches benefit from similarly high efficiency and much greater specificity. Accordingly, these cross-linkers have lately been successfully applied to study PPIs in living cells. Similarly, label transfer techniques make use of engineered ligases (like bacterial lipoic acid ligase, LplA) that catalyze covalent conjugation of diverse probes onto the special peptide tag. Such reactions are predominantly used to detect PPIs, since the label transfer is usually not connecting interacting partners.[48] One approach, however, utilizes the LplA mutant to specifically label the protein of interest with a fluorinated aryl azide photoaffinity probe.[49] Although this method allows a very efficient and robust labeling of proteins with photo-cross-linkers, it has been not yet applied for answering biological questions.

An interesting set of methods has arisen from the combination of the dimerizer and the cross-linking approaches. Similarly to CIDs, these synthetic cross-linker molecules are able to bind simultaneously to two protein domains or tags, which are incorporated into the proteins of interest. However, in this case specific and efficient covalent cross-linking only occurs when the two proteins are in close proximity, that is, interacting with each other. A technique introduced by Johnsson and co-workers utilizes the self-labeling proteins SNAP-tag and CLIP-tag, which are engineered mutants of human O^6-alkylguanine-DNA alkyltransferase and have non-overlapping substrate specificity, reacting with benzylguanine (BG) and benzylcytosine (BC) derivatives, respectively (Figure 4, Table 2).[50] Initially, homobifunctional small molecules consisting of two BG derivatives connected by a flexible linker of different length were introduced (CoDi1, CoDi2, CoDi3).[50a] Proof-of-principle experiments in living cells demonstrated that these molecules induced dimerization of two SNAP-tag fusion proteins in a proximity-dependent manner.[50a] In this respect, these cross-linkers act similarly to CIDs. Further development led to the S-CROSS system[50b] based on bifunctional cross-linkers consisting of BG and/or BC derivatives connected through a fluorophore-containing linker for detection. S-CROSS permitted selective, directional, and covalent cross-linking of two interacting proteins, selection between strong and weak interactions, and studies on the stability of protein complexes. Owing to the available variety of homo- and heterofunctional cross-linkers equipped with different fluorophores, it was even possible to analyze multiple interacting partners in a single experiment. Unfortunately, this technique was so far only used in cell lysates. Another possible drawback comes along with the necessity to fuse the proteins of interest to additional domains of significant size, which might negatively influence protein activity.

To circumvent this limitation, an alternative approach for the detection and modulation of PPIs in living cells was developed by our group.[51] This novel cross-linking technique is based on the formation of stable, covalent complexes between a dimeric biarsenical derivative (xCrAsH, a homobifunctional cross-linker) and two proteins containing the unique tetracysteine sequence motif FLNCCPGCCMEP (4cys-tag; Figure 4, Table 2).[52] Three important features make xCrAsH particularly attractive compared to other dimerizing or cross-linking techniques: reversibility (by addition of membrane-permeant dithiols), specific and efficient interactions in living cells, and the use of only a very small peptide tag. In contrast to the larger domains required for other CID approaches, the 4cys-tag mimics a protein loop, which significantly reduces the likelihood for disrupting protein function, even when inserted as an internal fragment.[53] Despite its advantages, further improvement of this technique is necessary. In particular, the use of heterobifunctional cross-linkers should be developed to assure directionality and improved specificity of the approach. Also fluorogeneity that reports on successful cross-linking would be very beneficial.

Besides the xCrAsH technique, several other cross-linking approaches based on bisarsenical probes have been reported (Table 2). ReAsH and FlAsH in combination with

a split tetracysteine tag have been previously used to monitor the oligomeric state of proteins or to connect two parts of a protein structure.[54] However, applications of this technique are limited, because the split motives need to be in very close proximity for sufficient cross-linking to occur. Other systems utilized semi-specific, trifunctional cross-linkers, such as a DOPA-biotin-FlAsH chimera[55] or the photo-cross-linker TRAP (targeted release affinity probe),[56] which are covalently bound to a protein of interest containing the 4cys-tag. Upon periodate oxidation or irradiation interacting partners were trapped, and subsequently identified based on the label transfer mechanism. However, only TRAP is applicable in living cells.[56]

6. Ways to Go

The field of dimerizers and cross-linkers has been rapidly expanding and now constitutes an established research toolbox. The use of these small molecules to sense and manipulate PPIs led to the dissection of protein function in important cellular events, such as signal transduction and transcription regulation.

