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Dynamic modifications of biomacromolecules: mechanism and chemical interventions

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Biological macromolecules (proteins, nucleic acids, polysaccharides, etc.) are the building blocks of life, which constantly undergo chemical modifications that are often reversible and spatial-temporally regulated. These dynamic properties of chemical modifications play fundamental roles in physiological processes as well as pathological changes of living systems. The Major Research Project (MRP) funded by the National Natural Science Foundation of China (NSFC)—"Dynamic modifications of biomacromolecules: mechanism and chemical interventions" aims to integrate cross-disciplinary approaches at the interface of chemistry, life sciences, medicine, mathematics, material science and information science with the following goals: (i) developing specific labeling techniques and detection methods for dynamic chemical modifications of biomacromolecules, (ii) analyzing the molecular mechanisms and functional relationships of dynamic chemical modifications of biomacromolecules, and (iii) exploring biomacromolecules and small molecule probes as potential drug targets and lead compounds.

chemical biology, biomacromolecule, dynamic modification, modification analysis, chemical intervention

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Introduction

"Dynamic chemical modifications of biomacromolecules" is an emerging and highly active area at the interface of chemistry and life sciences

The discovery of "central dogma" is a fundamental breakthrough in life sciences that unveiled the molecular mechanism of inheritance and the evolution of biological species (Crick, 1970). However, with the completion of the Human Genome Project at the beginning of the 21st century, people realized that although the evolution of individual life depends mainly on gene sequence, its complexity and diversity cannot be simply explained by the "central dogma". As the basic "components" of life, biomacromolecules including proteins, nucleic acids, polysaccharides and lipids support diverse life processes, with themselves undergoing dynamic chemical modifications and regulations in the cell (Davis, 2004; Prabakaran et al., 2012). The dynamic chemical modifications of these macromolecules play a key role in the regulation of cell fate, and are crucial during the occurrence and development of diseases. Understanding the

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underlying mechanism of dynamic biomacromolecule modifications has become one of the most attractive frontiers in life sciences, as well as the most active research direction at the interface of chemistry, life sciences and medicine (Bode and Dong, 2004; Mann and Jensen, 2003; Wold, 1981).

Growing evidences have shown that the chemical modification of biomacromolecules is a ubiquitous way for regulating life processes. Its important physiological value and pathological significance have been widely recognized and valued. Research in this field is progressing rapidly and has greatly promoted the development of life sciences (e.g. the rise of epigenetics). However, as the molecular basis and regulatory component in emerging fields such as epigenetics (Goldberg et al., 2007; Heim and Binder, 2012), the investigation of chemical modifications on biomacromolecules is still in its infancy. For example, we still know little about the temporal and spatial specificity and bidirectional reversibility of various chemical modifications, the corresponding biological functions and physiological significance of many chemical modifications are still unclear, and the relationships between dynamic modifications and biological functions remain largely unknown (Figure 1) (Goldberg et al., 2007; Mann and Jensen, 2003).

The complexity of dynamic biomacromolecule modifications was first attributed to the diversity of chemical modifications. The common chemical modifications on nucleic acids (e.g. DNA and RNA) are methylation, hydromethylation and among others (Cedar and Bergman, 2009). For proteins, there are dozens of chemical modifications such as methylation, acetylation, phosphorylation, glycosylation and ubiquitination (Walsh et al., 2005). In recent years, with the development of advanced analytical techniques, particularly mass spectrometry, more chemical modifications have been identified, such as formylation, carboxylation and acylation on nucleic acid and protein molecules. Secondly, these chemical modifications have high spatial and temporal specificity. The time and space of specific chemical modifications inside cells are precisely regulated. It has high selectivity in the spatial distribution, sequence, site, nucleic acid bases and protein side chains, which all correspond to different biological effects. Thirdly, these chemical modifications are reversible and are catalyzed by different enzymes, such as methyltransferase, demethylase, acetylase and deacetylase, to name a few. Together, chemical modifications of biomacromolecules have many dynamic properties, such as diversity, spatial-temporal specificity and reversibility. They constitute a complex network within cells and performs a wide range of biological functions. Meanwhile, the abnormality of this dynamic modification process is often accompanied by the occurrence of diseases (Gregorich and Ge, 2014; Magi et al., 2006).

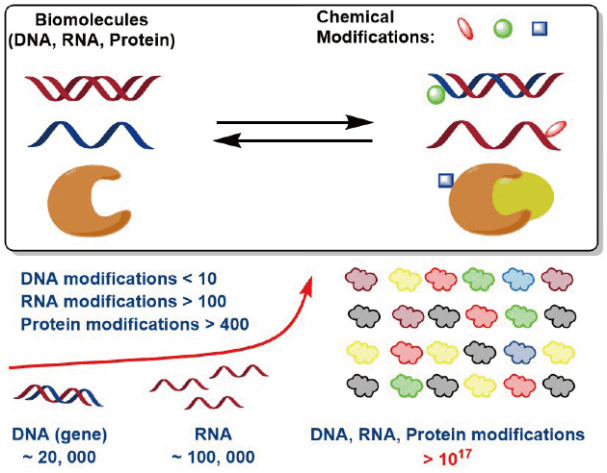

Figure 1 The dynamic chemical modifications of biomacromolecules support the complexity of life beyond "central dogma".

A chemical biology-enabled interdisciplinary approach for studying dynamic modifications of biomacromolecules

With the development of a series of "omics"-based technology in the past 20 years, biologists have accumulated a large amount of biological data, which not only renewed our understanding of the complexity of life, but also opened many questions that are difficult to address using traditional biological methods (Mann and Jensen, 2003). Further understanding of life processes has been increasingly dependent on the continuous input of new ideas, theories and research tools from chemistry, physics and other scientific disciplines. Interdisciplinary research has become extremely critical, and its advantages in solving frontier problems have become increasingly evident. Embarking on chemical theory, methods and technology, chemical biology has become an attractive new frontier that provided new research models and powerful methodological tools for biological research, and is becoming an important driving force to promote future life science and biomedicine. Meanwhile, these chemical approaches driven by biological questions, also significantly promoted the innovation of chemistry as well as the deep integration of chemistry with life sciences, biomedicine and other disciplines (Schenone et al., 2013).

Taking the dynamic chemical modification of biomacromolecules as an example, a great deal of biological data has been obtained by genomics, proteomics and epigenetic techniques. However, the number and type of proteins whose molecular mechanisms have been really resolved and/or confirmed as drug targets remain very limited. The key reason lies in the lack of specific methods for labeling, capturing and detecting the dynamic chemical modifications of biomacromolecules, and the lack of techniques for studying how these dynamic chemical modifications regulate the functions of the substrate molecules. It still remains difficult to analyze the corresponding molecular mechanisms

at the cellular and even animal level. With the increasing importance of epigenetic regulation, these "bottlenecks" have greatly restricted the development of this field and the transformation to drug research and development (Rix and Superti-Furga, 2008).

