国家蛋白质科学中心·北京(凤凰中心)
National Center for Protein Sciences · Beijing
Beijing Proteome Research Center
北京蛋白质组研究中心

第六届中国计算蛋白质组学研讨会（CNCP）

定量蛋白质组学在泛素化修饰检测与功能研究中的应用

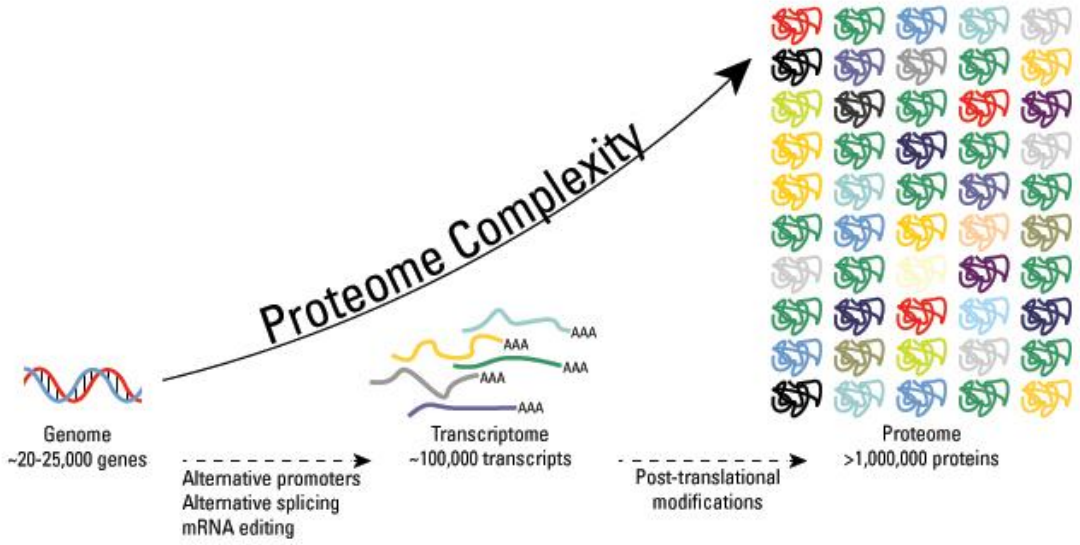
（Quantitative Proteomics for Ubiquitination Detection and Functional Researches）

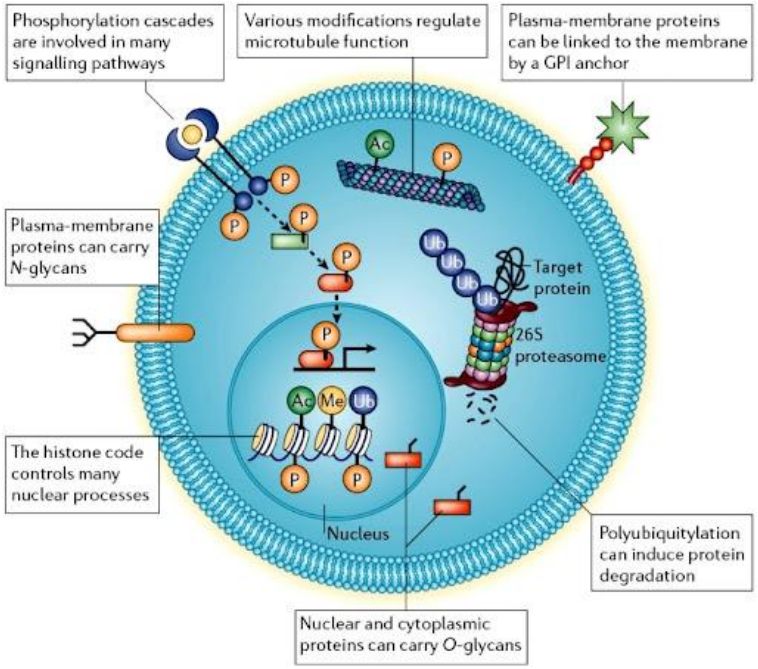
李衍常

国家蛋白质科学中心·北京

2021. 08. 25

蛋白质翻译后修饰（PTM）





More than **500 PTMs** are listed in the Uniprot database - with phosphorylation, lysine acetylation, ubiquitination, and proteolytic processing being the most prominent ones.

https://www.creative-proteomics.com/services/proteomic-analysis-of-post-translational-modifications-service.htm

蛋白质的降解需要能量（ATP）

问题：细胞内蛋白质是如何降解的？

- 1953年，Melvin Simpson利用放射性同位素实验发现了“细胞内蛋白质降解需要能量，即ATP水解反应”的现象。

Review: Nobel Lecture

Ubiquitin at Fox Chase*

I Rose*,[1]

My interest in protein breakdown as a research problem began in 1955 at about the time I joined the Biochemistry Department of Yale University. It was known that proteins break down intracellularly in the mature animal.[1,2] In 1955, I learned from Melvin Simpson about experiments he had published 2 years earlier,[3] showing that a variety of conditions that should lower the ATP level of liver slices (anaerobiosis, cyanide, 2,4-dinitrophenol) decreased the rate of liberation of labeled methionine from protein of rat liver slices. Simpson and I had just joined the Biochemistry Department and we had down the hall labs from each other. Simpson's research goal

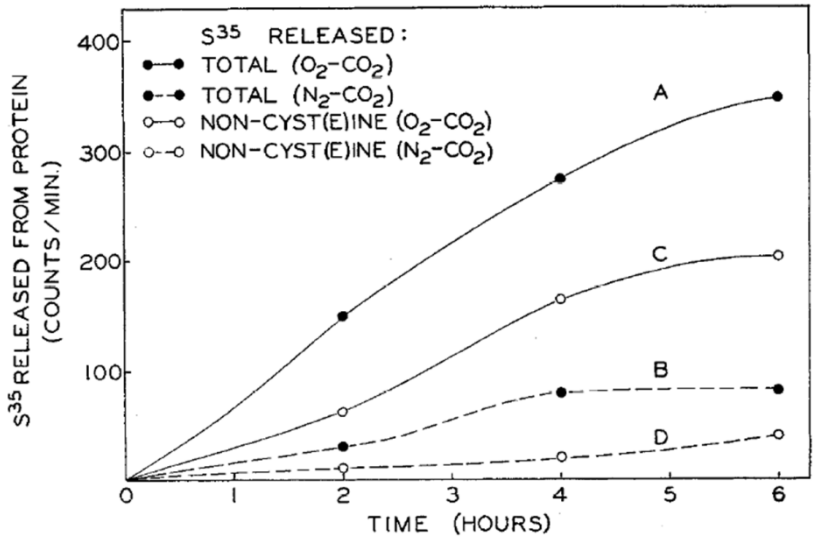


FIG. 1. The effect of anaerobiosis on the release of total S^{35} and of non-cyst(e)ine-S^{35} from the proteins of rat liver slices.

Rat liver

Simpson, M.V. (1953) J. Biol. Chem. 251, 143-154

溶酶体并非唯一的蛋白质降解场所

- 60年代初期，溶酶体被认为是蛋白质的降解场所；但是其非选择性降解方式与蛋白质半衰期千差万别的现象存在矛盾。

Protein Turnover and Lysosome Function

SOME ASPECTS OF THE INTRACELLULAR BREAKDOWN OF EXOGENOUS AND ENDOGENOUS PROTEINS

Brian Poole, Shoji Ohkuma
Michael Warburton

The Rockefeller University
New York

Annual Reviews
www.annualreviews.org/aronline

Table 1 Proteins degraded most rapidly in rat liver

Enzyme	Half-life (hr)[a]
1. Ornithine decarboxylase	0.2
2. δ-Aminolevulinate synthetase	
(soluble)	0.33
(mitochondrial)	1.1
3. RNA polymerase I	1.3
4. Tyrosine aminotransferase	2.0
5. Tryptophan oxygenase	2.5
6. Deoxythymidine kinase	2.6
7. β-Hydroxy-β-methylglutaryl coenzyme-A reductase	3.0
8. Serine dehydratase	4.0
9. Amylase	4.3
10. PEP carboxykinase	5.0
11. Aniline hydroxylase	5.0
12. Glucokinase	12
13. RNA polymerase II	12
14. Dihydrooratase	12
15. Glucose-6-phosphate dehydrogenase	15
16. 3-Phosphoglycerate dehydrogenase	15

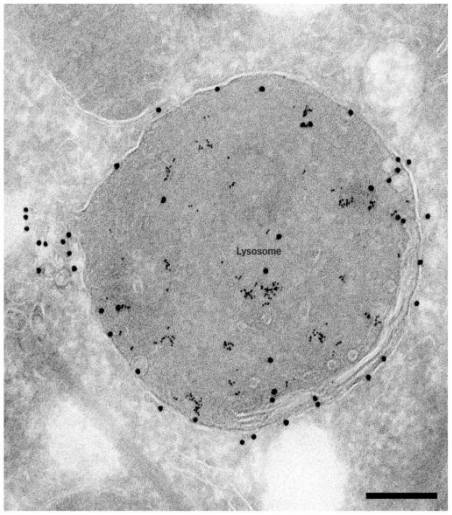
[a] For original references for these values, see (5, 14, 15). [These data for half-lives were obtained by a variety of techniques by different experimenters. Therefore, the precise values may not be always comparable and may be subject to different types of methodological problems (1).]

catalyzes a critical reaction in gluconeogenesis. Similarly glucokinase (34) determines the rate of hepatic glucose uptake, while glucose-6-phosphate dehydrogenase (35) regulates flux through the hexose monophosphate shunt (35). The level of thymidylate kinase (36) controls the salvage pathway for pyrimidine biosynthesis (37, 38), and its level correlates closely with cell growth rate (37, 38). Also included in this group are δ-aminolevulinate synthetase (39), which regulates heme biosynthesis, and hydroxymethylglutaryl CoA reductase (40, 41). This latter enzyme, which limits the rate of cholesterol production, shows diurnal variations and changes markedly in response to dietary influences (42).

Goldberg, A.L., and St. John, A.C. (1976) Annu. Rev. Biochem. 45, 747-803.

溶酶体并非唯一的蛋白质降解场所

- 随着溶酶体抑制剂的开发，经过抑制剂处理的细胞仍然存在恒定的蛋白质降解现象。



Nature Reviews | Molecular Cell Biology

TABLE II. Digestion of Endogenous and Exogenous Macrophage Proteins

Medium	Amount Digested in Two Hours (%)		
	Control cells	Cells fed dead macrophages	
	Endogenous	Endogenous	Exogenous
Control	4.0	2.4	37
Chloroquine 100 μM	3.3 (-17%)	2.3 (-4%)	12 (-68%)

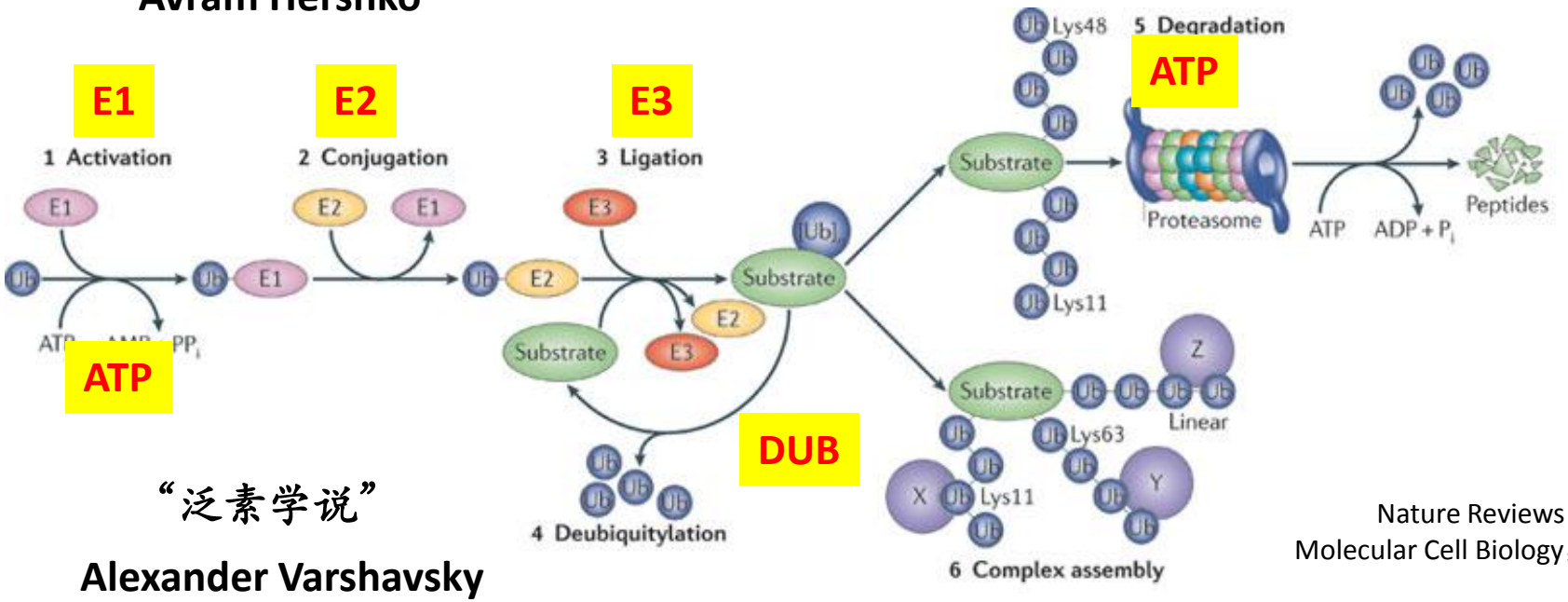
真核细胞内存在另外一种蛋白质降解途径

泛素-蛋白酶体系统

“泛素假说”

Avram Hershko

Alfred L. Goldberg



“泛素学说”

Alexander Varshavsky

Nature Reviews
Molecular Cell Biology，2011

坚实的生物学现象，是发现重要生命规律或理论的基础

2000年，拉斯克基础医学奖

2000 LASKER AWARDS:

2000 Albert Lasker Basic Medical Research Award

Ubiquitin system for regulated protein degradation

Aaron Ciechanover
Technion-Israel Institute of Technology

Avram Hershko
Technion-Israel Institute of Technology

Alexander Varshavsky
California Institute of Technology

2004年，诺贝尔化学奖

The Nobel Prize in Chemistry 2004

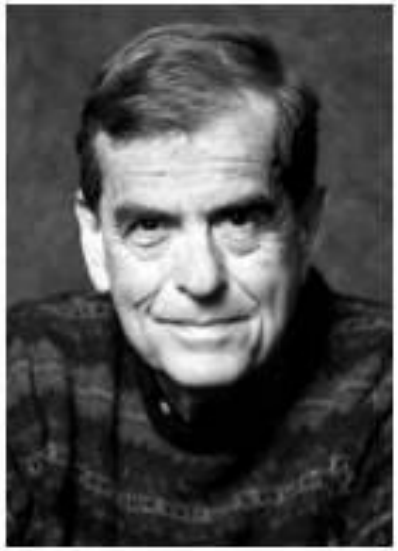


Photo: D. Porges

Aaron Ciechanover

Prize share: 1/3

Photo: D. Porges

Avram Hershko

Prize share: 1/3

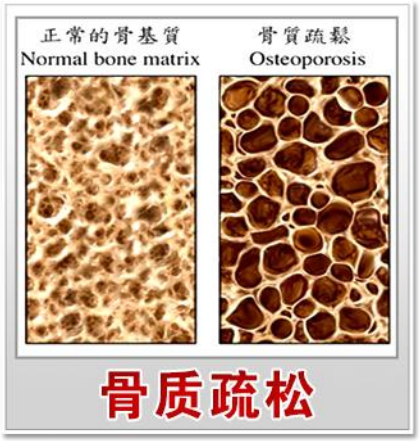
Irwin Rose

Prize share: 1/3

The Nobel Prize in Chemistry 2004 was awarded jointly to Aaron Ciechanover, Avram Hershko and Irwin Rose *"for the discovery of ubiquitin-mediated protein degradation"*.