While the well-established FK506/rapamycin systems have been extensively developed and widely applied, a worthwhile goal would be to simultaneously regulate multiple protein networks by using tools that are completely orthogonal to each another. Besides the alternative methods described herein, a few more potential pairs of small molecules and protein binding motives wait for further exploration including bacterial enzymes (β-lactamase[57]), proteins stabilized by spontaneous intramolecular isopeptide bonds (the SpyTag system[58]), plant proteins with their ligands,[59] and artificial molecules interacting with short tags (e.g. the boric acid derivative RhoBo,[60] other biarsenical derivatives like AsCy3,[61] and many more). The continuous improvement of such systems will not only broaden the spectrum of applications but also lead to better biocompatibility within living cells by reducing or eliminating off-target effects, such as the interaction of CIDs with endogenous proteins.

The most reoccurring limitation for intracellular cross-linking is the permeability of the extraneously applied small molecules. Another critical issue is the impact of the tag size on protein function as well as the invasiveness of the approach to obtain recombinant proteins. In this aspect, genetically encoded unnatural amino acids (i.e. photo-cross-linkers) have the highest potential. This technique requires, however, additional modifications and improvements to for example increase the incorporation rate in mammalian cells.[62] For size reasons, small peptide tags such as 4cys-tag seem to be also favorable. Large additional domains (see CID systems) are usually restricted to N- or C-terminal fusions, whereas short tags can in principle be incorporated at internal sites of the protein of interest.[53] Here, a combinatorial approach with novel technologies to modify endogenous proteins in mammalian cells and experimental animals may develop into a completely new generation of dimerizers and cross-linkers. Last but not least, new methods are urgently required that would on one side improve the delivery and on the other side ensure stable localization in the cell's interior. Finally, most of the described techniques were so far presented in proof-of-concept studies. A more general acceptance by the cell biology community will require prominent applications in physiologically relevant cell systems and in model organisms, such as *Xenopus laevis*, *C. elegans*, or zebrafish. For this purpose, the ultimate aim is to create small molecules that not only detect and modify PPIs, but also allow the simultaneous visualization of the interactions in living organisms.

In conclusion, it is fair to state that numerous cross-linking tools are at hand and we now need strongly interdisciplinary efforts to make them help answering important biological questions. In this respect, the most exciting border is yet to cross—an application as therapeutic or diagnostic agents in patients or in cells from patients.

A.R. is a fellow of the Peter und Traudl Engelhorn-Stiftung and the EMBL Interdisciplinary Postdoc Programme. Work of the group is funded by the EMBL, the ESF (EuroMembrane TraPPs, Schu 943/7-1), the DFG (Transregio 83), the Helmholtz Association (SBCancer and LungSysII), the German Lung Research Center, and the EU (Integrated project LIVIMODE).