This complex nature of problems from life sciences has brought new opportunities for chemical biology to probe these fundamental questions underlying life processes and to develop corresponding chemical intervention strategies through new chemical reactions, labeling and detection techniques, and small molecules. Chemical biology has also been a link connecting the basic research of the upstream life sciences and the downstream area in drug development. It is uniquely positioned to bring innovation in research tools and methods to discover and obtain more information about the dynamic chemical modifications, to explore the molecular mechanism of the dynamic process, and to analyze its intrinsic relationship with human diseases.

Unmet challenges in studying dynamic chemical modifications of biomacromolecules

In view of the chemical nature and dynamic properties of biomacromolecule modifications, the development of novel chemical biology tools and the acquisition of chemically modified probe molecules have been increasingly appreciated, which may help to address the following unmet challenges towards the study of dynamic biomacromolecule modifications:

(1) Chemical modifications of biological macromolecules mainly include protein and nucleic acid modifications, which are ubiquitous in cells and affect its expression and/or function by modifying proteins or nucleic acids. For example, protein phosphorylation alone can occur independently on seven different types of amino acids, and so can dephosphorylation (Rubin and Rosen, 1975). The dynamic characteristics and complexity of chemical modification of biological macromolecules are evident. It remains challenging to label the biological macromolecules and their chemical modifications at specific time and space to achieve visualization and dynamic characterization. At the same time, spatial-temporally resolved detection, high throughput and multi-parameter analysis as well as high-precision imaging of dynamic modification are still challenging and urgently needed for studying dynamic modification of biomacromolecules (Mann and Jensen, 2003).

(2) Many new types of biomacromolecule modifications have been identified in recent years, which greatly expanded the chemical space that can be directly utilized by nature as the modification elements (Janke and Chloë Bulinski, 2011). In view of these new modifications, the study of their intracellular spatial distribution and dynamic regulation has attracted extensive attention, and has bred important scientific breakthroughs and opportunities. With the discovery of many important dynamic modifications as well as the characterization of their distribution spectra, it is urgent to carry out in-depth studies on the chemical basis, molecular mechanisms and functional roles of these modifications, focusing on their dynamic generation, elimination, recognition and regulation. The dynamic characteristics and regulation of biomacromolecule modifications will also need to be revealed at different scales, such as subcellular, cellular, tissue and intact animal levels (Snider and Omary, 2014).

(3) Small molecule probes are one of the most representative chemical biology tools, which have a great impact on a wide range of fields ranging from basic science to clinical translations. This area has triggered intensive collaborations between biologists and chemists, with a great potential in regulating dynamic modifications on biomacromolecules and life processes they participate. Therefore, chemical intervention of disease-related biomacromolecule modifying enzymes and their action networks, elucidation of the structural mechanism of interaction between small molecules and biomacromolecules, discovery of regulatory and modification sites, and acquisition of intervention leading compounds are the future directions in this exciting area (Bunnage et al., 2015).

The initiation and organization of the NSFC Major Research Plan on studying dynamic modifications of biomacromolecules

In order to meet the above-mentioned grand challenges, we believe that only by taking full advantage of interdisciplinary research, can we effectively promote chemical biology as a leading approach to study chemical modifications of biomacromolecules. The study will be focused on the core issues of this scientific frontier as well as the development of original and irreplaceable new technologies and methods, which will promote the current study from *static* to *dynamic*, from *in vitro* to *in vivo*, from *qualitative* to *quantitative*, from *simple subjects* to *complex systems*. This approach will ultimately make the function of biomacromolecules visible, measurable and controllable in living systems. To achieve these exciting goals, NSFC launched a Major Research Project (MRP) aiming to accelerate the study of dynamic chemical modifications of biomacromolecules by adopting various chemical biology-enabled approaches.

The fundamental question in the area is to discover and clarify the chemical nature and dynamic properties of biological macromolecules with important functions, to reveal their molecular regulatory mechanisms and biological functional relationships, and to achieve targeted chemical intervention of these dynamic modifications. This Major Research Plan aims to develop specific labeling and detec-

tion tools for dynamic chemical modification of biological macromolecules, to analyze the molecular mechanism and functional relationship of dynamic chemical modification of biological macromolecules, to provide potential intervention molecules and new targets for drug research and development, and to improve the understanding and regulation of life processes.

This research plan will organize the joint efforts from scientists in chemistry, biology, medicine, mathematics, materials, information and other disciplines in the following three areas with a central key question for each area (Figure 2).

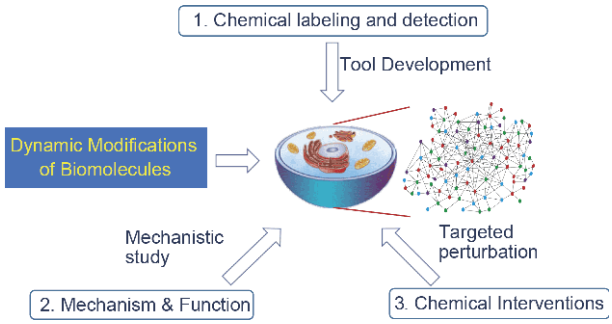

Figure 2 The organization of the NSFC Major Research Plan on studying dynamic modifications of biomacromolecules.

Chemical labeling and detection of dynamic modifications of biomacromolecules

Scientific question: how to perform dynamic characterizations of chemical modifications of biomacromolecules?

The research focuses include discovering and elucidating the chemical basis and dynamic process of biomacromolecule modifications with important biological functions. Through high-throughput, multi-module analysis technologies, spatial-temporal resolved detection and high-precision imaging, the dynamic modification of biomacromolecules can be realized, and the underlying mechanism of many dynamic modifications can be revealed at cellular, molecular and atomic scales.

The aim of this direction is to reveal the nature and regulation mechanism of dynamic biomacromolecule modifications via chemical labeling and detection methods at the molecular, subcellular, cellular and animal levels. Research directions will be emphasized on developing general methods and research platforms for high sensitivity dynamic discovery, high throughput and multi-parameter analysis and detection of macromolecule modifications; developing new methods, new technologies and new instruments for spatial-temporal resolved detection and high precision imaging of biomacromolecule modifications.

Deciphering molecular mechanisms and functions of dynamic biomacromolecule modifications

Scientific question: what are the underlying connections and mechanisms between dynamic modifications of biomacromolecules and their biological functions?