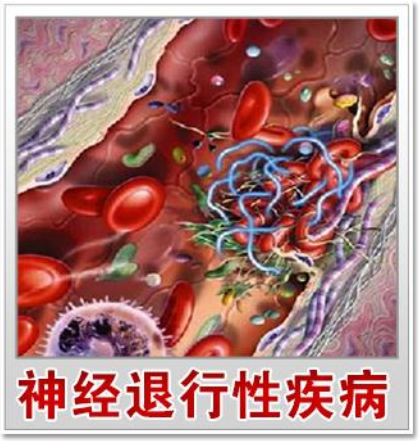
泛素化系统与疾病


肿瘤



骨质疏松

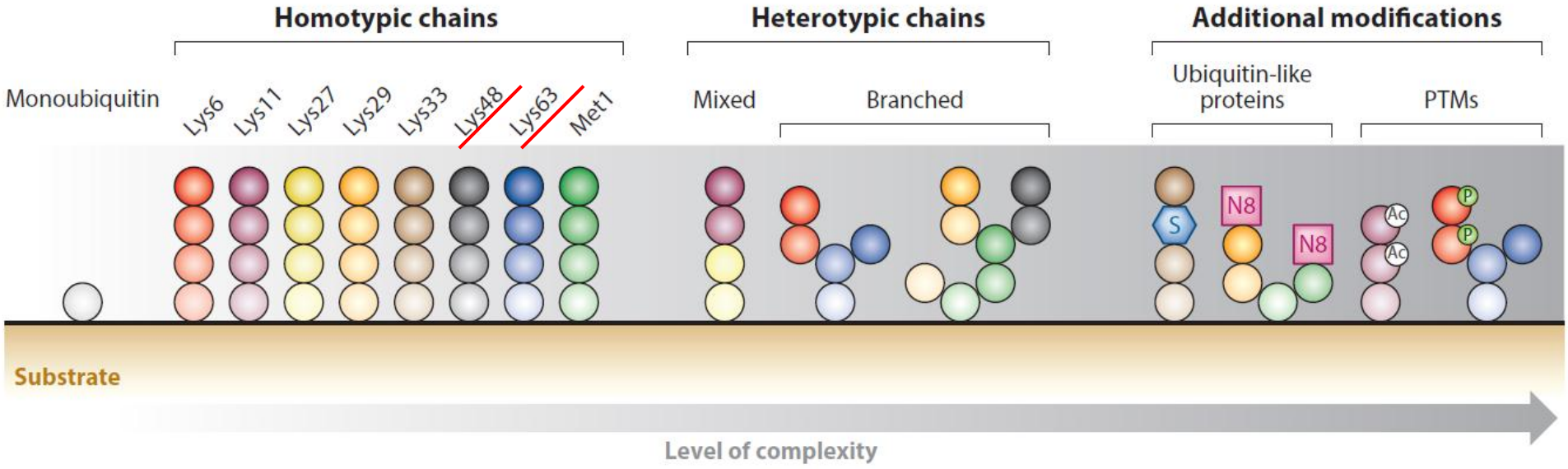

自身免疫病


神经退行性疾病

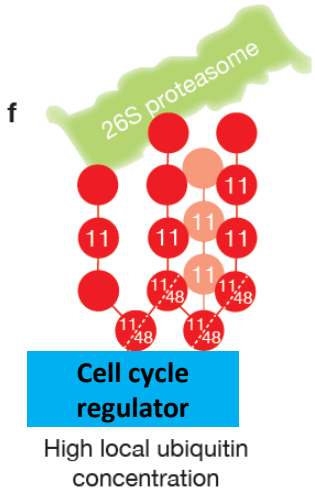
UPS控制细胞内**大多数蛋白质**的稳态调控

Pathway	Deregulated protein	Type of deregulation	Substrate	Modification	Tumour type/disease
Cell cycle	MDM2 **E3**	SNP in the promotor region	p53	Polyubiquitylation	Non-small-cell lung cancer, soft-tissue carcinoma, colorectal cancer
	HAUSP **DUB**	Downregulation	p53, MDM2	De-ubiquitylation	Non-small-cell lung cancer
	SCF (SKP2)	Upregulation of SCF	p27 (KIP)	Polyubiquitylation	Malignant melanoma, lymphoma
	APC		Cyclin B, securin	Polyubiquitylation	Colorectal cancer
DNA repair	FANCL	Defect of PHF9	FANCD2	Monoubiquitylation	Fanconi anaemia-related cancers
NFκB signalling	CYLD **DUB**	Mutation	IKKγ	De-ubiquitylation	Cylindromatosis
	IAP2	Mutation	BCL10	Polyubiquitylation	MALT lymphomas

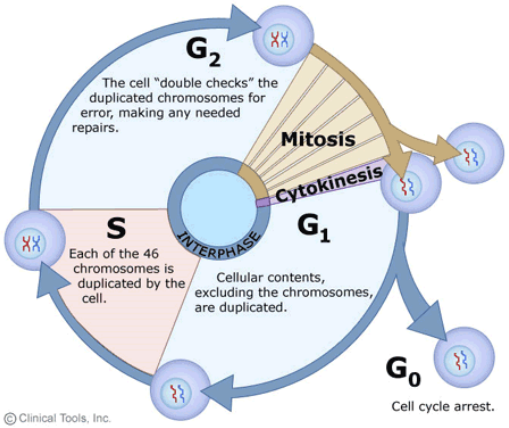
泛素链的复杂性为功能的多样性提供结构基础



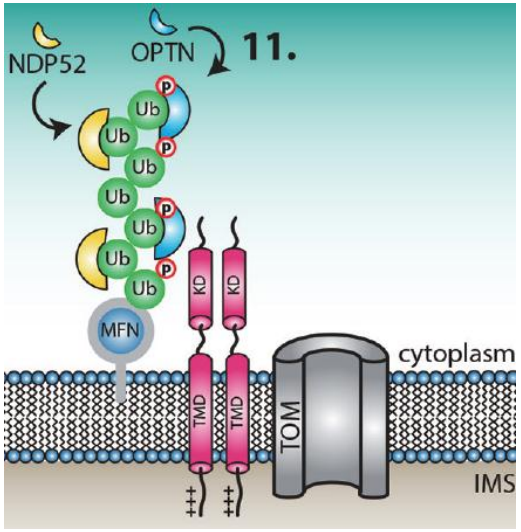
K48/K11 chains



Cell cycle

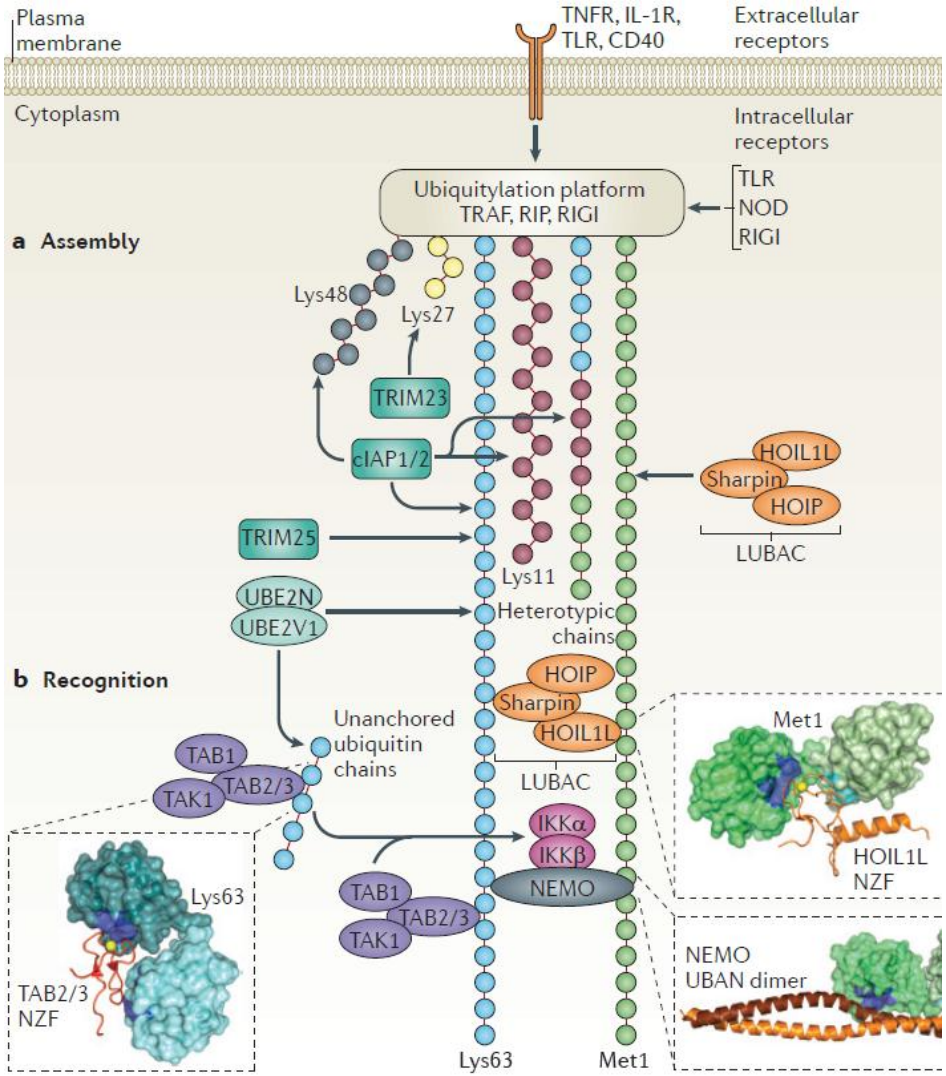


Mitophagy

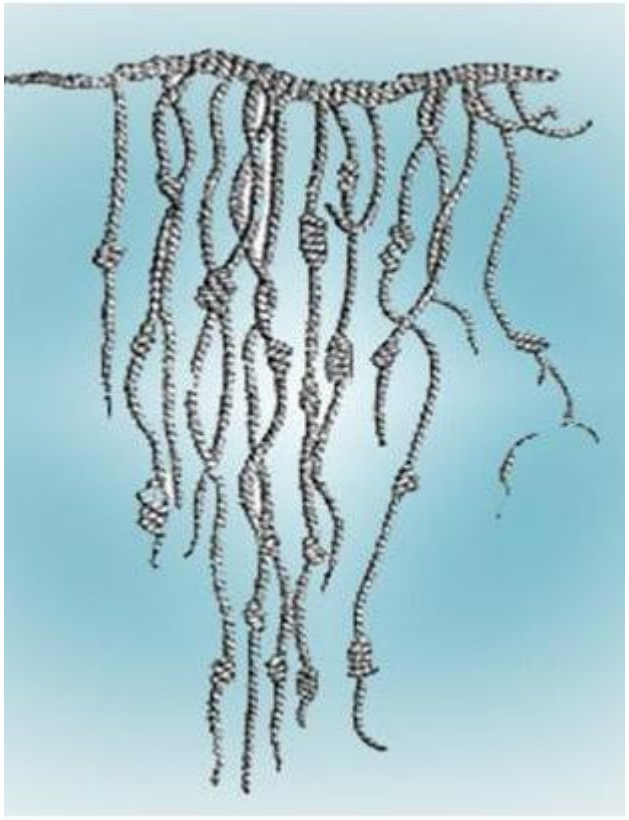


Meyer, H.J. and M. Rape. Cell, 2014.

泛素密码（Ubiquitin code）



上古无文字，结绳以记事。

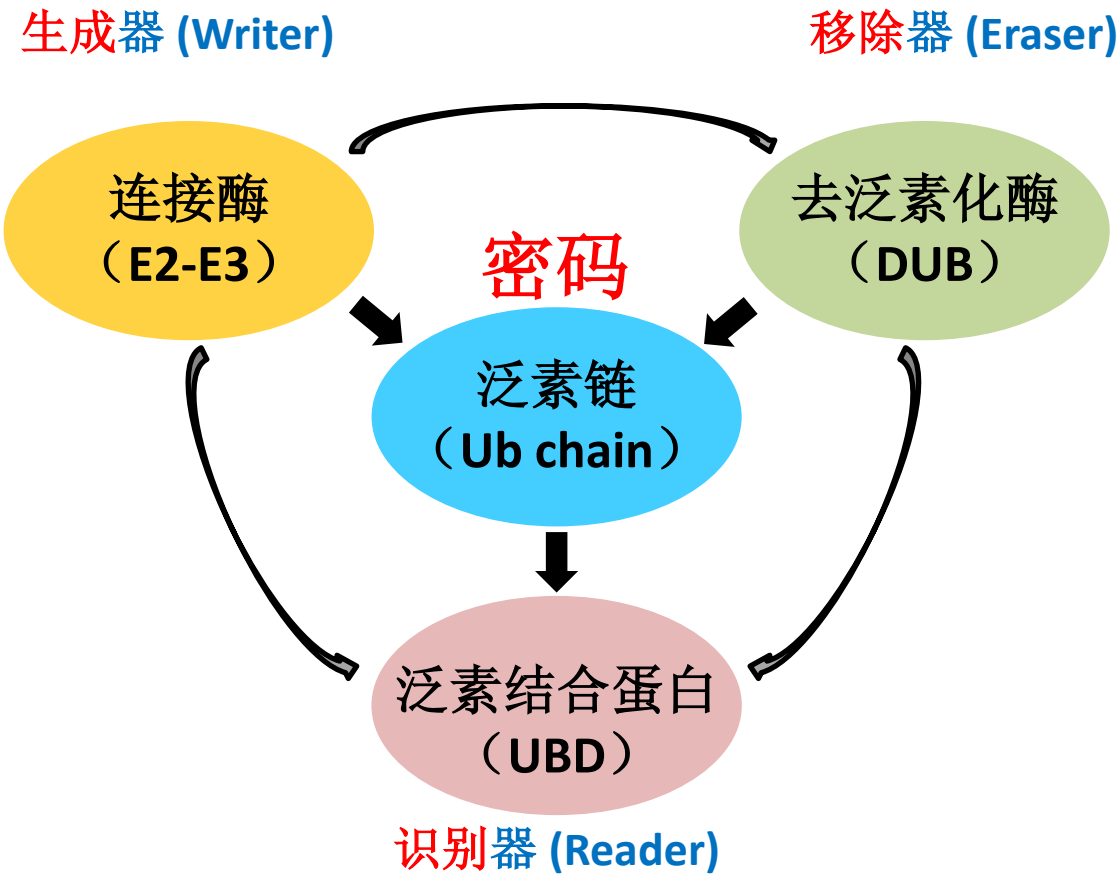


《易．系辞下》：
“上古结绳而治”