Received: March 2, 2012
Published online: June 11, 2012

[1] a) V. Villalobos, S. Naik, D. Piwnica-Worms, *Annu. Rev. Biomed. Eng.* **2007**, *9*, 321 – 349; b) E. Phizicky, P. I. Bastiaens, H. Zhu, M. Snyder, S. Fields, *Nature* **2003**, *422*, 208 – 215.
[2] C. Choudhary, M. Mann, *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 427 – 439.
[3] a) A. Fegan, B. White, J. C. Carlson, C. R. Wagner, *Chem. Rev.* **2010**, *110*, 3315 – 3336; b) J. D. Klemm, S. L. Schreiber, G. R. Crabtree, *Annu. Rev. Immunol.* **1998**, *16*, 569 – 592.
[4] a) M. A. Farrar, J. Alberol-Ila, R. M. Perlmutter, *Nature* **1996**, *383*, 178 – 181; b) Z. Luo, G. Tzivion, P. J. Belshaw, D. Vavvas, M. Marshall, J. Avruch, *Nature* **1996**, *383*, 181 – 185.
[5] a) T. Inoue, W. D. Heo, J. S. Grimley, T. J. Wandless, T. Meyer, *Nat. Methods* **2005**, *2*, 415 – 418; b) H. Haruki, J. Nishikawa, U. K. Laemmli, *Mol. Cell* **2008**, *31*, 925 – 932; c) M. S. Robinson, D. A. Sahlender, S. D. Foster, *Dev. Cell* **2010**, *18*, 324 – 331.
[6] a) Z. L. Xu, H. Mizuguchi, T. Mayumi, T. Hayakawa, *Gene* **2003**, *309*, 145 – 151; b) T. Clackson, *Gene Ther.* **2000**, *7*, 120 – 125; c) V. M. Rivera, G. P. Gao, R. L. Grant, M. A. Schnell, P. W. Zoltick, L. W. Rozamus, T. Clackson, J. M. Wilson, *Blood* **2005**, *105*, 1424 – 1430.
[7] N. Kley, *Chem. Biol.* **2004**, *11*, 599 – 608.
[8] a) K. Baker, C. Bleczinski, H. Lin, G. Salazar-Jimenez, D. Sengupta, S. Krane, V. W. Cornish, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 16537 – 16542; b) P. Peralta-Yahya, B. T. Carter, H. Lin, H. Tao, V. W. Cornish, *J. Am. Chem. Soc.* **2008**, *130*, 17446 – 17452; c) H. Lin, H. Tao, V. W. Cornish, *J. Am. Chem. Soc.* **2004**, *126*, 15051 – 15059.
[9] a) V. M. Rivera, X. Wang, S. Wardwell, N. L. Courage, A. Volchuk, T. Keenan, D. A. Holt, M. Gilman, L. Orci, F. Cerasoli, Jr., J. E. Rothman, T. Clackson, *Science* **2000**, *287*, 826 – 830; b) C. T. Rollins, V. M. Rivera, D. N. Woolfson, T. Keenan, M. Hatada, S. E. Adams, L. J. Andrade, D. Yaeger, M. R. van Schravendijk, D. A. Holt, M. Gilman, T. Clackson, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 7096 – 7101; c) A. Volchuk,

M. Amherdt, M. Ravazzola, B. Brugger, V. M. Rivera, T. Clackson, A. Perrelet, T. H. Sollner, J. E. Rothman, L. Orci, *Cell* **2000**, *102*, 335 – 348.

[10] P. D. Braun, K. T. Barglow, Y. M. Lin, T. Akompong, R. Briesewitz, G. T. Ray, K. Haldar, T. J. Wandless, *J. Am. Chem. Soc.* **2003**, *125*, 7575 – 7580.

[11] M. A. Sellmyer, K. Stankunas, R. Briesewitz, G. R. Crabtree, T. J. Wandless, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2703 – 2705.

[12] J. E. Gestwicki, G. R. Crabtree, I. A. Graef, *Science* **2004**, *306*, 865 – 869.

[13] a) J. H. Bayle, J. S. Grimley, K. Stankunas, J. E. Gestwicki, T. J. Wandless, G. R. Crabtree, *Chem. Biol.* **2006**, *13*, 99 – 107; b) S. D. Liberles, S. T. Diver, D. J. Austin, S. L. Schreiber, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 7825 – 7830; c) K. Stankunas, J. H. Bayle, J. E. Gestwicki, Y. M. Lin, T. J. Wandless, G. R. Crabtree, *Mol. Cell* **2003**, *12*, 1615 – 1624; d) K. J. Liu, J. R. Arron, K. Stankunas, G. R. Crabtree, M. T. Longaker, *Nature* **2007**, *446*, 79 – 82.

[14] M. R. Pratt, E. C. Schwartz, T. W. Muir, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 11209 – 11214.

[15] a) J. S. Schneekloth, Jr., F. N. Fonseca, M. Koldobskiy, A. Mandal, R. Deshaies, K. Sakamoto, C. M. Crews, *J. Am. Chem. Soc.* **2004**, *126*, 3748 – 3754; b) J. S. Schneekloth, Jr., C. M. Crews, *ChemBioChem* **2005**, *6*, 40 – 46.

[16] D. M. Janse, B. Crosas, D. Finley, G. M. Church, *J. Biol. Chem.* **2004**, *279*, 21415 – 21420.

[17] a) H. D. Mootz, E. S. Blum, A. B. Tyszkiewicz, T. W. Muir, *J. Am. Chem. Soc.* **2003**, *125*, 10561 – 10569; b) A. B. Tyszkiewicz, T. W. Muir, *Nat. Methods* **2008**, *5*, 303 – 305.

[18] D. M. Spencer, T. J. Wandless, S. L. Schreiber, G. R. Crabtree, *Science* **1993**, *262*, 1019 – 1024.