The research focuses include (1) understanding the chemical reaction, recognition and regulation mechanism of dynamic modification of biomacromolecules; (2) discovering and identifying new dynamic modification and regulation factors of biomacromolecules; (3) revealing the molecular basis of their functional implications; (4) clarifying their dynamic characteristics in physiological or pathological processes.

With the help of a series of innovative chemical biology methods, structural analysis and high-resolution imaging techniques, the chemical basis of dynamic modifications of biomacromolecules will be revealed, with the underlying physiological and pathological roles clarified. Multi-scale and multi-dimensional studies from atoms, molecules, cells to intact animals will be carried out. The identification and dynamic functional analysis of chemical modifications on biomacromolecules such as nucleic acids, proteins and glycolipids will be carried out by means of bioorthogonal chemical labeling and metabolic engineering. The molecular mechanisms and chemical basis for the generation, elimination, recognition and regulation of chemical modifications will be unveiled. The temporal and spatial distribution and dynamic characteristics of bio-macromolecule modifications in living systems will be studied and the underlying regulation mechanisms will be illustrated.

Chemical intervention of dynamic biomacromolecule modifications

Scientific question: how to explore the chemical intervention strategies to perturb the dynamic modification process of biomacromolecules?

Small molecule tools have the advantages of high stability, specificity and real-time control of biological functions. Developing chemical methods and small molecule tools to selectively perturb the dynamic regulation of protein machines will help to illustrate the dynamic processes and molecular mechanisms of chemical modifications of biomacromolecules under living conditions. The mechanism of small molecule intervention in the dynamic biological macromolecule modification will be elucidated from the molecular, cellular and tissue levels. The establishment of new targets is another major goal, which is to discover and confirm new targets for chemical interventions, with the potential value as drug targets that will promote the innovation in drug discovery.

The research focuses include (1) developing small chemical molecule tools for biomimetic simulation, selective intervention, and precise control of the addition, removal or

recognition of dynamic modification of biomolecules; (2) deepening understanding of the chemical nature of dynamic regulation of biomacromolecules modifications; (3) establishing stable and reliable systems for discovery and characterization of small molecule tools aiming at these key protein machines (enzymes, protein-protein interactions, protein recognition nucleic acids, etc.) to obtain high selectivity and clear mechanism of action. In particular, the rich natural product resources in China and the active ingredients from traditional Chinese medicine will be subjected to high-throughput screening to identify highly selective and controllable small molecule tools. Aiming at the urgent needs of new targets in drug discovery in China, this specific area also includes systematically verifying the pharmacological functions of key protein machines as drug targets, as well as promoting the discovery of new drug targets.

Representative directions and projects funded by the Major Research Plan

Since its initiation in 2017, NSFC has focused its support on projects that plan to address the three afore-mentioned scientific questions. There are 89 projects being funded so far, ranging from synthetic approaches to construct biomacromolecules with defined chemical modifications, to probing and perturbing these chemical modifications under living conditions. Here we will summarize the aim and content of some representative directions and highlight projects which have already made significant progress.

Protein post-translational modifications

Research topics in the NSFC program cover a broad range of protein post-translational modifications (PTMs). In addition to classic modifications such as histone methylation and acetylation, protein phosphorylation, nitrosylation and lipidation, more than half of the funded projects are focusing on more recently discovered modifications, including histone succinylation, β-hydroxybutyrylation, aminoacylation, and crotonylation. Together, these studies aim to elucidate the cellular functions of various PTMs and the molecular mechanisms underlying their regulations. Below, we describe research projects in each category of protein PTMs.

Histone modifications

This category includes various modifications on histone proteins, such as methylation (P5 and P24, Table S1 in Supporting Information), acetylation (P89), succinylation (P36), and β-hydroxybutyrylation (P61). Many of these modifications occur primarily on lysine residues, but some have been observed on the arginine side chain as well. Because modifications on histone proteins could alter their interaction with DNA in the nucleosome, they often have important implications in regulating gene expression (Cedar and Bergman, 2009; Zentner and Henikoff, 2013). Due to its importance, more than one third (5 out of 14) funded projects focus on histone modifications.

Identifying novel protein modifications

As more protein PTM forms have been unearthed, many scientists wonder how many proteoforms there are. To answer this question, it would require continuing efforts in detecting novel protein modifications, particularly for those at low abundances. Development of bottom-up mass spectrometry-based proteomic profiling techniques over the past decade has brought us closer to reaching this goal (Bandeira et al., 2007). Armed with advanced techniques, several funded projects in this NSFC program are exploring novel protein PTMs (P2, P34, P41, P49, P66, P67).

Protein modifications in liquid-liquid phase separation

In addition to the identification of new protein modifications, the study of PTM-mediated new biological mechanism has also attracted much attention. For example, the liquid-liquid phase separation phenomenon (LLPS) has gained increasing attention in recent years (Aguzzi and Altmeyer, 2016). These microscopic, granular and membrane-less structures can assemble within living cells in a highly dynamic and reversible manner, often in response to external stimuli (e.g. oxidative stress) or internal cues (e.g. gene activation). Many protein PTMs have been implicated in regulating the formation of LLPS. At present, two funded projects are exploring the linkage between PTM and LLPS (P57, P70).

Proteomic and computational tools to study protein modifications

Protein methylation is another prevalent and functionally indispensable modification in biology (Black et al., 2012). Histone methylations have drawn much attention because of its profound impact on gene transcription and chromosome stability. Despite progress in functional characterization of individual histone methylation, chemical tools to globally profile protein methylations in proteomes are still lacking, especially for those non-histone methylations. Another technical bottleneck is to precisely quantify the modification stoichiometry of different types of methylations (e.g. mono-, di- and tri-methylations on substrate proteins). Scientists funded by this MRP have adopted both proteomic (P6, P8, P76) and computational approach (P10, P21) to study PTMs such as methylation.

Recently, the research teams lead by Professor Peng Chen and Professor Chu Wang at Peking University adopted a computer-aided approach to develop a universal protein activation method in living systems. This strategy was based on the concept of “proximal decaging” on any given enzyme

pocket that was enabled by integrating the genetically encoded chemical decaging reaction with computational protein design (Wang et al., 2019). The Chen group has previously created bioorthogonal cleavage reactions that allowed the *in situ* decaging of active sites of enzymes families such as kinases and PLP-dependent enzymes (Li and Chen, 2016). By combining the protein computational modeling tools from the Wang group, they turned to computer to guide them to identify the incorporation sites of the photo-caged tyrosine analogue (ONBY) that are in close proximity to the actual active site of a protein of interest. The enzyme function can be temporally blocked by this proximal ONBY but readily activatable upon photo-triggered decaging, achieving the on-demand protein activation with high temporal resolution in living systems (Figure 3). They demonstrated the applicability of this method on an array of protein families including luciferases, kinases, GTPases, caspases, demethylases as well as metalloenzymes, which provide a powerful toolkit for time-resolved study of protein functions, interactions and PTMs within the native cellular context.