古秘鲁人的结绳文字（*Quipus*）

泛素链作为信息载体(messenger)蕴含丰富的生命内涵

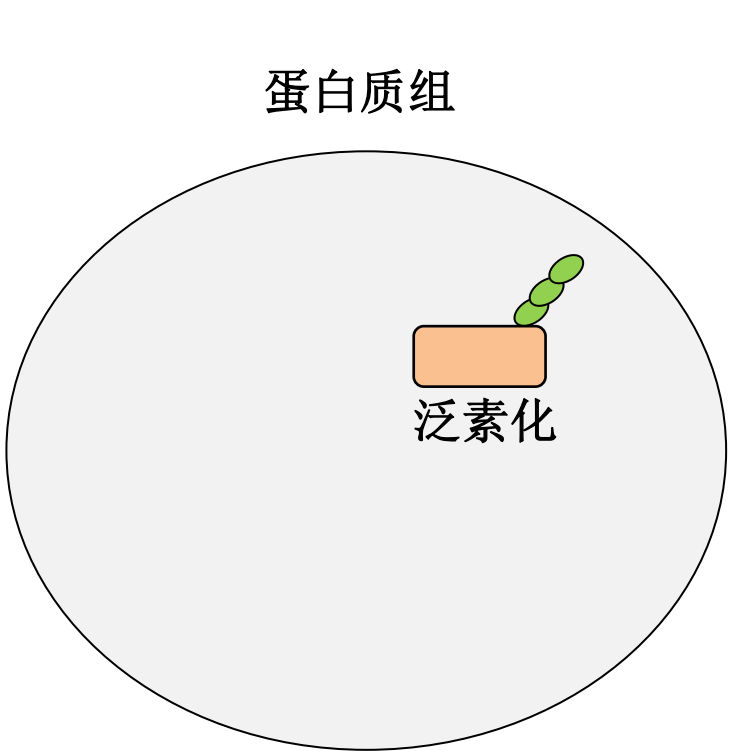
“泛素密码”解析的核心问题



1. 发现泛素链(经典与非经典)的新功能

2. 关键调控酶与泛素链的特异性关系研究

蛋白质组学在泛素化研究中的关键环节



- 丰度低，占蛋白质组的~1%
- 泛素链易被去泛素化酶移除
- 泛素化蛋白易被蛋白酶体降解

富集

检测

调控

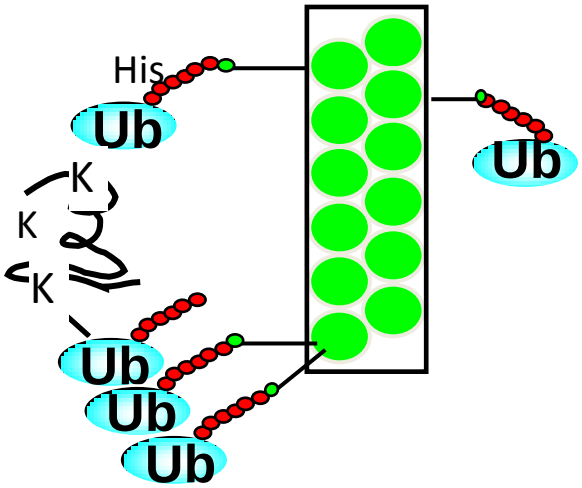
功能

修饰位点

泛素链类型

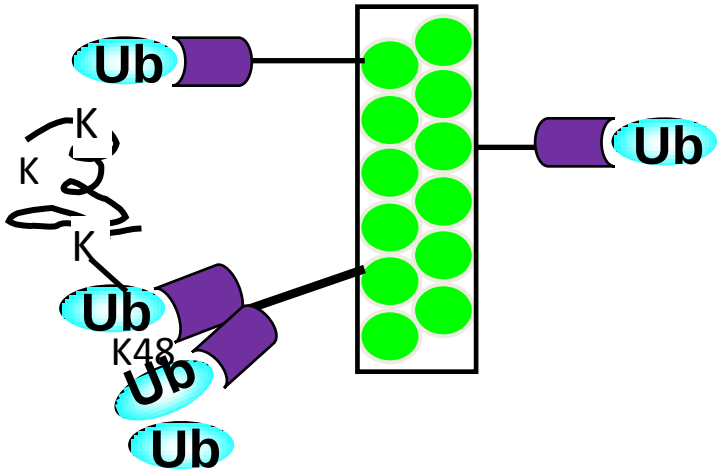
经典的泛素化蛋白富集策略

Tagged Ub

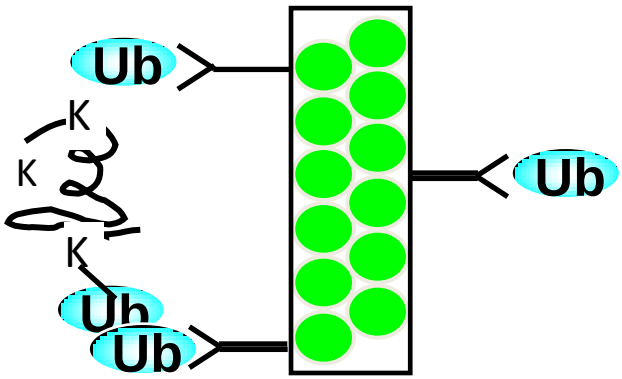


Tag: 6x His, Biotin, Flag, Myc, HA

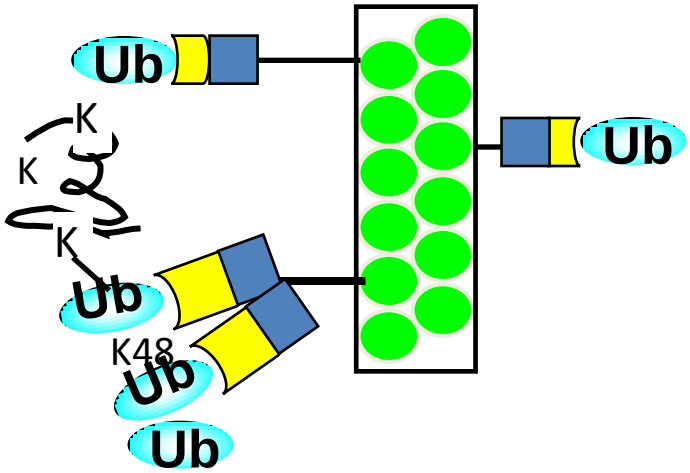
Ub binding protein,Ab



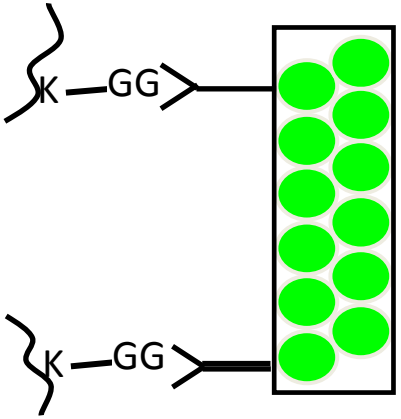
Ub Ab



Ub binding domain (UBD)



K-GG Ab



基于泛素结合结构域（UBD）的富集策略

EMBO reports

scientific report

Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities

Roland Hjerpe[1], Fabienne Aillet[1], Fernando Lopitz-Otsoa[1], Valerie Lang[1], Patrick England[2,3] & Manuel S. Rodriguez[1,4+]

[1]Proteomics Unit, CIC bioGUNE, CIBERehd, Bizkaia Technology Park, Derio, Spain, [2]Institut Pasteur, Plate-forme de Biophysique des Macromolécules et de leurs Interactions, and [3]CNRS, URA2185, Paris, France, and [4]Biochemistry Department, University of the Basque Country, Leioa, Bizkaia, Spain

Post-translational modification with ubiquitin is one of the most important mechanisms in the regulation of protein stability and function. However, the high reversibility of this modification is the main obstacle for the isolation and characterization of ubiquitylated proteins. To overcome this problem, we have developed tandem-repeated ubiquitin-binding entities (TUBEs) based on ubiquitin-associated (UBA) domains. TUBEs recognize tetra-ubiquitin with a markedly higher affinity than single UBA domains, allowing poly-ubiquitylated proteins to be efficiently purified from cell extracts in native conditions. More significant is the fact that TUBEs protect poly-ubiquitin-conjugated proteins, such as p53 and IκBα, both from proteasomal degradation and de-ubiquitylating activity present in cell extracts, as well as from existing proteasome and cysteine protease inhibitors. Therefore, these new 'molecular traps' should become valuable tools for purifying endogenous poly-ubiquitylated proteins, thus contributing to a better characterization of many essential functions regulated by these post-translational modifications.

Keywords: purification; ubiquitin; proteasome; UBA; DUBs

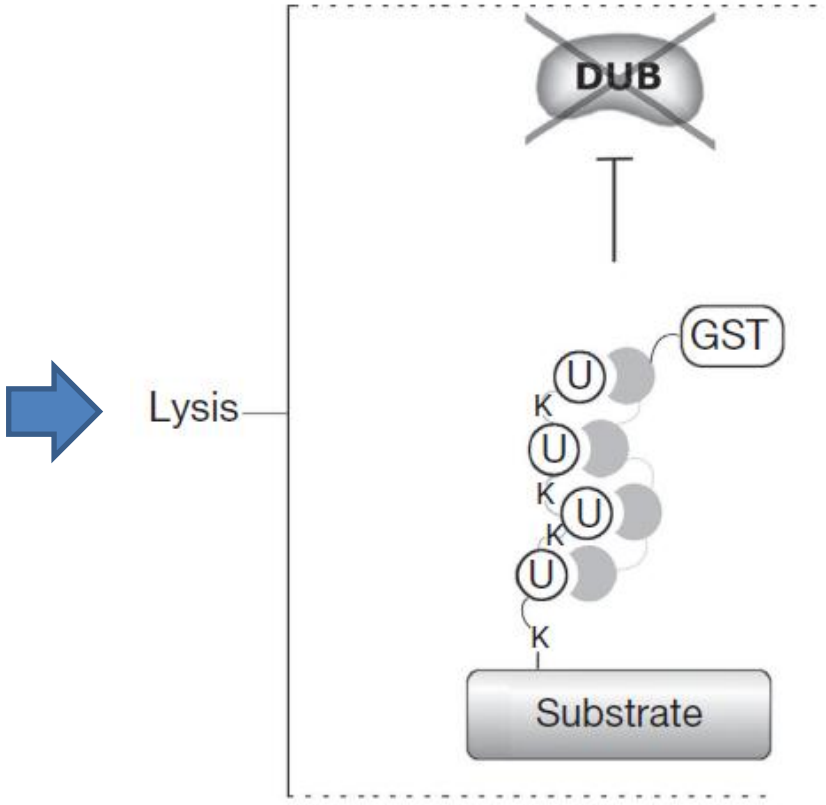
EMBO reports (2009) 10, 1250–1258. doi:10.1038/embor.2009.192

INTRODUCTION

Post-translational modification offers immense possibilities for proteins to modulate their properties. In addition to a variety of (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase).

Substrate proteins might be modified by a single ubiquitin at one acceptor site (mono-ubiquitylation), by several ubiquitins at different acceptor sites (multiple mono-ubiquitylation) or by poly-ubiquitin chains resulting from the modification of ubiquitin on any of its seven lysines. In general, chains linked through lysine 48 (Lys 48) are considered to mediate proteasomal degradation, whereas those coupled through lysine 63 (Lys 63) are involved in diverse processes such as signal transduction and DNA repair (Ikeda & Dikic, 2008). Further complexity arises from the existence of forked ubiquitin chains (Tagwerker *et al*, 2006) and heterogeneous chains incorporating ubiquitin-like (Ubl) molecules (Tatham *et al*, 2008). Similarly to phosphorylation, ubiquitylation is a reversible process, as de-ubiquitylating enzymes (DUBs) continuously de-conjugate ubiquitylated substrates by different catalytic mechanisms (Nijman *et al*, 2005). However, most known DUBs are cysteine proteases.

Various strategies have been used to analyse the ubiquitylated proteome—or 'ubiquitome' (Hjerpe & Rodriguez, 2008b). However, both in studies spanning the entire ubiquitome and in those focusing on a single protein, reliable detection or characterization of protein ubiquitylation is hampered by the de-conjugation mediated by DUBs. A well-known solution to this

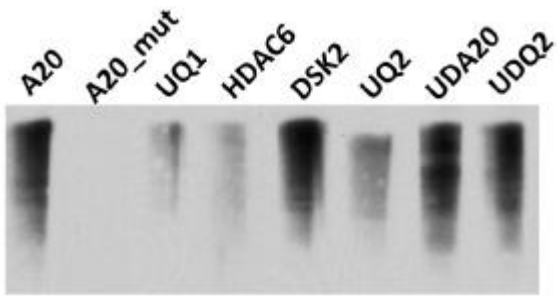


Roland Hjerpe, et al. EMBO Reports,2009.

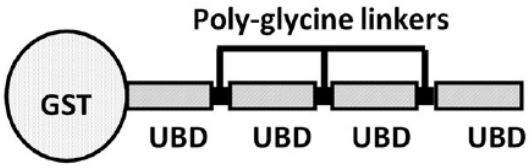
UBD 能够保护Ub chains不被DUB切割

串联杂合UBD（ThUBD）高效富集泛素化蛋白

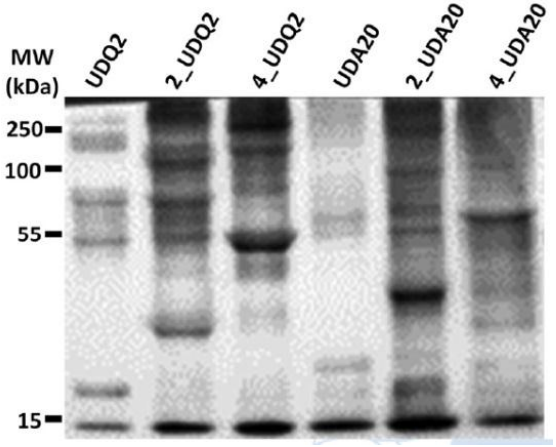
A



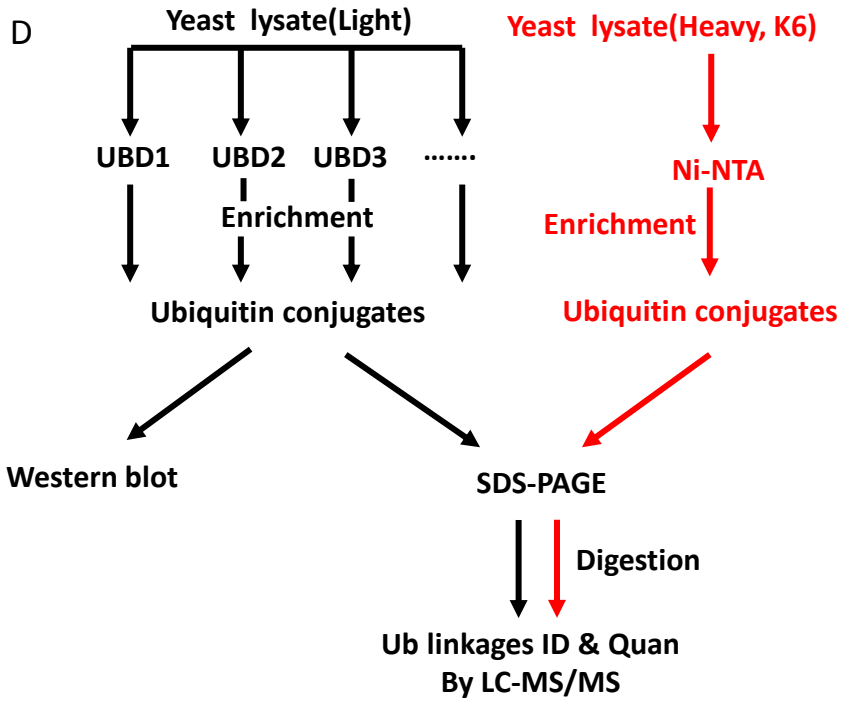
B



C



D



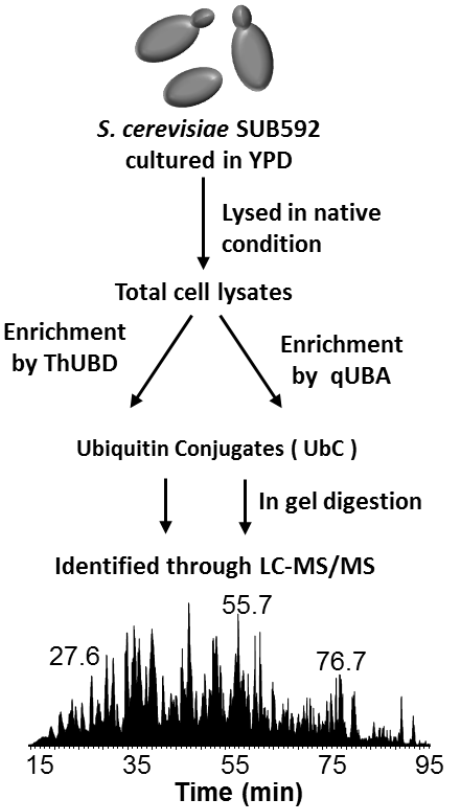
E

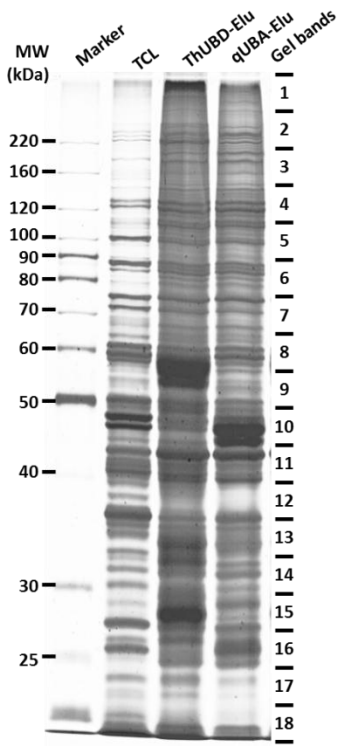
Ubiquitin chain enriched by different Ubiquitin binding domains[a]

	K6	K11	K27	K29	K33	K48	K63
Ni_denature	1.0±0.05	1.0±0.12	1.0±0.13	1.0±0.07	1.0±0.19	1.0±0.05	1.0±0.06
A20	1.2±0.12	2.0±0.08	1.7±0.46	0.8±0.08	1.2±0.33	2.3±0.10	2.6±0.08[b]
A20_mut	ND[c]	1.1±0.18	1.3±0.26	0.2±0.01	ND	0.5±0.02	ND
UQ1	1.8±0.19	3.2±0.36	0.9±0.03	0.6±0.08	1.2±0.29	0.6±0.01	0.8±0.03
HDAC6	0.4±0.13	1.1±0.21	0.6±0.07	ND	0.5±0.12	0.6±0.03	1.8±0.14
DSK2	2.0±0.32	1.3±0.05	2.3±0.14	1.2±0.50	2.7±0.61	2.5±0.20	1.6±0.14
UQ2	1.6±0.32	3.0±0.17	1.6±0.15	0.4±0.03	1.8±0.28	0.9±0.02	2.0±0.12
UDA20	2.6±0.25	3.3±0.25	2.4±0.04	1.4±0.26	2.9±0.45	2.7±0.10	2.9±0.18
UDQ2	1.0±0.07	1.1±0.17	1.3±0.12	0.7±0.07	1.6±0.43	1.2±0.06	1.8±0.05

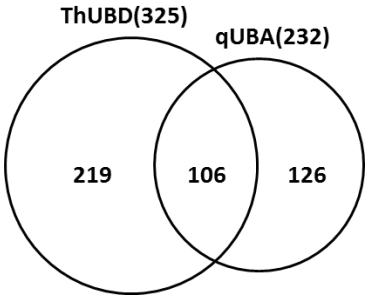
串联杂合UBD（ThUBD）高效富集泛素化蛋白

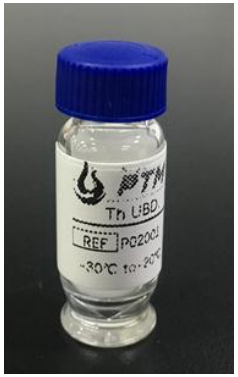
A





B

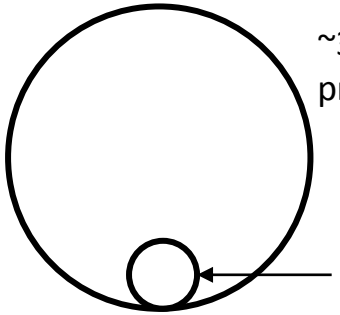




F1000Prime RECOMMENDED

Michele Pagano,
New York University,
Chair, Biochemistry

Gao Yuan*, Li Yanchang*, *et al*, **Mol Cell Proteomics**,2016.