[19] a) T. Keenan, D. R. Yaeger, N. L. Courage, C. T. Rollins, M. E. Pavone, V. M. Rivera, W. Yang, T. Guo, J. F. Amara, T. Clackson, M. Gilman, D. A. Holt, *Bioorg. Med. Chem.* **1998**, *6*, 1309 – 1335; b) T. Clackson, W. Yang, L. W. Rozamus, M. Hatada, J. F. Amara, C. T. Rollins, L. F. Stevenson, S. R. Magari, S. A. Wood, N. L. Courage, X. Lu, F. Cerasoli, Jr., M. Gilman, D. A. Holt, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 10437 – 10442.

[20] a) D. M. Spencer, I. Graef, D. J. Austin, S. L. Schreiber, G. R. Crabtree, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 9805 – 9809; b) V. O. Mallet, C. Mitchell, J. E. Guidotti, P. Jaffray, M. Fabre, D. Spencer, D. Arnoult, A. Kahn, H. Gilgenkrantz, *Nat. Biotechnol.* **2002**, *20*, 1234 – 1239; c) M. E. Pownall, B. E. Welm, K. W. Freeman, D. M. Spencer, J. M. Rosen, H. V. Isaacs, *Dev. Biol.* **2003**, *256*, 89 – 99.

[21] M. A. Farrar, S. H. Olson, R. M. Perlmutter, *Methods Enzymol.* **2000**, *327*, 421 – 429.

[22] a) M. G. Mohi, K. Arai, S. Watanabe, *Mol. Biol. Cell* **1998**, *9*, 3299 – 3308; b) A. M. O'Farrell, Y. Liu, K. W. Moore, A. L. Mui, *EMBO J.* **1998**, *17*, 1006 – 1018.

[23] E. L. Knight, A. J. Warner, A. Maxwell, S. A. Prigent, *Oncogene* **2000**, *19*, 5398 – 5405.

[24] a) L. Sanz, M. T. Diaz-Meco, H. Nakano, J. Moscat, *EMBO J.* **2000**, *19*, 1576 – 1586; b) T. L. Ung, C. Cao, J. Lu, K. Ozato, T. E. Dever, *EMBO J.* **2001**, *20*, 3728 – 3737.

[25] H. F. Zhao, J. Boyd, N. Jolicoeur, S. H. Shen, *Hum. Gene Ther.* **2003**, *14*, 1619 – 1629.

[26] J. A. Burlison, B. S. Blagg, *Org. Lett.* **2006**, *8*, 4855 – 4858.

[27] a) S. J. Kopytek, R. F. Standaert, J. C. Dyer, J. C. Hu, *Chem. Biol.* **2000**, *7*, 313 – 321; b) J. C. Carlson, S. S. Jena, M. Flenniken, T. F. Chou, R. A. Siegel, C. R. Wagner, *J. Am. Chem. Soc.* **2006**, *128*, 7630 – 7638.

[28] a) J. Chen, X. F. Zheng, E. J. Brown, S. L. Schreiber, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 4947 – 4951; b) J. Choi, J. Chen, S. L. Schreiber, J. Clardy, *Science* **1996**, *273*, 239 – 242.

[29] a) "Controlling Protein – Protein Interactions Using Chemical Inducers and Disrupters of Dimerization": T. Clackson in *Chemical Biology. From Small Molecules to Systems Biology and Drug Design*, Wiley-VCH, Weinheim, **2007**; b) M. Putyrski, C. Schultz, *FEBS Lett.*, DOI:10.1016/j.febslet.201204061.

[30] a) W. D. Heo, T. Inoue, W. S. Park, M. L. Kim, B. O. Park, T. J. Wandless, T. Meyer, *Science* **2006**, *314*, 1458 – 1461; b) B. C. Suh, T. Inoue, T. Meyer, B. Hille, *Science* **2006**, *314*, 1454 – 1457; c) Z. Szentpetery, P. Varnai, T. Balla, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 8225 – 8230.

[31] a) M. Putyrski, C. Schultz, *Chem. Biol.* **2011**, *18*, 1126 – 1133; b) S. Terrillon, M. Bouvier, *EMBO J.* **2004**, *23*, 3950 – 3961.

[32] J. Johnston, J. Tazelaar, V. M. Rivera, T. Clackson, G. P. Gao, J. M. Wilson, *Mol. Ther.* **2003**, *7*, 493 – 497.