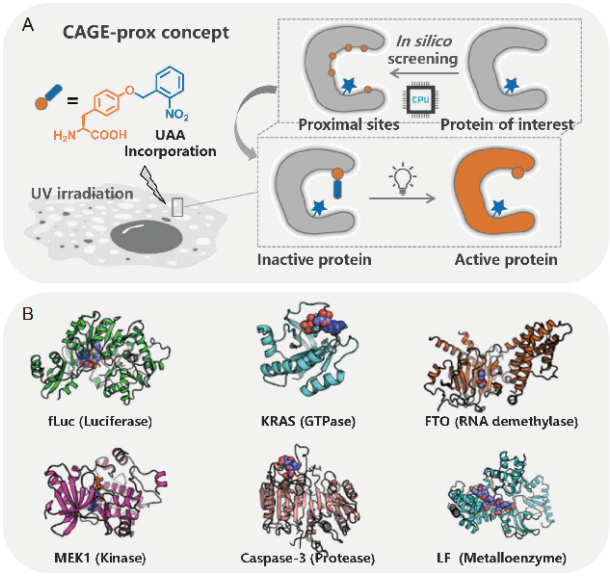

Figure 3 The computer-aided, proximal decaging (CAGE-prox) technique as a universal protein activation strategy for time-resolved study of protein functions, interactions and PTMs in living systems. A, CAGE-prox concept and workflow. B, Representative proteins of various types of enzymes activated using GAGE-prox.

Glycosylation and lipidation

New tool developments in glycosylation research

Glycosylation is one major class of post-translational modifications that are playing crucial roles in biology; however, its functional studies have generally lagged behind other PTMs such as phosphorylation and acetylation due to the diverse structures and properties of glycosylation (Ohtsubo and Marth, 2006). Therefore, new chemical tools are desperately needed to aid characterization of this important class of PTMs at the molecular level, and several proposals are focusing on this specific area (P28, P60, P68, P80, P81).

Profiling and functional characterization of O-GlcNAc modifications in biology

O-GlcNAc is a wide-spread type of intracellular protein glycosylation that has been shown with numerous functional roles in biology. Several proteomics methods have been developed in recent years to globally profile substrates of O-GlcNAcylation in proteomes in site-specific and quantitative manners (Wells et al., 2001). However, they have yet waited to be applied in more specific biological systems. Three research teams have independently proposed to develop and apply chemoproteomic strategies to identify key events of O-GlcNAcylation in the area of stem cell and embryo development research (P35, P44, P72), which can further direct functional study on how such a monosaccharide modification can regulate complex biological processes of cell fate development.

Protein lipidation

Protein lipidation is a unique type of PTM because it drastically changes the hydrophobicity of modified proteins (Casey, 1995; Hang and Linder, 2011). Such changes could lead to rapid re-localization to specific subcellular compartments (e.g. plasma membrane), thus allowing the initiation of cell signaling pathways. Protein lipidation has been implicated in a wide range of biological processes, from cancer to learning and memory. Scientists aim to identify previously unknown long-chain fatty acyl transferases in a high-throughput manner (P23), as well as to locally profile protein lipidation at specific subcellular compartments with high spatial resolutions (P31), which could help to elucidate the molecular mechanism of cell growth regulation and reveal potential anti-tumor targets. In addition, cholesterol is the most abundant steroid lipids in cell and its main function is to regulate the fluidity of cell membranes. Non-covalent interactions of cholesterol and its binding proteins have been extensively profiled by chemical proteomics tools. However, although the first substrate of covalent cholesterol modification was identified as the Hedgehog (Hh) protein back in 1996, little is known about the covalent cholesterol modification on other proteins and the physiological/pathological implications. A project funded by this MRP aims to systematically study covalent cholesterol modifications on proteins (P42).

Prof. Bao-Liang Song and his colleagues from Wuhan University recently developed a cholesterol-based chemical probe (Xiao et al., 2017). Through unbiased biochemical enrichment and mass spectrometry identification, they found that Smoothened (Smo) is covalently modified by cholesterol. They performed thorough functional study to elucidate the role of this specific modification in the Hedgehog sig-

naling and embryonic development. Based on this study, they proposed here to further optimize the cholesterol probe and the proteomic pipeline for discovering more novel substrate proteins of covalent cholesterol modifications. They also aim to reveal the mechanism by which Smo is modified by cholesterol and unveil the function of cholesterylation. These studies will provide a foundation for developing small molecule compounds that might have anti-tumor properties by perturbing the process of cholesterol modification in living cells (Figure 4).

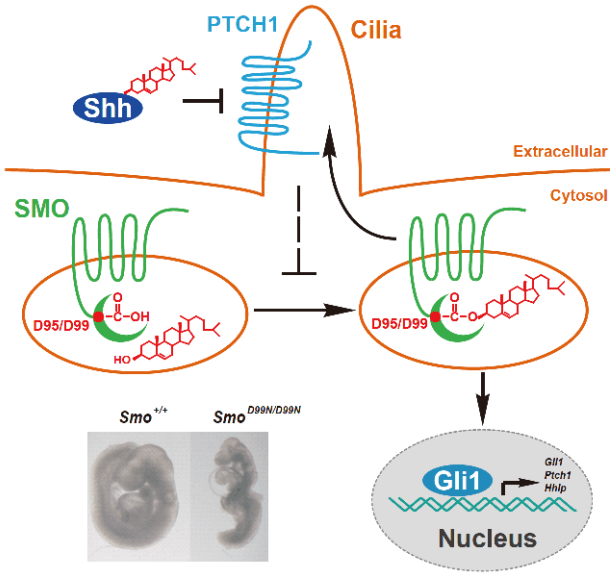

Figure 4 Cholesterol can covalently modify the D95 of human SMO or D99 of mouse SMO. This cholesterylation is inhibited by PTCH1 and enhanced by Shh that is also linked to cholesterol. The homozygous D99N/D99N knock-in mouse showed severe developmental defects and is embryonic lethal.