~3000s ubiquitinated proteins

~200s modified sites

基于泛素化蛋白层面的富集，能否提高修饰位点的鉴定能力？

基于镜像酶的泛素化修饰位点高效鉴定技术

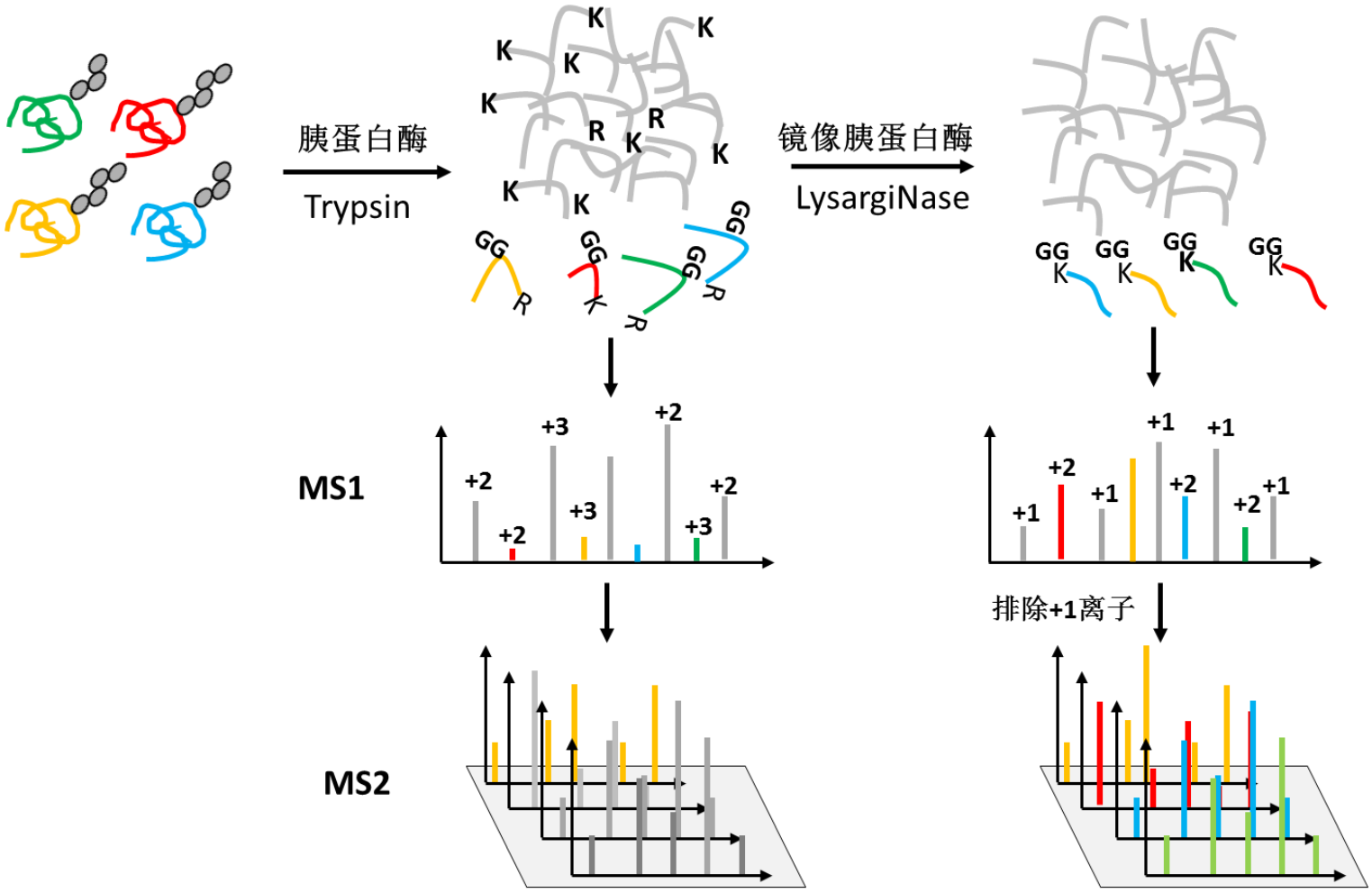
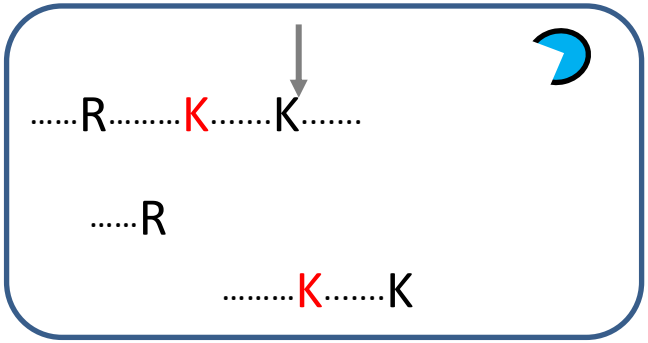


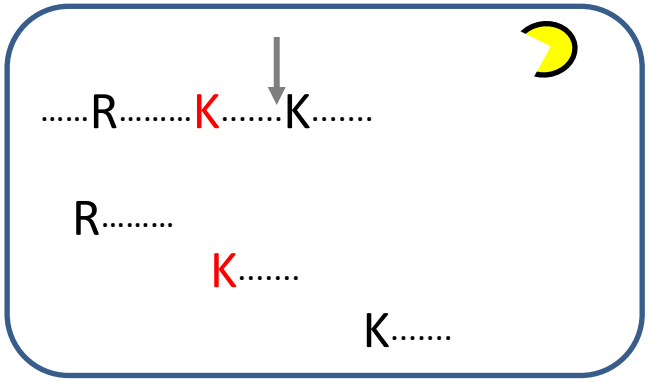
图1. 胰蛋白酶及其镜像酶高效鉴定泛素化修饰位点示意图

高效稳定的胰蛋白酶镜像酶

Trypsin digestion



LysargiNase digestion



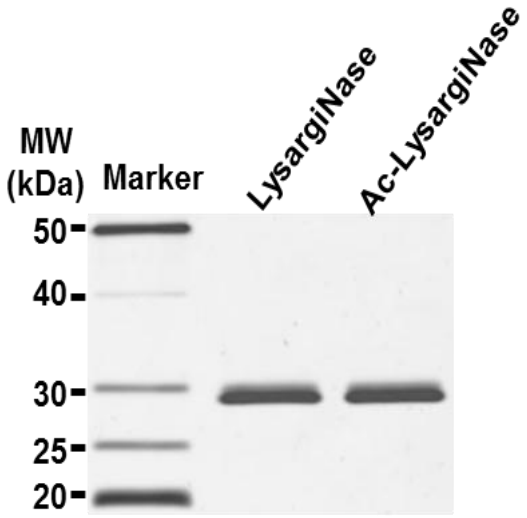
Trypsin

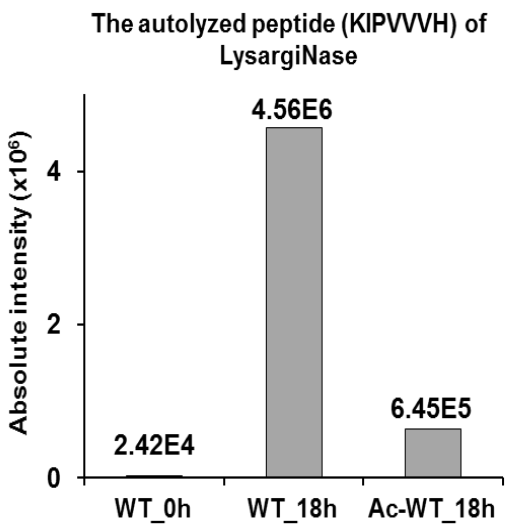
LysargiNase

K methylated lysine

K un-modified lysine

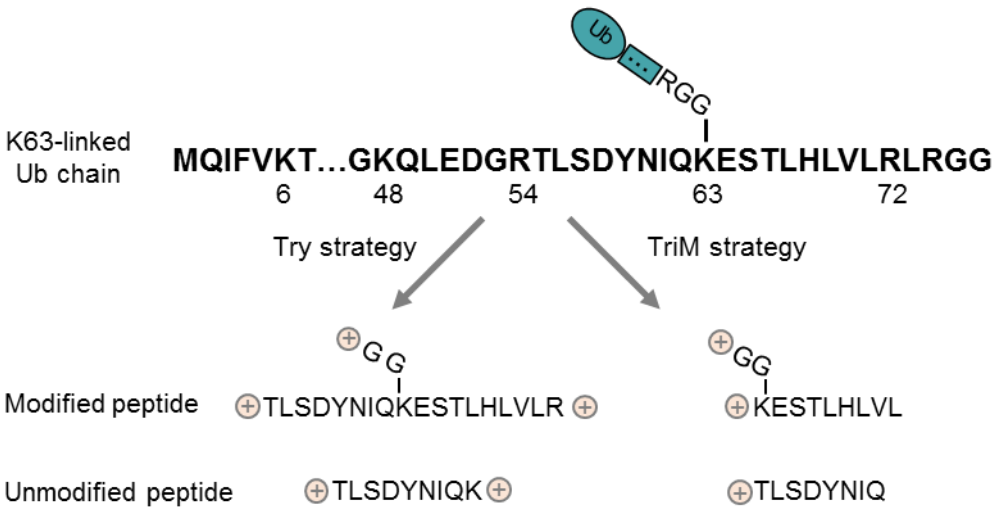
Huesgen *et al. Nat Methods. 2015* .
Tallant, C., *et al. J Biol Chem*, 2006.

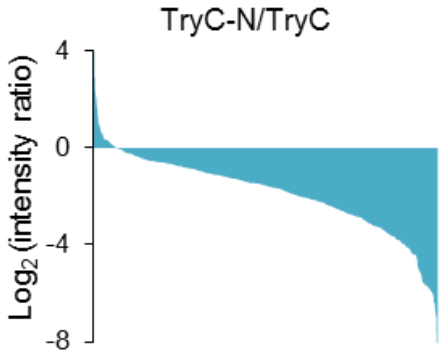


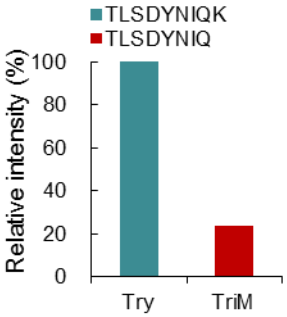


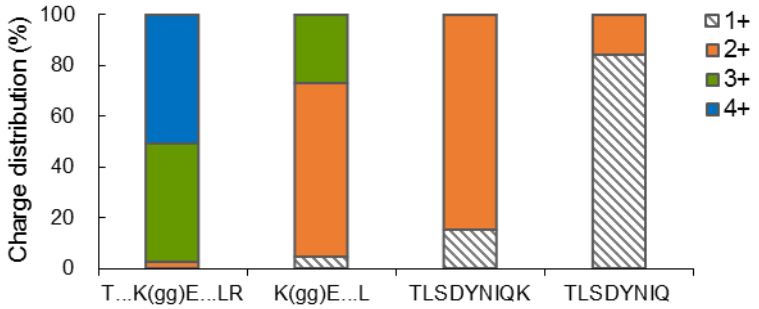
Yang Hao#, Li Yanchang #, et al., ***Mol Cell Proteomics,*** 2019 (#共一, Highlight).

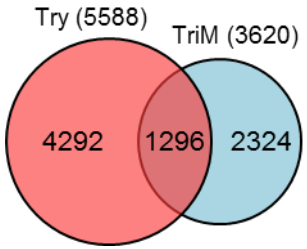
串联镜像酶切策略有效降低电荷和非修饰干扰

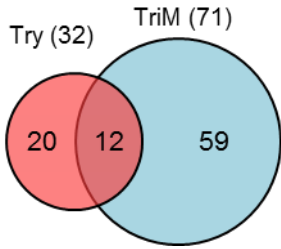








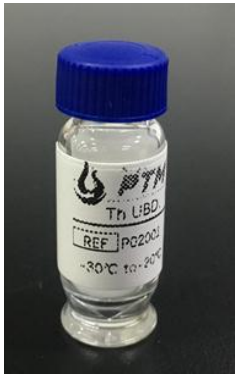


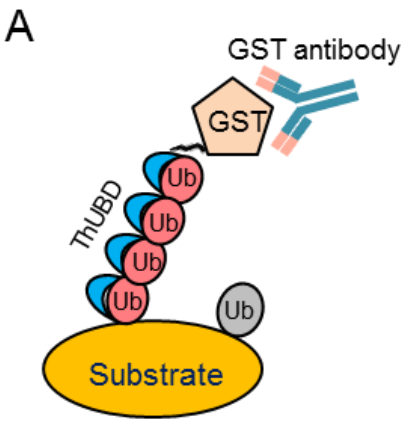


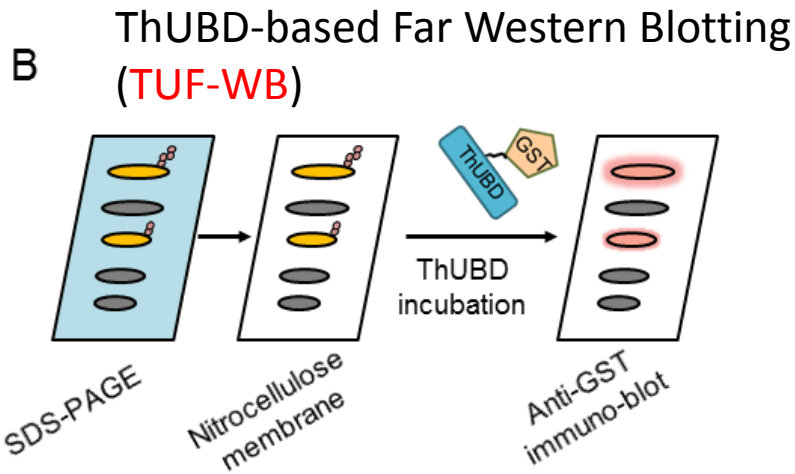
泛素化位点鉴定效率提升2–3倍

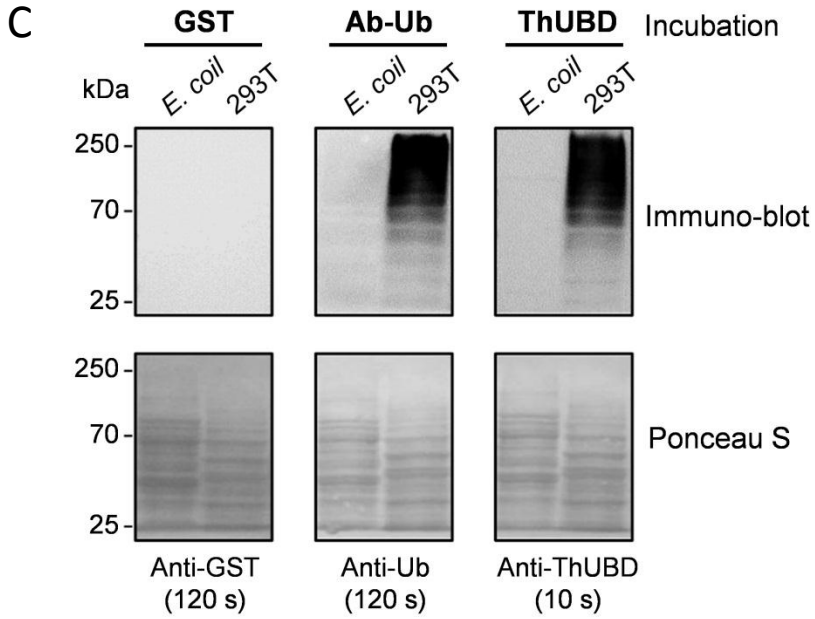
Xiao Weidi, …, Li Yanchang* and Xu Ping*, ***Anal Chem,*** 2019.