[33] a) J. J. Kohler, C. R. Bertozzi, *Chem. Biol.* **2003**, *10*, 1303 – 1311; b) J. J. Kohler, J. L. Czlapinski, S. T. Laughlin, M. W. Schelle, C. L. de Graffenried, C. R. Bertozzi, *ChemBioChem* **2004**, *5*, 1455 – 1458.

[34] a) N. Umeda, T. Ueno, C. Pohlmeyer, T. Nagano, T. Inoue, *J. Am. Chem. Soc.* **2011**, *133*, 12 – 14; b) A. V. Karginov, Y. Zou, D. Shirvanyants, P. Kota, N. V. Dokholyan, D. D. Young, K. M. Hahn, A. Deiters, *J. Am. Chem. Soc.* **2011**, *133*, 420 – 423.

[35] F. S. Liang, W. Q. Ho, G. R. Crabtree, *Sci. Signaling* **2011**, *4*, rs2.

[36] a) H. N. Lin, W. M. Abida, R. T. Sauer, V. W. Cornish, *J. Am. Chem. Soc.* **2000**, *122*, 4247 – 4248; b) W. M. Abida, B. T. Carter, E. A. Althoff, H. Lin, V. W. Cornish, *ChemBioChem* **2002**, *3*, 887 – 895; c) K. Baker, D. Sengupta, G. Salazar-Jimenez, V. W. Cornish, *Anal. Biochem.* **2003**, *315*, 134 – 137; d) S. S. Gallagher, L. W. Miller, V. W. Cornish, *Anal. Biochem.* **2007**, *363*, 160 – 162.

[37] a) E. A. Althoff, V. W. Cornish, *Angew. Chem.* **2002**, *114*, 2433 – 2436; *Angew. Chem. Int. Ed.* **2002**, *41*, 2327 – 2330; b) J. L. Czlapinski, M. W. Schelle, L. W. Miller, S. T. Laughlin, J. J. Kohler, V. W. Cornish, C. R. Bertozzi, *J. Am. Chem. Soc.* **2008**, *130*, 13186 – 13187.

[38] a) S. L. Hussey, S. S. Muddana, B. R. Peterson, *J. Am. Chem. Soc.* **2003**, *125*, 3692 – 3693; b) S. S. Muddana, B. R. Peterson, *Org. Lett.* **2004**, *6*, 1409 – 1412.

[39] a) S. Athavankar, B. R. Peterson, *Chem. Biol.* **2003**, *10*, 1245 – 1253; b) W. Weber, W. Bacchus, M. Daoud-El Baba, M. Fussenegger, *Nucleic Acids Res.* **2007**, *35*, e116.

[40] S. Gendreizig, M. Kindermann, K. Johnsson, *J. Am. Chem. Soc.* **2003**, *125*, 14970 – 14971.

[41] A. Levskaya, O. D. Weiner, W. A. Lim, C. A. Voigt, *Nature* **2009**, *461*, 997 – 1001.

[42] M. Yazawa, A. M. Sadaghiani, B. Hsueh, R. E. Dolmetsch, *Nat. Biotechnol.* **2009**, *27*, 941 – 945.

[43] M. J. Kennedy, R. M. Hughes, L. A. Peteya, J. W. Schwartz, M. D. Ehlers, C. L. Tucker, *Nat. Methods* **2010**, *7*, 973 – 975.

[44] a) G. T. Hermanson, *Bioconjugate Techniques*, Elsevier, Amsterdam, **2008**; b) *Molecular Probes Handbook. A Guide to Fluorescent Probes and Labeling Technologies, Vol. 11*, Life Technologies, Carlsbad, **2010**.

[45] K. A. Kelly, N. Bardeesy, R. Anbazhagan, S. Gurumurthy, J. Berger, H. Alencar, R. A. Depinho, U. Mahmood, R. Weissleder, *PLoS Med.* **2008**, *5*, e85.

[46] a) N. Hino, Y. Okazaki, T. Kobayashi, A. Hayashi, K. Sakamoto, S. Yokoyama, *Nat. Methods* **2005**, *2*, 201 – 206; b) I. S. Farrell, R. Toroney, J. L. Hazen, R. A. Mehl, J. W. Chin, *Nat. Methods* **2005**, *2*, 377 – 384.

[47] M. Suchanek, A. Radzikowska, C. Thiele, *Nat. Methods* **2005**, *2*, 261 – 267.

[48] S. A. Slavoff, D. S. Liu, J. D. Cohen, A. Y. Ting, *J. Am. Chem. Soc.* **2011**, *133*, 19769 – 19776.