Synthesis and reactions enabled post-translational modification studies

Chemical protein synthesis

Currently it remains difficult to obtain post-translationally modified protein samples with high homogeneity and usable quantities by traditional methods such as natural extraction or biological expression owing to the dynamic reversibility of PTMs. Chemical protein synthesis, which relies on the chemoselective ligation of peptides, provides an important alternative pave to obtain protein samples and probe molecules bearing site-specific post-translational modifications (Kochendoerfer and Kent, 1999). In order to meet the challenges to prepare post-translationally modified proteins with increased size and complexity, such as hydrophobic lipoproteins, glycosylated proteins and non-classical ubiquitinated proteins, it is necessary to further develop new chemical synthetic methods of proteins to prepare specific post-translationally modified proteins and their probes for their biochemical and biophysical studies (P43, P71). In addition, the development of new active molecules to specifically modulate post-translational modification processes is needed to study their regulatory mechanisms. A series of projects related to protein chemical synthesis have been initiated towards the development of new chemically synthetic methods such as peptide hydrazide ligation (P22) and serine/threonine ligation (P33) for post-translational modification proteins, the development of chemically modified protein probes (e.g. glycosylation (P37), lipidation (P4)) and fluorescent labeling (P63), as well as the discovery of active molecules for functional regulatory of proteins (P55).

Prof. Lei Liu's group from Tsinghua University has developed several new chemical ligation methods to readily produce ubiquitinated proteins, including the cysteine-aminoethylation assisted chemical ubiquitination (CAACU) strategy for post-translationally modified nucleosomes (Chu et al., 2019). These modified nucleosomes have been applied for their biochemical and biophysical studies by Cryo-electron microscopy and other advanced techniques (Ai et al., 2019). In addition, Liu group developed K27-di-Ub single-molecule Förster resonance energy transfer (smFRET) probes with fluorescent donor and acceptor (Cy3/Cy5) to study the molecular recognition mechanism of Ub-interacting proteins (such as UCHL3) with K27-linked Ubs. The smFRET distributions of probes before and after UCHL3 recognition revealed that UCHL3 had a "compact to open" configuration transition in the process of UCHL3 recognizing K27 ubiquitin chain. This work demonstrates that protein chemical synthesis has broad application prospects in solving the dynamic regulation of post-translational modifications (Figure 5) (Pan et al., 2019).

New bioorthogonal reactions

The development of light triggered bioorthogonal reactions that rely on visible light as the source (P19), or in conjunction with biocompatible photocatalysts (P26), is an important research direction in chemical biology. These reactions allowed bioorthogonal labeling and imaging proteins under living conditions, orthogonal control of protein expression, photo-GABAB receptor activity as well as subcellular decaging in mitochondria (Ramil and Lin, 2014). In addition, the *in situ* photo-triggered bioorthogonal reaction has been developed to specifically label and study protein PTMs (P56).

Prof. Yan Zhang's group from Nanjing University developed a visible light triggered bioorthogonal photocycloaddition reaction. They designed this new type of bioorthogonal reaction based on the unique reactivity of the phenanthrenequinone (PQ) at its $\pi\pi^*$ excited state in polar solvent with electron-rich alkenes such as vinyl ether (VE). This visible light initiated bioorthogonal photo-click cycloaddition makes it possible to label proteins in living cells

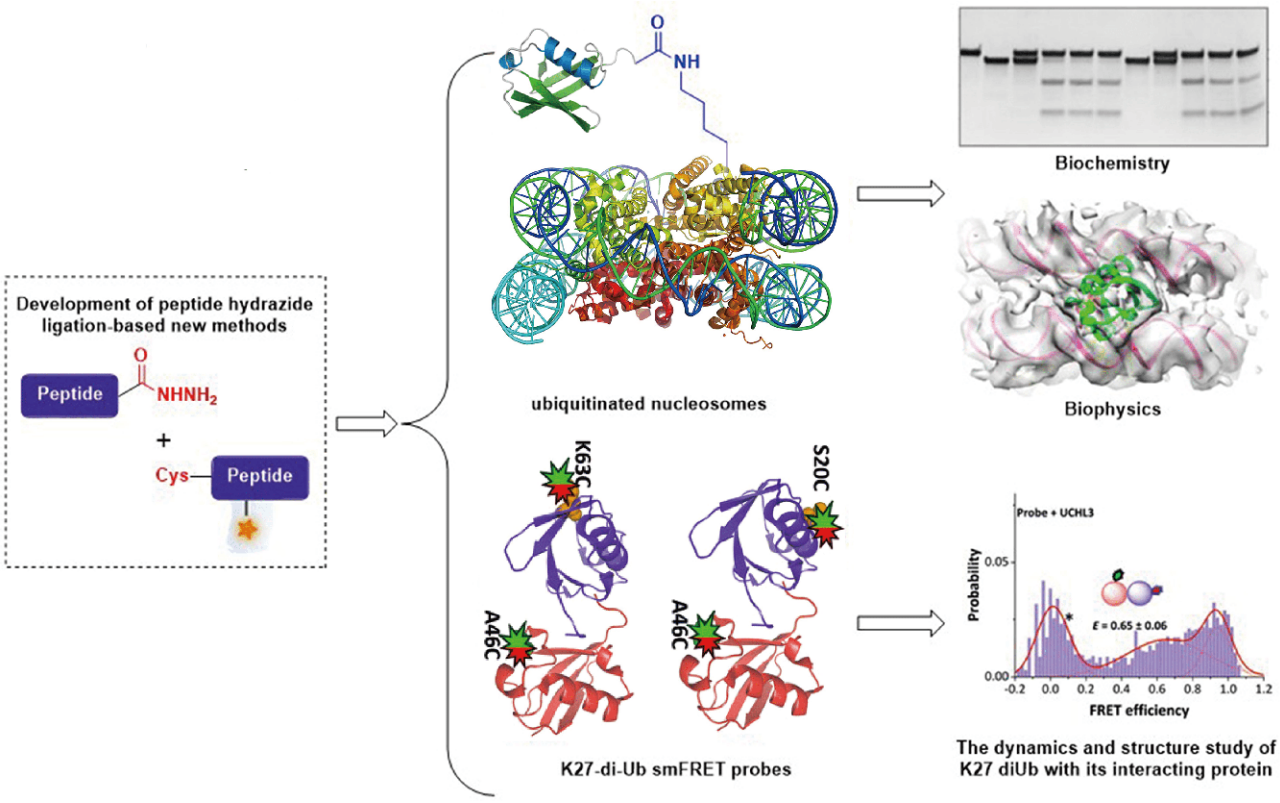

Figure 5 Chemical protein synthesis of post-translationally modified proteins/probes and their representative applications for the studies of the dynamic regulation mechanisms of ubiquitin chains and ubiquitinated nucleosomes.

with temporal and spatial control (Figure 6). In addition, the orthogonality of this photo-click cycloaddition with the strain-promoted azide alkyne cycloaddition (SPAAC) also allowed simultaneous use of these two bioorthogonal reactions for orthogonal labeling of two biomolecules without crosstalking (Li et al., 2018).