串联杂合UBD高效无偏性泛素化信号检测技术

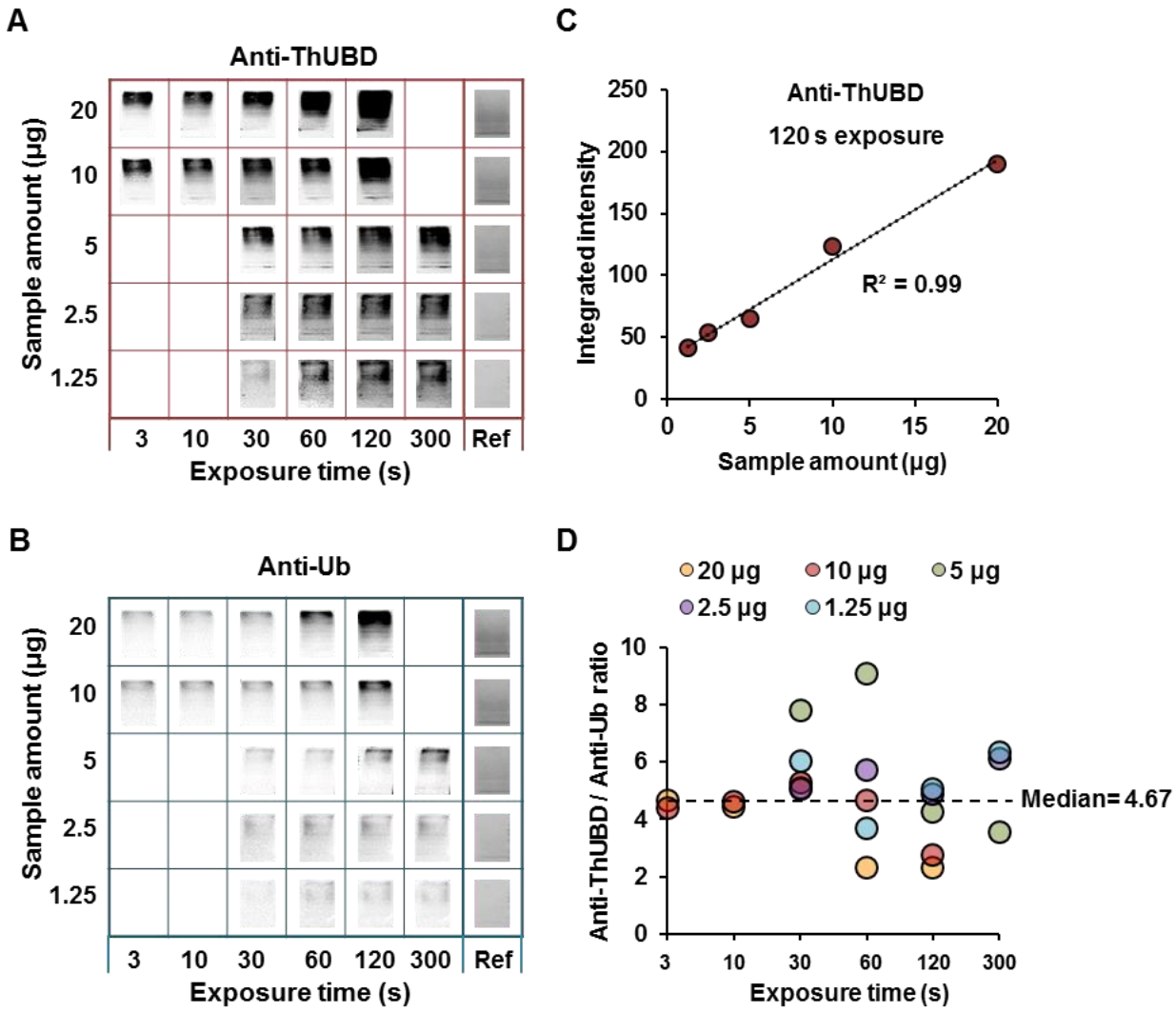




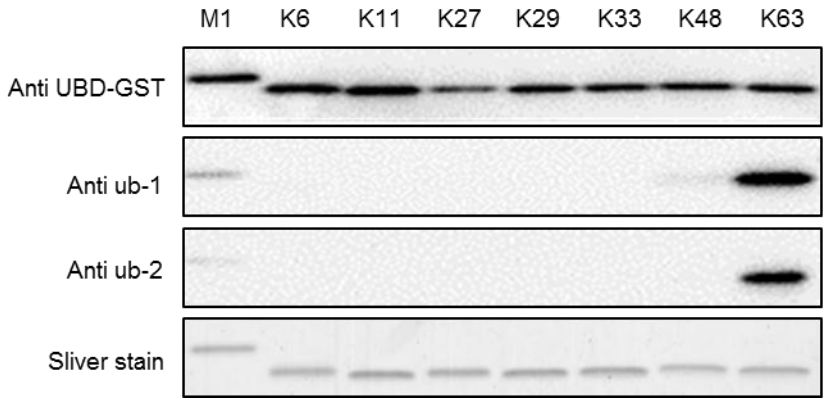


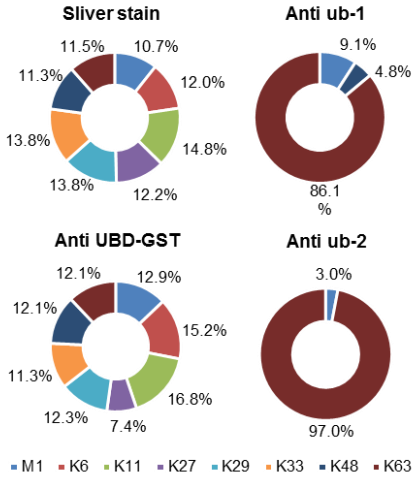


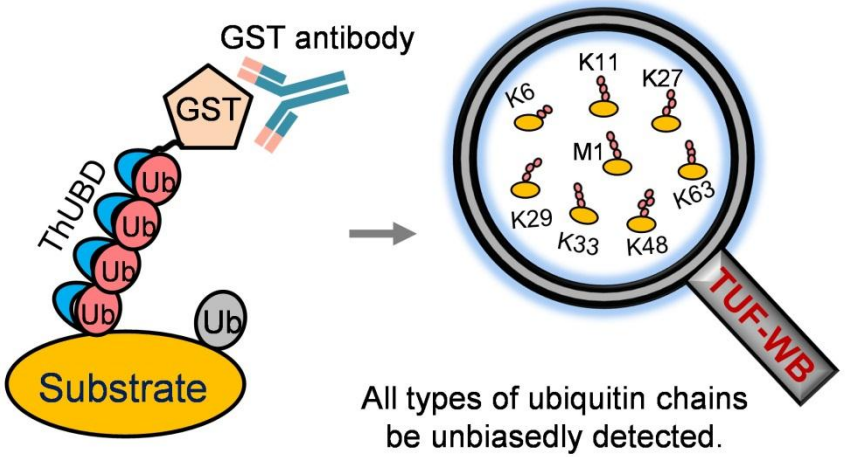
TUF-WB相比抗体法灵敏度提升近5倍



串联杂合UBD高效无偏性泛素化信号检测技术







TUF-WB技术实现对泛素链的高灵敏、无偏性检测

Xiao Weidi, …, Li Yanchang* and Xu Ping*, ***Anal Chem***, 2020.

高灵敏泛素化信号检测探针技术路线

①

辣根过氧化物酶（HRP）
标记ThUBD

②

荧光素（Flu）
标记ThUBD

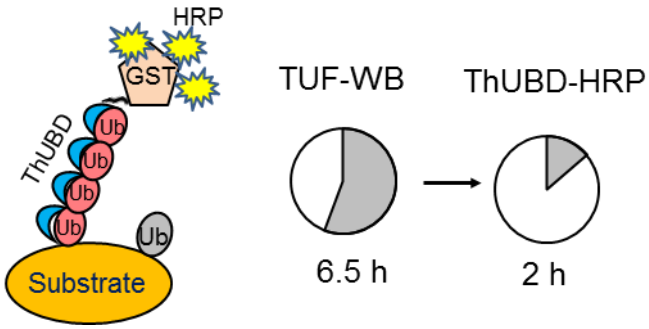
检测标记效率

检测方式：
化学发光

检测方式：
荧光（488nm，绿色）

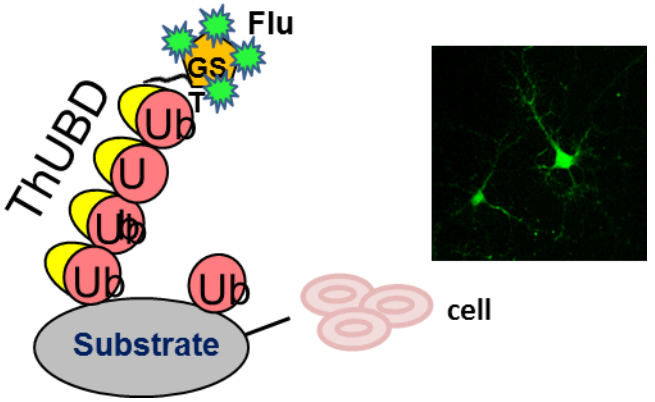
检测特异性、灵敏度

简便、灵敏，提升10倍以上，
免疫组化



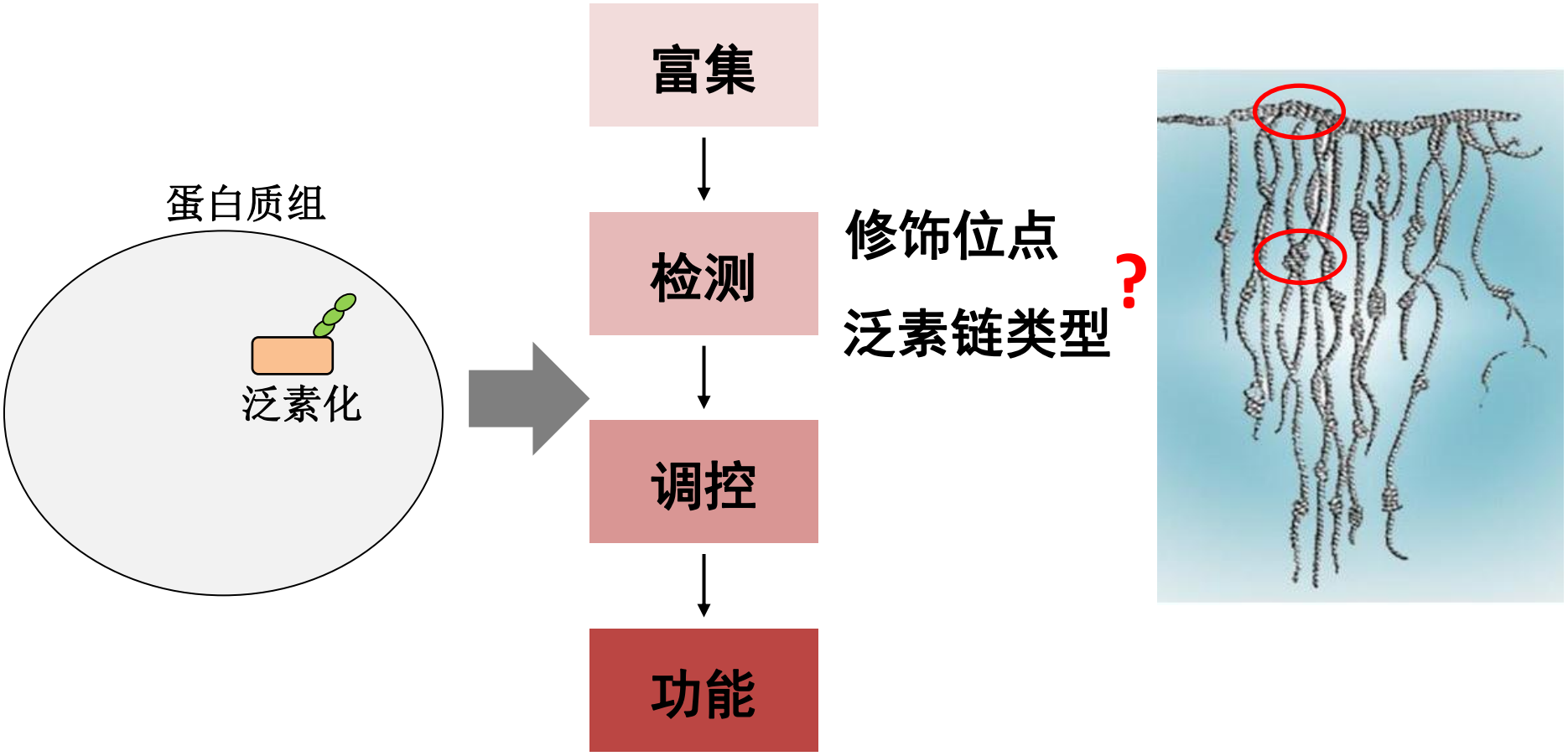
(Prepared)

简便、灵敏，细胞原位



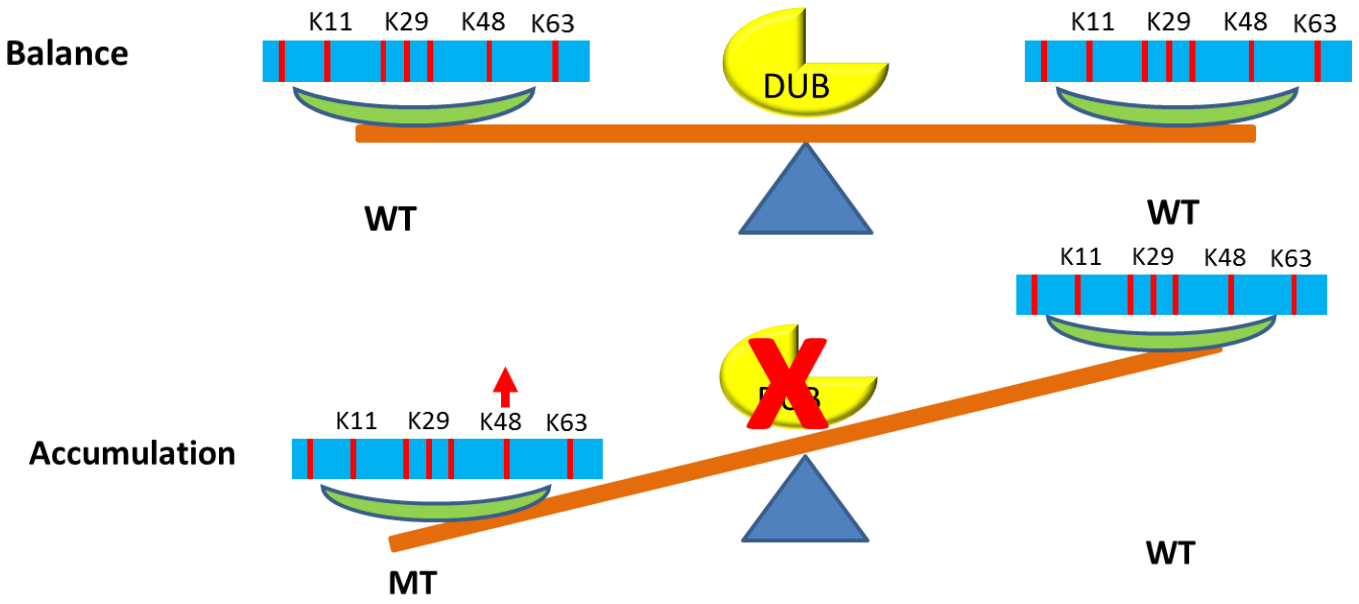
(Prepared)