[49] H. Baruah, S. Puthenveetil, Y. A. Choi, S. Shah, A. Y. Ting, *Angew. Chem.* **2008**, *120*, 7126 – 7129; *Angew. Chem. Int. Ed.* **2008**, *47*, 7018 – 7021.

[50] a) G. Lemercier, S. Gendreizig, M. Kindermann, K. Johnsson, *Angew. Chem.* **2007**, *119*, 4359 – 4362; *Angew. Chem. Int. Ed.* **2007**, *46*, 4281 – 4284; b) A. Gautier, E. Nakata, G. Lukinavicius,

K. T. Tan, K. Johnsson, *J. Am. Chem. Soc.* **2009**, *131*, 17954 – 17962.

[51] A. Rutkowska, C. H. Haering, C. Schultz, *Angew. Chem.* **2011**, *123*, 12867 – 12870; *Angew. Chem. Int. Ed.* **2011**, *50*, 12655 – 12658.

[52] B. R. Martin, B. N. Giepmans, S. R. Adams, R. Y. Tsien, *Nat. Biotechnol.* **2005**, *23*, 1308 – 1314.

[53] C. Hoffmann, G. Gaietta, M. Bunemann, S. R. Adams, S. Oberdorff-Maass, B. Behr, J. P. Vilardaga, R. Y. Tsien, M. H. Ellisman, M. J. Lohse, *Nat. Methods* **2005**, *2*, 171 – 176.

[54] a) B. Krishnan, L. M. Gierasch, *Chem. Biol.* **2008**, *15*, 1104 – 1115; b) N. W. Luedtke, R. J. Dexter, D. B. Fried, A. Schepartz, *Nat. Chem. Biol.* **2007**, *3*, 779 – 784; c) R. A. Scheck, A. Schepartz, *Acc. Chem. Res.* **2011**, *44*, 654 – 665.

[55] B. Liu, C. T. Archer, L. Burdine, T. G. Gillette, T. Kodadek, *J. Am. Chem. Soc.* **2007**, *129*, 12348 – 12349.

[56] P. Yan, T. Wang, G. J. Newton, T. V. Knyushko, Y. Xiong, D. J. Bigelow, T. C. Squier, M. U. Mayer, *ChemBioChem* **2009**, *10*, 1507 – 1518.

[57] a) S. Mizukami, S. Watanabe, Y. Akimoto, K. Kikuchi, *J. Am. Chem. Soc.* **2012**, *134*, 1623 – 1629; b) S. Watanabe, S. Mizukami, Y. Akimoto, Y. Hori, K. Kikuchi, *Chem. Eur. J.* **2011**, *17*, 8342 – 8349.

[58] B. Zakeri, J. O. Fierer, E. Celik, E. C. Chittock, U. Schwarz-Linek, V. T. Moy, M. Howarth, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 690 – 697.

[59] S. R. Cutler, *Sci. Signaling* **2011**, *4*, pe13.

[60] T. L. Halo, J. Appelbaum, E. M. Hobert, D. M. Balkin, A. Schepartz, *J. Am. Chem. Soc.* **2009**, *131*, 438 – 439.

[61] H. Cao, Y. Xiong, T. Wang, B. Chen, T. C. Squier, M. U. Mayer, *J. Am. Chem. Soc.* **2007**, *129*, 8672 – 8673.

[62] a) L. Davis, J. W. Chin, *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 168 – 182; b) T. Plass, S. Milles, C. Koehler, C. Schultz, E. A. Lemke, *Angew. Chem.* **2011**, *123*, 3964 – 3967; *Angew. Chem. Int. Ed.* **2011**, *50*, 3878 – 3881; c) T. Plass, S. Milles, C. Koehler, J. Szymański, R. Mueller, M. Wießler, C. Schultz, E. A. Lemke, *Angew. Chem.* **2012**, *124*, 4242 – 4246; *Angew. Chem. Int. Ed.* **2012**, *51*, 4166 – 4170.

[63] P. J. Belshawl, D. M. Spencer, G. R. Crabtree, S. L. Schreiber, *Chem. Biol.* **1996**, *3*, 731 – 738.

[64] E. J. Licitra, J. O. Liu, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 12817 – 12821.

[65] P. J. Belshaw, S. N. Ho, G. R. Crabtree, S. L. Schreiber, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 4604 – 4607.