Nucleic acid modifications

Nucleic acids (DNA and RNA) are vital for cell functioning, and therefore for life. They keep track of hereditary information so that the cell can maintain itself and perform any specialized functions. Accordingly, the structural and mechanism elucidation of biologically important nucleic acids (and their protein complexes) and the precise manipulation of their activities have become indispensable in life sciences. The MRP is intended to encompass contributions from various approaches to influence biological action of nucleic acids, with a focus on chemical modulation and mechanism studies. This contains methods for elucidating molecular mechanisms for dynamic regulation of nucleic acids, including epigenetic modifications and non-coding RNAs. In addition, delivery systems for CRISPR/Cas9-based genome editing, as well as small organic molecules and aptamers as molecular recognition elements in bioanalytical applications are also a part of this topic.

RNA epigenetics

RNA is an essential player in almost all biological processes, and has an ever-growing number of roles in regulating cellular growth and organization. RNA functions extend far beyond just protein coding and has shown to function in numerous signaling events and transcriptional regulation. Mammalian messenger RNA (mRNA) and long noncoding RNA (lncRNA) contain tens of thousands of post-transcriptionally chemical modifications, which represents another level of epigenetic regulation of gene expression, comparable to DNA methylation and histone modification (Holoch and Moazed, 2015). Projects funded by this MRP aim to dissect how the complex network of RNA-modification enzymes work and the direct recognition of modified-RNA binding proteins to induce subsequent reactions within the cell is a real, but exciting, challenge for the RNA community. Investigating these biological questions will fuel the discovery of new mechanisms of epigenetic regulation of gene expression (P3, P46, P48, P65), and the development of single-cell analyses (P14), higher throughput genomic and epigenomic assays (P15) with increasingly higher resolution.

Among the 150+ modified RNA nucleotide variants identified, the N^6-methyl-adenosine (m^6A) is the most prevalent epigenetic mark in eukaryotic mRNA. About 25% of the mRNA in human cells contains m^6A modifications, and these modifications are mainly concentrated in the non-

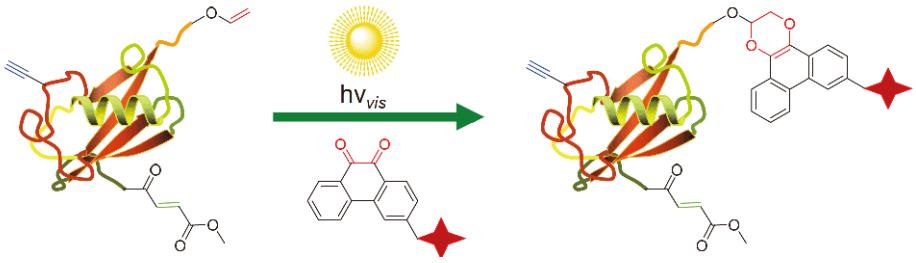

Figure 6 Site-selective labeling of protein by the visible light initiated bioorthogonal photo-click cycloaddition.

coding region of the 3′ end of the mRNA and the near stop codon. In addition, the m^{6}A modification is regulated by a dynamical complex encompassed of m^{6}A-specific proteins, i.e., "writers", "readers" and "erasers", which create, interpret and remove the m^{6}A variation, respectively. The study of dynamic m^{6}A modification on RNA will help to understand the significance of m^{6}A modifications under various physiological and pathological settings (Roundtree and He, 2016). For example, a heterodimeric enzyme complex composed of METTL3 and METTL14 generates m^{6}A on mRNAs. However, the catalytic domains of these methyltransferases and the relationship between the specificity of their binding to RNA substrates and the enzyme activity will be studied (P3, P46, P48), which will help to reveal the dynamic modification and regulation of m^{6}A and provide the guidance for the development of corresponding chemical interventions.

DNA modifications

The most commonly studied eukaryotic DNA modification is methylation, which is a stable epigenetic mark that can be inherited through multiple cell divisions (Jones, 2002). Similarly, consistencies of DNA methylation among different regions reveal the status of cancer cells and their response to the microenvironment. Therefore, DNA methylation can be a useful molecular marker for cancer diagnosis and drug treatment. DNA adducts are another important collection of covalent modifications resulting from exposure to particular carcinogens. Thus, the level of DNA adducts can serve as a biomarker for a substantial exposure to carcinogens. In addition to being markers of carcinogen exposure, DNA adducts may also directly alter regulation of transcription of tumor suppressors or oncogenes. Thus, establishing, maintaining and modifying DNA modification patterns will deepen our understanding of the regulation and biological significance of these variants (P17, P25).

For example, 6mA modification is the most common type of modification in prokaryotic DNA. However, it did not receive sufficient attention because of its relatively low content in eukaryotes and lack of detection methods (Luo et al., 2015). Until recently, three groups independently described a genome-wide distribution map of 6mA in *Chlamydomonas*, *C. elegans*, and *Drosophila*, which brings enthusiasm to the discovery of 6mA as a new type of epigenetic marker. So far 6mA modification has been commonly found in a variety of genomes from fungi, algae, nematodes, insects, vertebrates and even mammals. However, the specific biological functions of 6mA are still very limitedly understood and will be investigated by projects funded by this MRP (P20), which will gain insight into the mechanism by which 6mA acts as a novel epigenetic marker for dynamic genetic regulation and how it affects gene expression in important biological processes.

New technologies in nucleic acid research

Technology development focused on generating tools to advance nucleic acid-based research for functional genomics and gene-expression analysis, to accelerate drug discovery, and to develop environmental-monitoring and clinical-diagnostic strategies for detecting and identifying low levels of infections to carcinogens. These include nucleic acid purification, detection, amplification and perhaps most inspiringly, manipulation techniques, tools and materials (P9, P18, P40). Nonetheless, the development of new technologies and further developments in nucleic acid chemistry are required.

For example, CRISPR-Cas is emerging as a powerful tool for engineering the genome in diverse organisms (Cong et al., 2013). Site-specific recruitment of epigenetic factors with a modular CRISPR/Cas system is a valuable tool that combines precise recognition of DNA sequences with the capability to modify epigenetic marks. The tolerance of the sgRNAs to chemical modifications has the potential to enable new strategies for genome engineering and the precise mechanistic elucidation of virus-related m^{6}A modification (P27, P75).