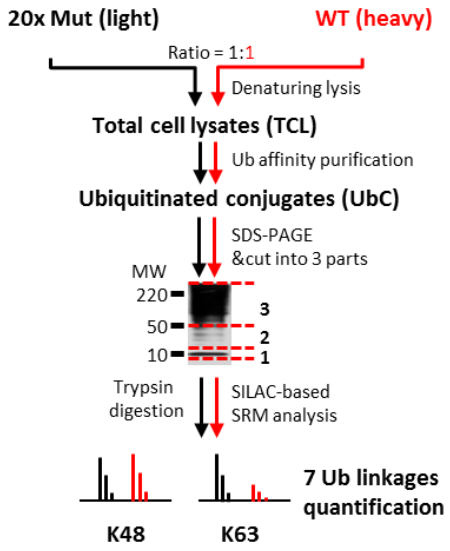
泛素化领域的挑战

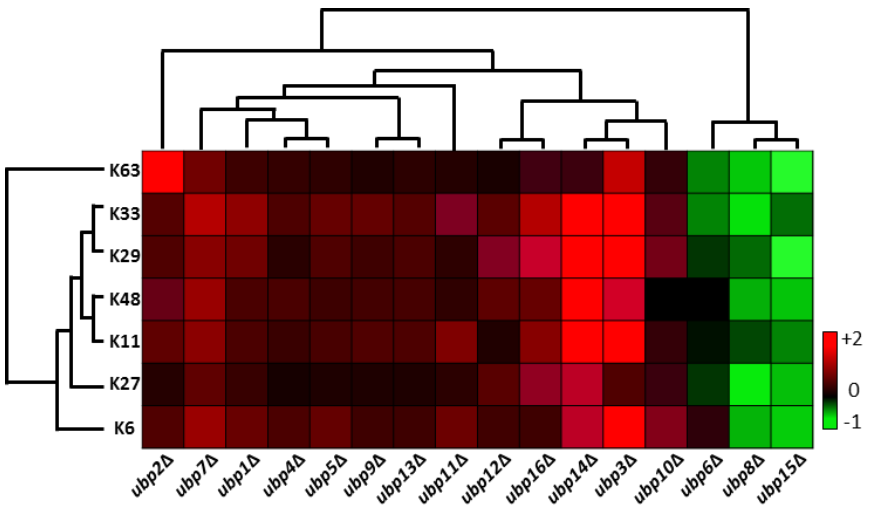


能否同时确定：“泛素链-底物修饰位点-调控酶-功能”？

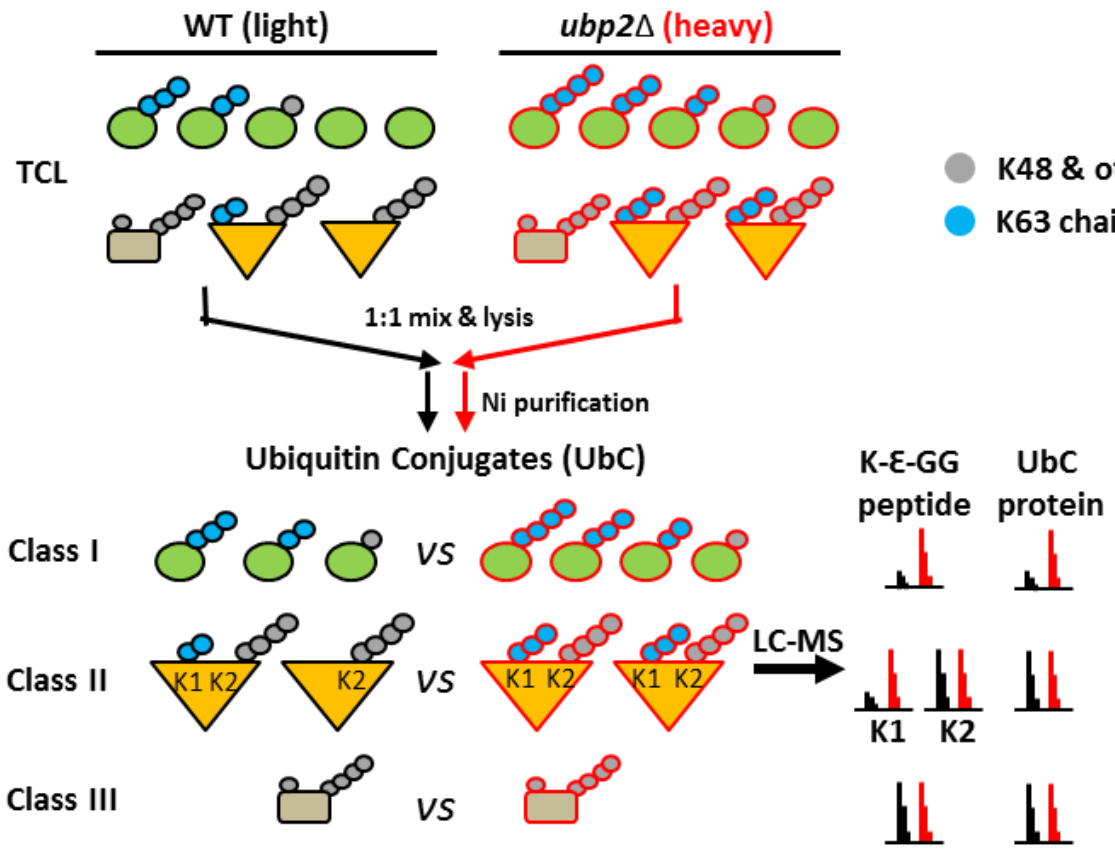
体内研究不同DUB对不同泛素链偏好性的策略





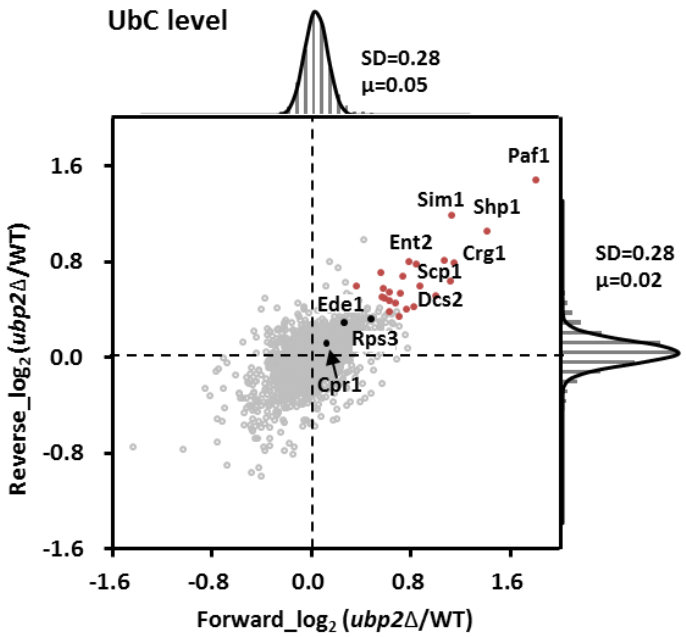


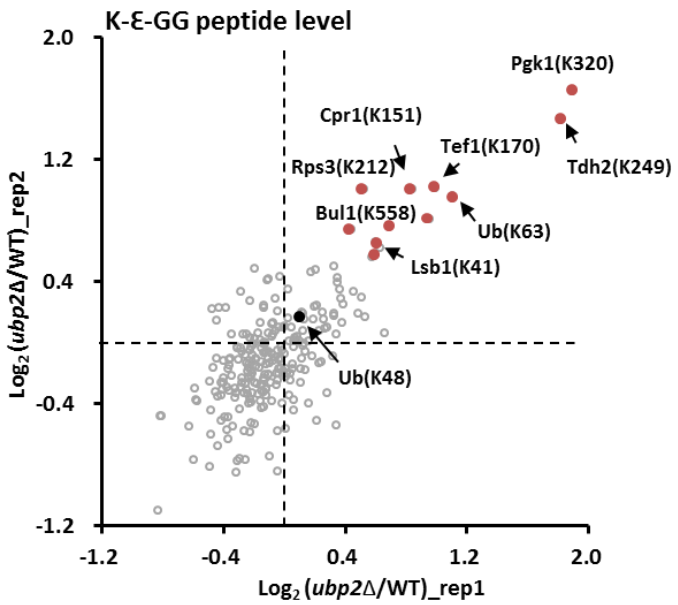
“泛素链-修饰位点-调控酶-功能”一体化研究策略

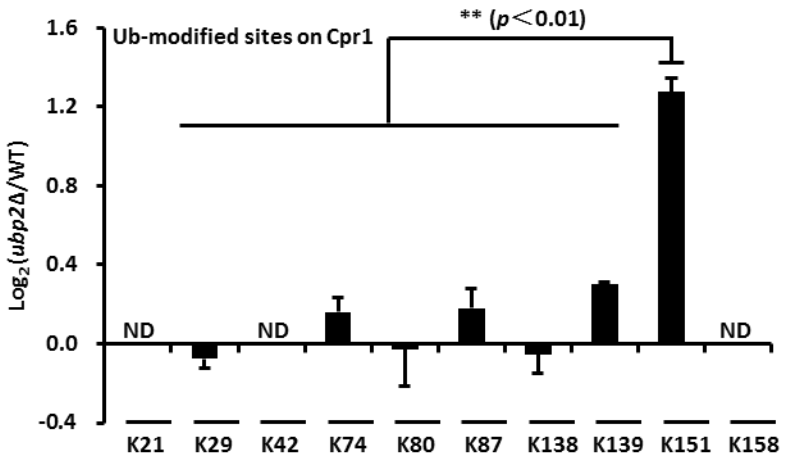


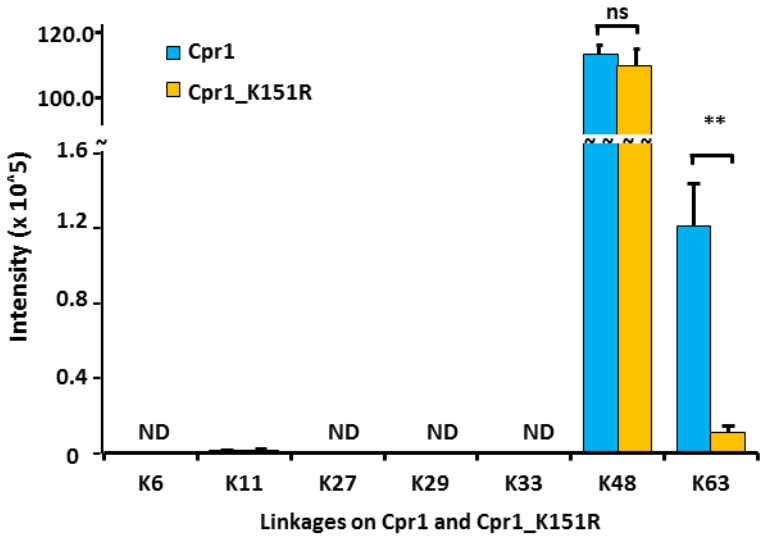
Class	UbC level	K-ε-GG site	Ubp2 regulated
I	+	–	Yes
	+	+	Yes
II	–	+	Yes
III	–	–	No

“修饰位点”精确筛选

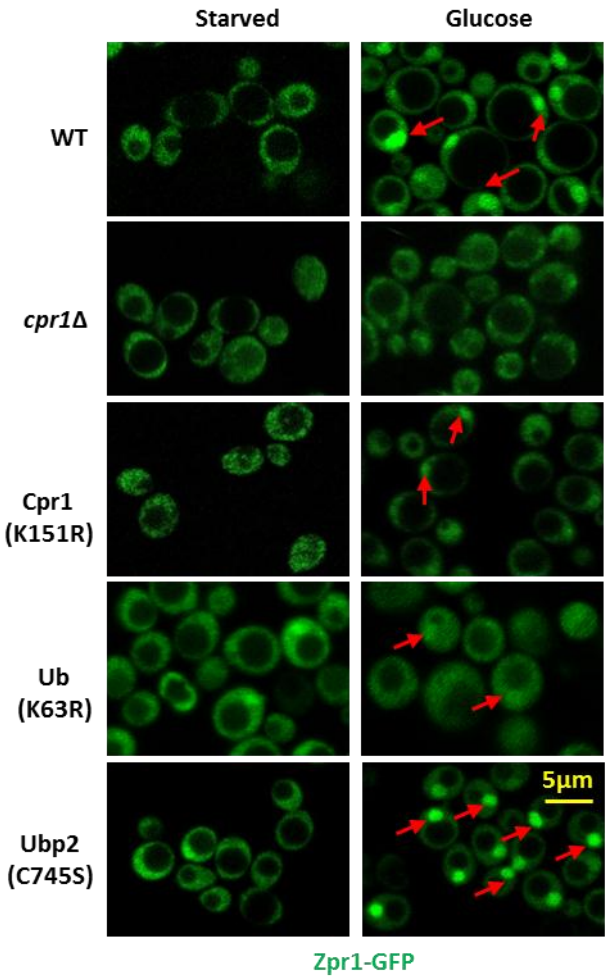

UbC level
SD=0.28
μ=0.05
SD=0.28
μ=0.02
Paf1
Sim1
Shp1
Ent2
Crg1
Scp1
Dts2
Ede1
Rps3
Cpr1
Reverse_log2 (ubp2Δ/WT)
Forward_log2 (ubp2Δ/WT)
1.6
0.8
0.0
-0.8
-1.6

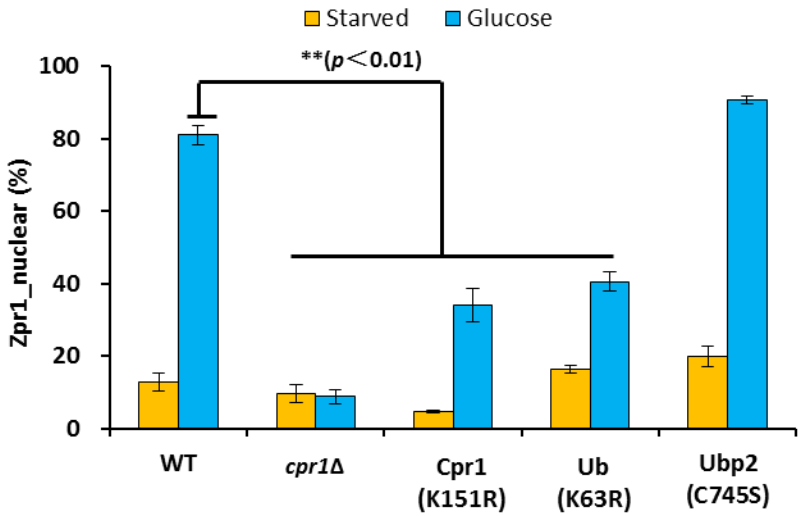

K-Ɛ-GG peptide level
Pgk1(K320)
Cpr1(K151)
Tef1(K170)
Tdh2(K249)
Rps3(K212)
Bul1(K558)
Ub(K63)
Lsb1(K41)
Ub(K48)
Log2 (ubp2Δ/WT)_rep2
Log2 (ubp2Δ/WT)_rep1
2.0
1.2
0.4
-0.4
-1.2

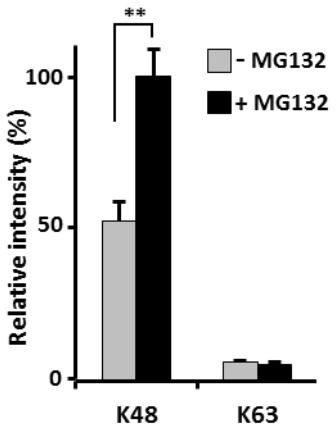

Ub-modified sites on Cpr1
** (p＜0.01)
Log2 (ubp2Δ/WT)
ND
K21
K29
K42
K74
K80
K87
K138
K139
K151
K158
1.6
1.2
0.8
0.4
0.0
-0.4

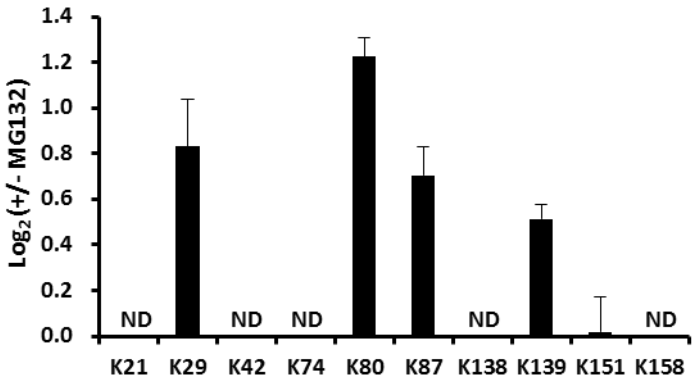

Cpr1
Cpr1_K151R
ns
**
Intensity (x 10^5)
120.0
100.0
1.6
1.2
0.8
0.4
0
ND
K6
K11
K27
K29
K33
K48
K63
Linkages on Cpr1 and Cpr1_K151R

“修饰位点”上泛素链功能的研究

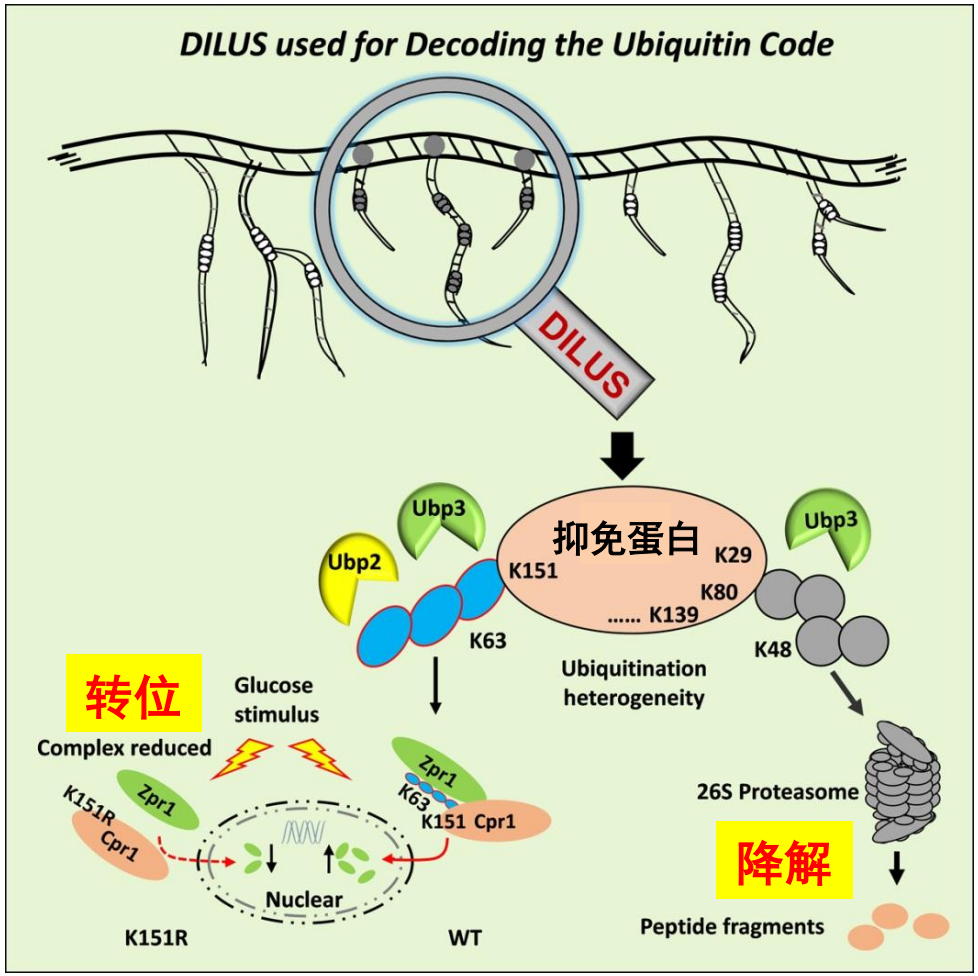








去泛素化酶与泛素链特异性关系研究



技术角度：

发展的DILUS为“泛素密码”解析提供一体化研究策略

生物学角度：

证明底物不同位点发生不同泛素链的修饰，受到特定去泛素化酶的精确调控，介导不同的生物学功能

Li Yanchang#, Lan Qiuyan#, et al., **iScience**，2020.

围绕“泛素密码”核心问题开展研究工作

● 利其器

技术体系

- 泛素化蛋白高效富集
- 泛素链高灵敏检测
- 修饰位点深度覆盖

高灵敏泛素化检测技术体系

Anal Chem, 2020
Anal Chem, 2019
Mol Cell Proteomics, 2019
Mol Cell Proteomics, 2016

泛素密码

● 善其事

泛素链新功能

K11泛素链的新功能

Mol Cell, 2019

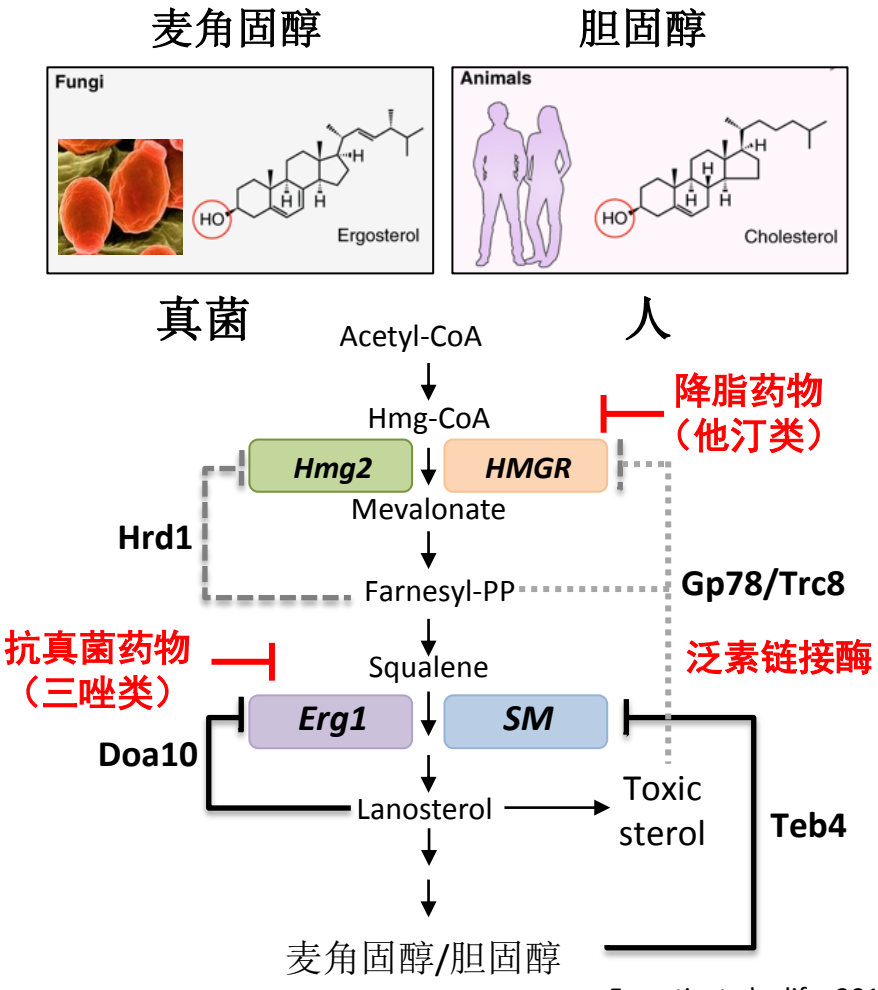
关键调控酶特异性关系

去泛素化酶与泛素链特异性关系研究

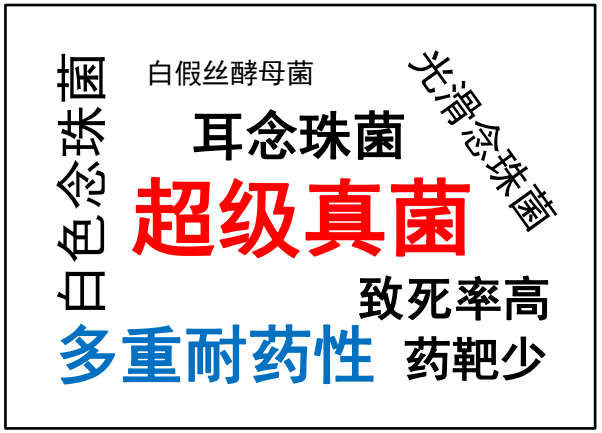
iScience,2019

JBC,2021

甾醇稳态研究的重要学术价值和临床意义



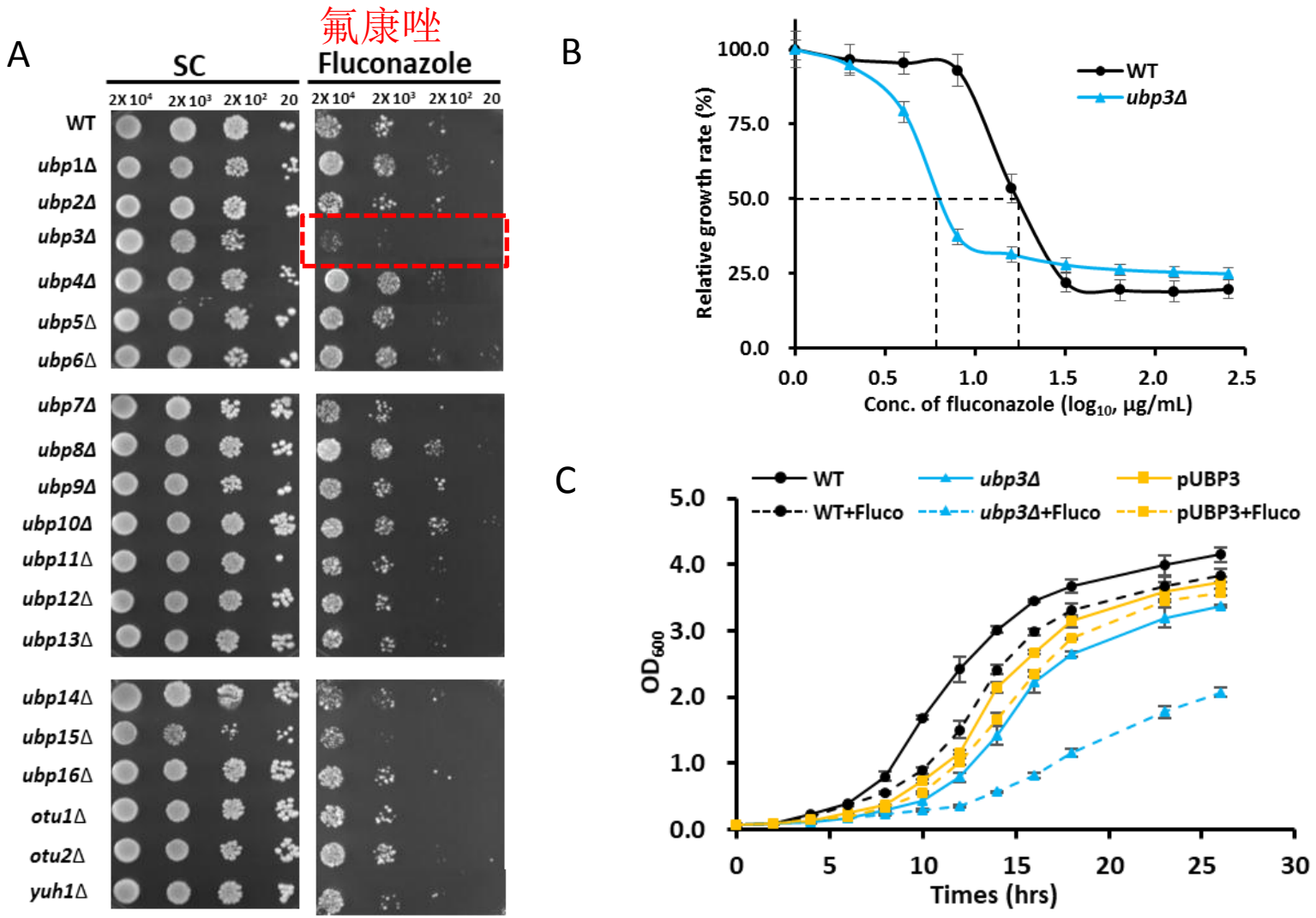
Foresti, et al. elife, 2013.