Current methods of manipulating RNA methylation are primarily based on modulating the expression of RNA methyltransferases or demethylase via bioengineering methods, which would cause broad epigenetic changes and activation of endogenous retroviruses (Goll and Bestor, 2005; Ooi and Bestor, 2008). However, these methods and reagents demethylate transcriptome globally, and it is difficult to study the effect of the specified RNA modification. Projects funded by this MRP will utilize small organic molecules and

precise chemical transformations to achieve highly efficient, selective and sensitive structural modification of RNA *in vitro* and in cells (P52). This approach has several advantages compared with traditional enzyme modulation, in which dynamic RNA modification could be modulated at different conditions with high efficiency, selectivity and sensitivity. Meanwhile, the CRISPR-Cas13b-based tool will be constructed that targets m^6A methylation of mRNA by fusing the dCas13 endonuclease with m^6A methyltransferases or demethylase (P59). This dCas13b tool with the capacity of targeting specific transcripts of interest will be able to regulate the mRNA translation and degradation.

Chemical probes and imaging methods for in situ biomacromolecule modifications analysis

Fluorescent probes for protein PTMs

Traditional biochemical tools for the analysis of PTMs suffer from drawbacks including the need for tedious manipulation, long detection time, high cost and the inability for the spatiotemporal detection of PTMs in live cells. Consequently, development of advanced chemical tools that could overcome these issues has become a dynamic research topic in recent years. In particular, chemical tools that enable the probing of biomolecular modifications directly at the cellular level with enhanced analytical precision and specificity are urgently needed for the better understanding of how these dynamic activities control cell fate (Ibraheem and Campbell, 2010). This would ultimately help us advance the diagnostic and therapeutic quality of human diseases (P73).

For example, ubiquitination is a dynamic and complex PTM that modulates the structure and function of proteins. Its dysfunction and abnormality are implicated in the initiation and development of many diseases (Radivojac et al., 2010). Projects funded by this MRP proposed a protocol in which high-affinity and selective peptides are screened by the phage-display technique for the targeted recognition of ubiquitin dimerization. The obtained peptide-fluorophore probes will be applied for the *in situ* fluorescence imaging of protein ubiquitination in live cells (P58). Fluorescent probes for other important protein PTMs including phosphorylation (P64), sulfation (P88), lipidation (P78), as well as mercapto-protein-relevant PTM biomarkers (P47) under oxidative stress will also be developed.

Fluorescent probes for epigenetic modifications

The three most well studied epigenetic mechanisms include DNA methylation, RNA regulation and histone modifications, which all regulate gene expression in a highly dynamic and reversible fashion. Imaging probes for detecting the activity of enzymes involved in epigenetic modifications and regulation will be developed, such as probes for DNA methyltransferases (DNMTs) and enhancer of zestehomolog 2 (EZH2, a methyltransferase that trimethylates histone H3 lysine27). This will offer a useful platform technique for the functional delineation of PPIs during the dynamic epigenetic processes (P39).

New bioanalytical methods for precise imaging of the dynamic activities of biomacromolecules

With the rapid advancement of both protein-based and small-molecule fluorescent probes, the development of novel imaging techniques that can improve the sensing precision of chemical probes is of equal importance (Lin et al., 2015). This requires the interdisciplinary cooperation among experts in the research fields of mathematics, biophysics, chemical biology and medical sciences. New biophysical methods such as single-molecule fluorescence microscopy that can better measure the folding, insertion and aggregation of α-synuclein on cell membrane will lay the foundation for the quantitative control and interference of α-synuclein aggregation at the cellular interface before and after PTMs (P7). A four-photon fluorescence imaging technique for the spatiotemporal tracking of α-synuclein biology will also be developed, with the ubiquitination of α-synuclein being probed in a rapid, non-invasive and highly permeable manner (P38).

Meanwhile, the rise of nanotechnology also significantly contributes to the sensitive detection of dynamic biomolecular activities that occur transiently or permanently in live cells. For example, a single-molecule probe based on gold nanorods will be developed for the delineation of the formation mechanism of nanomaterial-based protein corona using the single-particle dark-field imaging technique. The modification of nanorods with proteins in different physiological solutions and the internalization process of the formed protein corona by different cells will be examined, which will provide a new chemical tool for the study of the dynamic modification of nanoparticles by biomacromolecules (P82). Nanoelectrodes based on noble-metal organic frameworks will also be developed for the analysis of bioorthogonal reaction processes in cancer cells with the combination of fluorescence spectroscopy, surface-enhanced Raman spectroscopy and electrochemistry (P30). Finally, the nanopore technique will be optimized for better control of the motion of biomacromolecules that pass through the pore protein, achieving the more precise characterization of biomolecular PTMs and modification site at the single molecule level (P16).

Chemical interventions on biomacromolecule modifications

Chemical probes are selective small-molecule modulators of biomacromolecule functions that allow the user to ask mechanistic and phenotypic questions about their molecular target in biochemical, cell-based or animal studies. The

chemical probe-centered studies are becoming an increasingly interested field in chemical biology (Bunnage et al., 2013). Development and application of high-quality chemical probes significantly promote the cutting-edge studies in both fundamental biology and drug discovery (Arrowsmith et al., 2015).

Over the past decade, high-quality chemical probes have been recognized as a complement tool to traditional genetic approach to elucidate the roles of the targeted proteins in cells and tissues in a rapid and reversible manner. In addition, chemical probes not only monitor the tractability and translatability of given targets by small molecules but also provide lead compounds for drug discovery. The dynamic post-translational modifications such as ubiquitylation, phosphorylation and glycosylation provide key ways to manipulate the biological function of biomacromolecules. Hence, developing strategies and small molecules targeting the enzymes involved in these processes to artificially create, manipulate, intervene or eliminate the function of biomacromolecules will largely benefit the understanding of the biological function of biomacromolecules, especially for those so called "undruggable" and "untargetable" targets in cancer or other human diseases that are hard to be targeted directly by small molecules, such as RAS and MYC, and potentially facilitate drug and therapeutic design against human diseases associated with these post-translational modification processes (P77, P86).

Chemical intervention on dynamic ubiquitylation

Ubiquitylation is one of the most important post-translational modifications on biomacromolecules that normally relies on E1(activating)-E2(conjugating)-E3(ligasing) three enzyme cascades as well as the cofactor ATP to covalently attach single (mono-ubiquitination) or multiple (poly-ubiquitination) ubiquitin molecules specifically to the sidechain of a lysine on the target protein (Greer et al., 2010). Since ubiquitination alters the function and behavior of biomacromolecules significantly, projects funded by this MRP will look into small molecule inhibitors, particularly on E3 ligases such as UBE3A (P79) and LUBAC (P12). Meanwhile, deubiquitinating enzymes can remove the ubiquitin from the target protein, forming a dynamic ubiquitination/deubiquitinating regulation on the function and behavior of target protein, such as cellular localization, binding partner preference or degradation. Small molecule inhibitors will also be developed to target this important family of enzymes such as DUB (P29) and USP7 (P53). Finally, enzymes that mediate ubiquitin-like modifications such as sumoylation, neddylation and non-classical ubiquitination will also be targeted. Small molecule inhibitors for the Cbx4 enzyme responsible for HIF-1α sumoylation (P87), E3 ligase Cullin-RING (CRL) responsible for protein neddylation (P83), SidE/SidJ enzymes responsible for a non-classical ubiquitination/deubiquitination process in a bacteria pathogen *Legionella pneumophila* will also be developed (P13).

Chemical intervention on dynamic phosphorylation

Phosphorylation events mostly occur in numerous cellular signal transduction pathways (Cohen, 2002). Chemical intervention strategies towards many important kinases as well as phosphatases involved in diverse diseases will be pursued, which include the type 1 angiotensin II receptor (AT1R) (P32), Myh9 (the heavy chain of hexamer non-muscle myosin, Myosin IIa) (P69), and the protein phosphatase 2C (PP2C), an essential phosphatase in the ABA (abscisic acid) receptor-mediated signal transduction in plant cells (P51). Moreover, a semi-chemical synthesis strategy to efficiently obtain recombinant phosphorylated proteins such as Metalloproteinase-2 (TIMP-2, a natural metastasis suppressor that inhibits the matrix metalloproteinases MMP) is pursued to study the phosphorylation regulation mechanism (P74).

Chemical intervention on dynamic glycosylation modification

Glycosylation, the covalent attachment of glycosyl carbohydrate group(s) to a hydroxyl or other functional group of residues on the biomacromolecules, dynamically regulates its biofunctions (Moremen et al., 2012). For instance, CD33 is a sialic acid-dependent immunoglobulin-like lectin protein, and has been found to be specifically expressed in mononuclear myeloid immune cells as well as acute myeloid leukemia (AML) cells. The biofunction of CD33 is dynamically regulated by the labeling of sialic acid sugar chain ligands, and subsequently regulates the biological activity of the corresponding cells with unclear signaling pathway and mechanisms. Hence, CD33 has been considered as an important drug target for AML immunotherapy. A DNA-encoded sialic acid derivative library (DEALS) based high-throughput screening technology will be employed to the discovery of small molecules for chemical intervention of the dynamic glycosylation regulation of CD33 (P62).

Chemical intervention on epigenetics

Currently, compelling evidence has demonstrated that epigenetic regulation contributes to cancer progression and small molecules targeting the epigenetic modulators emerge as a novel approach to cancer therapies (Dawson et al., 2012). For example, the discovery of small molecules (JQ1) directly promotes a new field of targeting the BET protein, BRD4, for cancer treatment. Projects supported by this MRP will develop chemical activators of many important epigenetic regulatory enzymes such as SIRT6, a tumor suppressor, which is responsible for deacetylation of histone H3 Nε-acetyl-lysines 9 (H3K9ac) and 56 (H3K56ac). Selective SIRT6 activator can increase the deacetylase activity of SIRT6 via binding to an allosteric site and lead to a global

decrease of key acetylation histone (P11). In addition, as a key RNA demethylation enzyme, FTO dysregulation has been demonstrated to be associated with many diseases and small molecule inhibitors for this important enzyme are highly expected towards the development of intervention of many diseases (P50).

Chemical intervention on viruses

Viral infections cause a diverse range of human diseases through cytopathic effects and virus-induced immunopathology, which remains a severe threat to human health causing significant high mortality. It is also well recognized that several viral infections are closely associated with human cancer (Pettitt et al., 2013). In light of this public health concern, efforts have been made to achieve antiviral intervention through small molecules on targets covering any of the stages of virus life cycle (i.e., attachment, fusion, integration, replication, assembly and release). Projects funded by this MRP will use chemical approaches to precisely modify the viral envelop protein through utilizing unnatural amino acids to clarify the conformation changes during virus-host fusions, and develop small molecules for dynamic and targeted intervention on this process for further antiviral drugs (P1, P45).

Chemical intervention of comprehensive post-modifications

Indeed, the dynamic post-translational modification events mentioned above occur in a highly spatial-temporal organized fashion. Hence, studying the crosstalk regulation between different post-translational modifications is also critical for the understanding of these dynamic events. For example, Human DYRK2 is a dual specificity tyrosine-phosphorylation-regulated kinase 2 involved in the control of mitotic transition and cellular growth and development (Maddika and Chen, 2009; Taira et al., 2007). Moreover, DYRK2 has been found to be also involved in ubiquitylation process as a scaffold for an E3 ubiquitin ligase complex. Small molecule inhibitors against DYRK2 in myeloma will be developed by using structure-based rational drug design. This may link the two major dynamic reversible modifications of protein phosphorylation and ubiquitination, and will provide great significance to explore the intrinsic link between dynamic modification processes and original drug discovery and development (P85).

Parkin regulates mitochondrial autophagy involved in the pathogenesis of Parkinson's disease. The temporal and spatial distribution of the crosstalk of dynamic modifications such as serine/tyrosine phosphorylation and ubiquitination in Parkin under various autophagy induction conditions may help to reveal the effects of Parkin tyrosine phosphorylation on Parkin serine phosphorylation and ubiquitination activity (Ross and Poirier, 2004). Small molecules targeting Parkin can further help to study the chemical processes and regulatory mechanisms of Parkin's dynamic modification, as well as identify new drug targets and candidate small molecules that regulate mitochondrial autophagy (P54).

Finally, the recognition, regulation and chemical intervention on dynamic biomacromolecule modifications can be systematically carried out at the molecular, cellular, tissue and animal levels, with a particular interest on the "untargetable" proteins such as RAS superfamily and autophagy-related proteins. The goal is to discover new regulatory sites and chemical probes targeting these "untargetable" proteins based on their post-translational modifications by using a combination of computational biology, chemical biology, structural biology and pharmacology (Chatterjee and Köhn, 2013). This will provide innovative strategies and models of chemical biology research to overcome the bottleneck problems on these "untargetable" proteins, and enhance the link between dynamic modification regulation studies and molecularly targeted drug discovery (P84).

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SUPPORTING INFORMATION

Table S1 List of research projects that have been funded by the Major Research Project at NSFC

The supporting information is available online at http://life.scichina.com and https://link.springer.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.