抗真菌药物、降脂药物的重要靶点

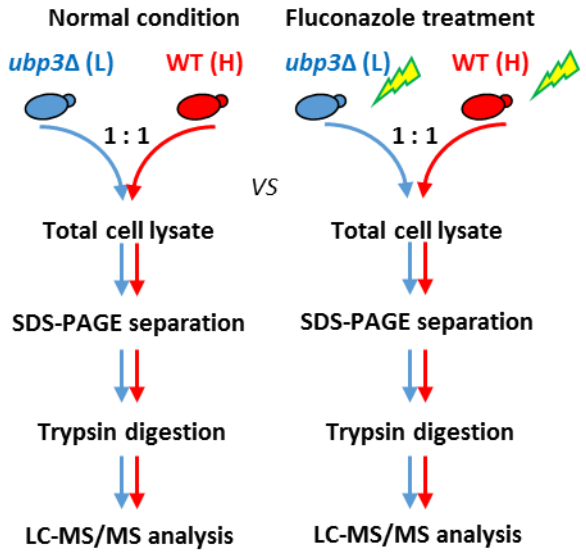
去泛素化酶在甾醇稳态的调控机制尚不清楚

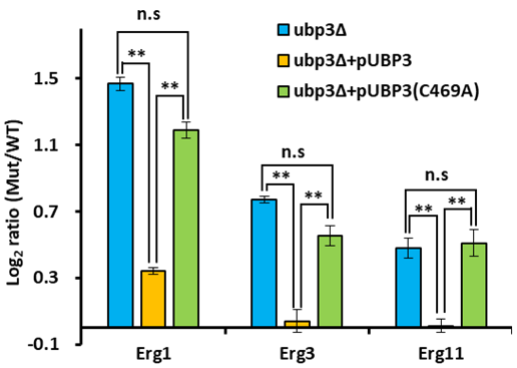
去泛素化酶Ubp3缺失对氟康唑敏感

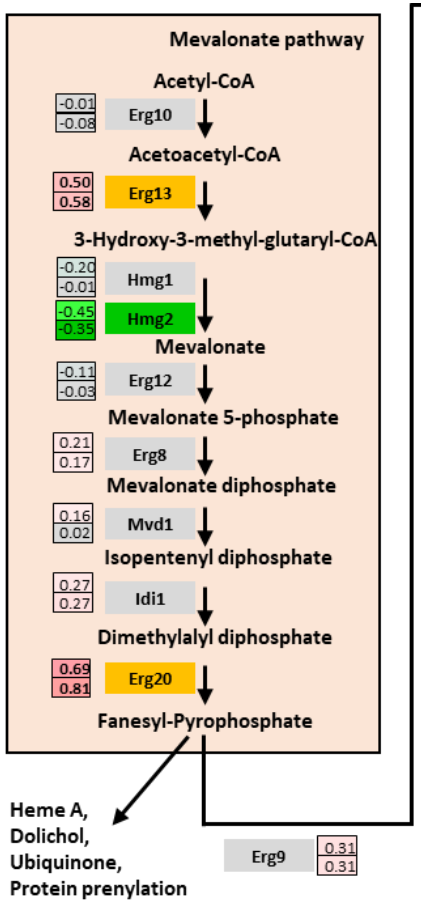


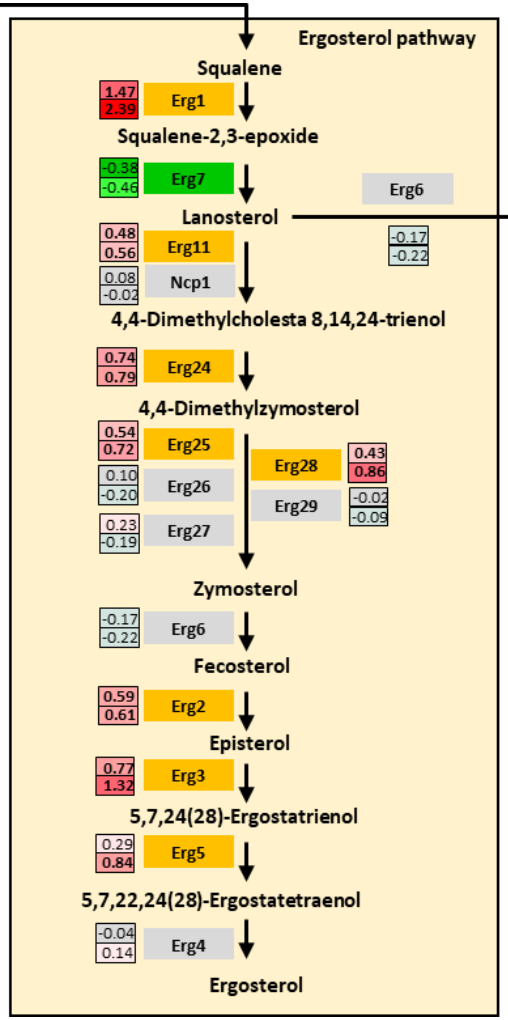
去泛素化酶Ubp3特异性参与甾醇稳态调控

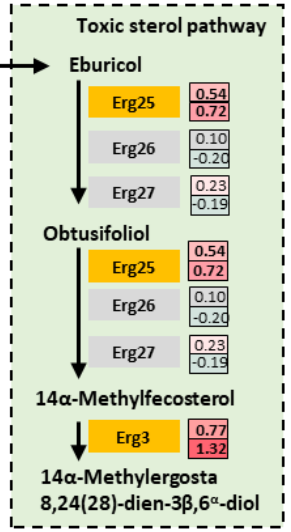
蛋白质组学揭示甾醇稳态通路全貌变化

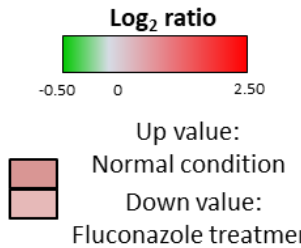




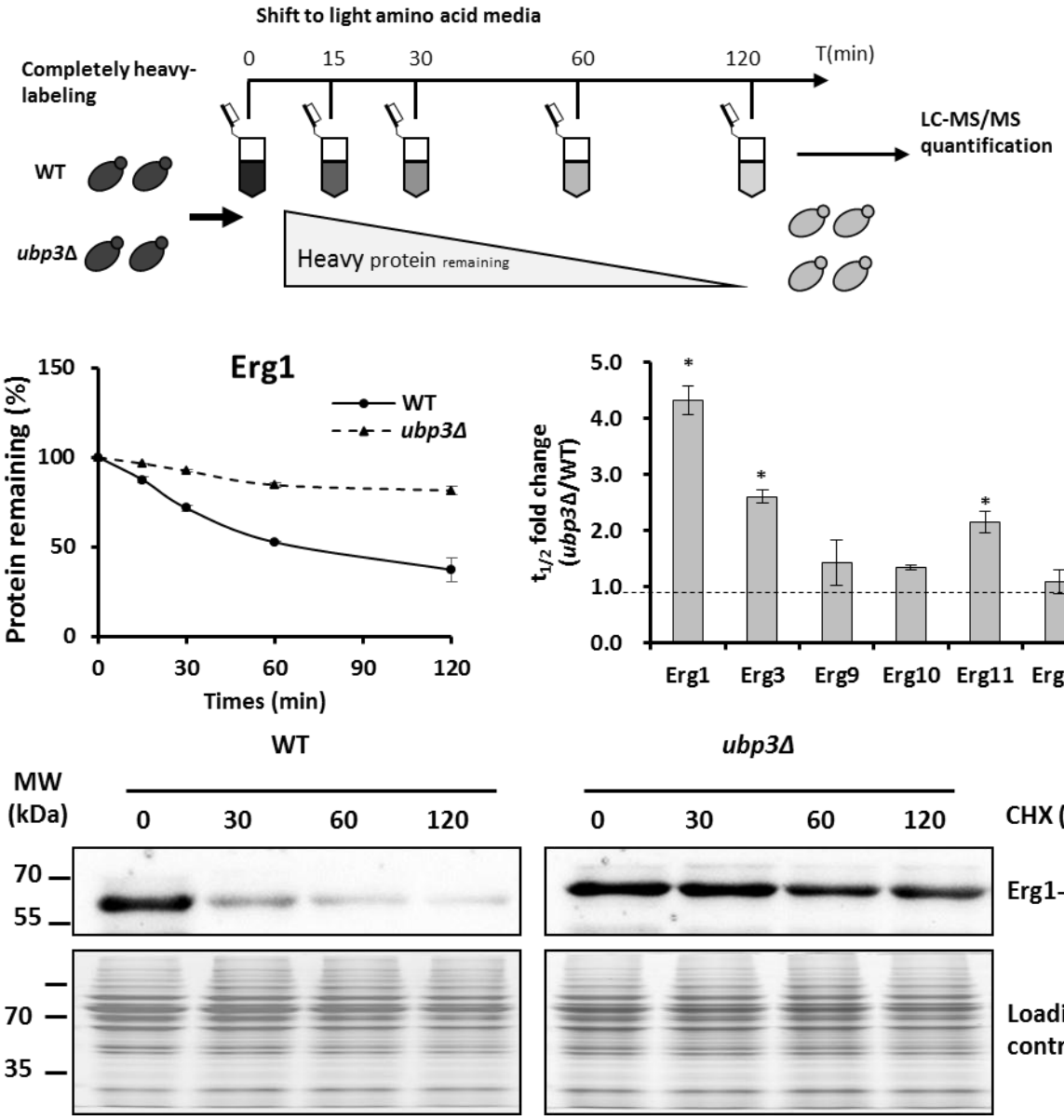




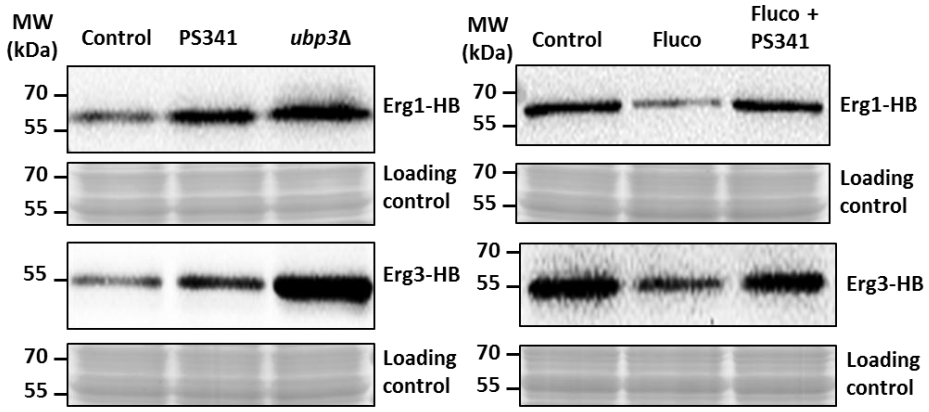


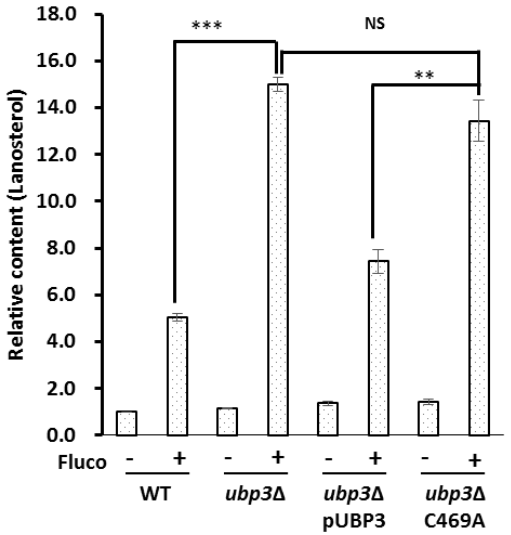


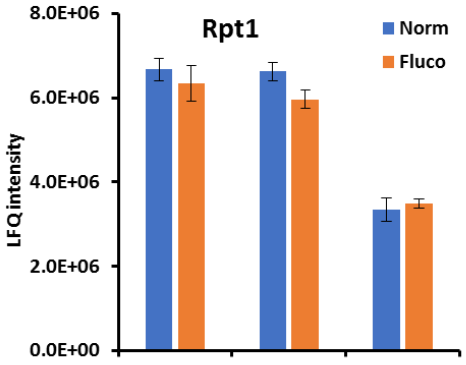
Ubp3缺失导致关键酶Erg1降解受阻

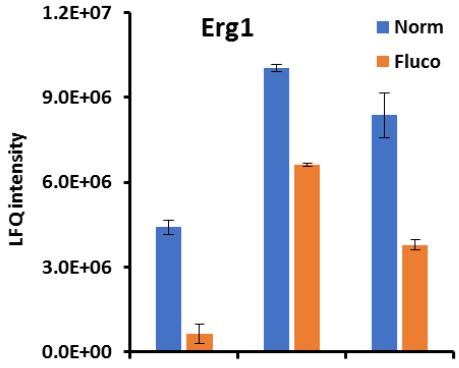


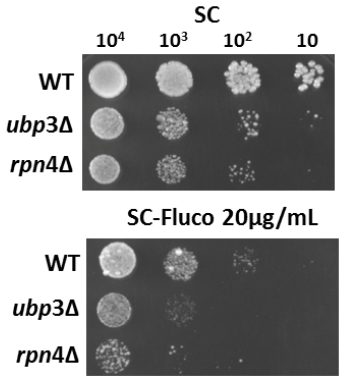
关键酶Erg1降解受阻累积毒性甾醇



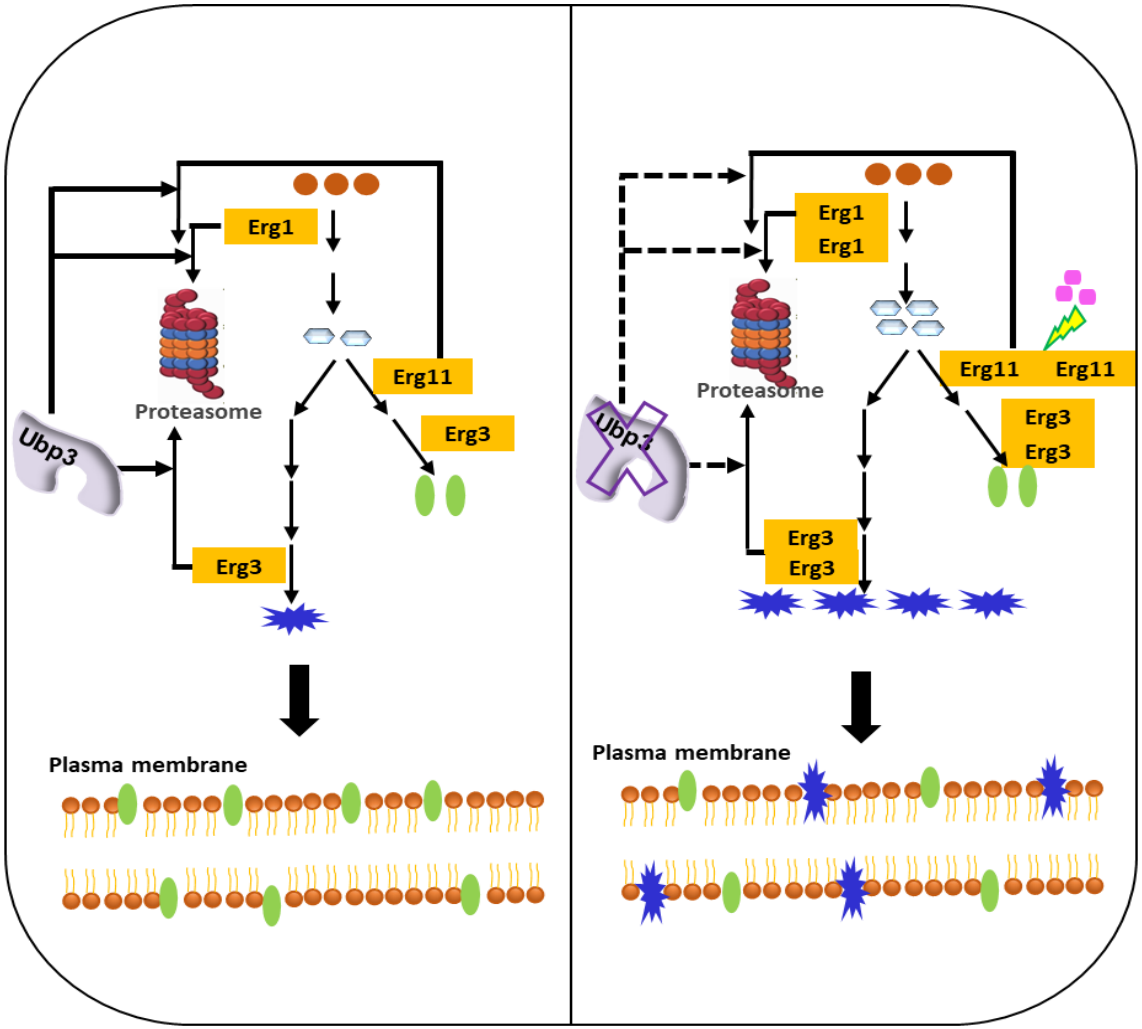






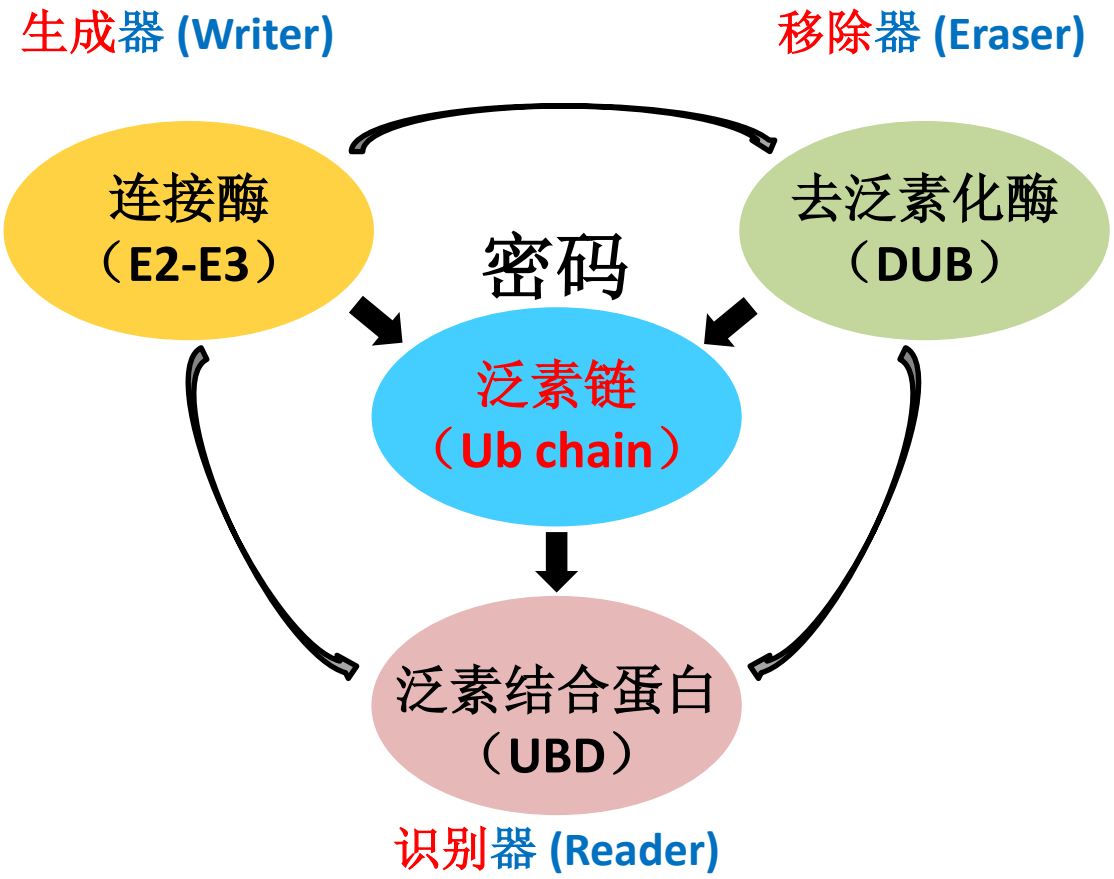


模型：去泛素化酶正向促进关键酶的降解



Lan Qiuyan, Li Yanchang*, et al. **J of Bio Chem**, 2021.

“泛素密码”解析的核心问题

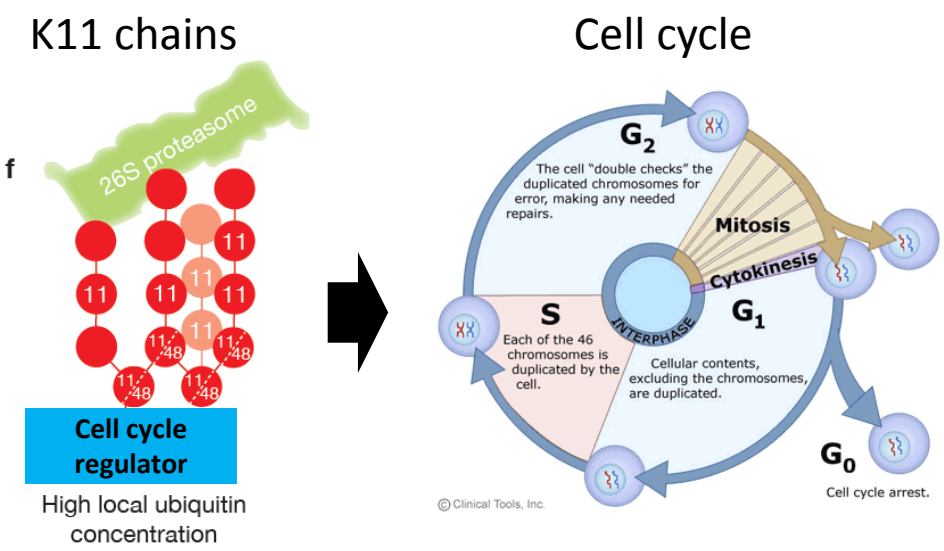


1. 发现泛素链(非典型)的新功能

2. 关键调控酶与泛素链的特异性关系研究

定量蛋白质组学发现K11泛素链的新底物

K11泛素链的已知功能



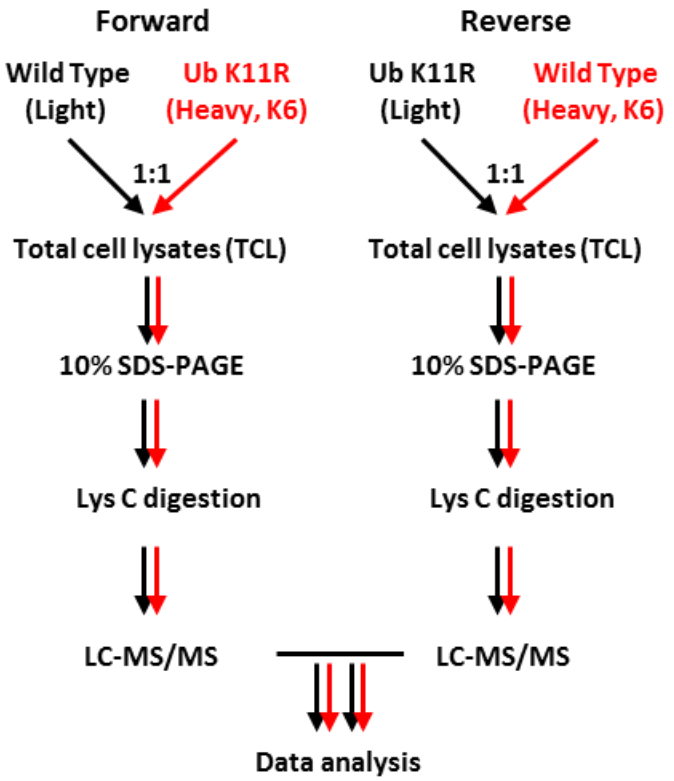
主要功能是介导蛋白质的降解，包括Cell cycle和ERAD关键蛋白

L Wang, et al. Nature, 2019.

Meyer, H.J. et al. Cell, 2014.

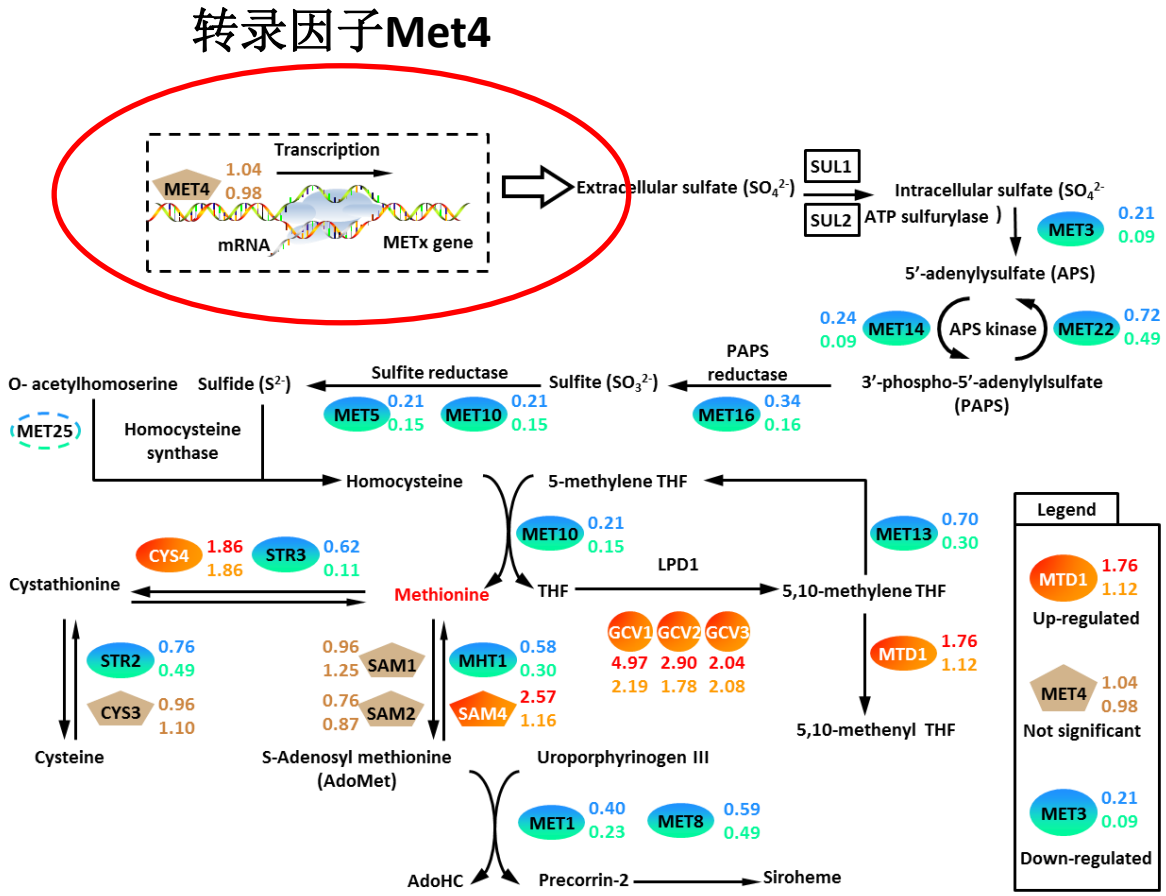
P Xu, et al. Cell, 2009.

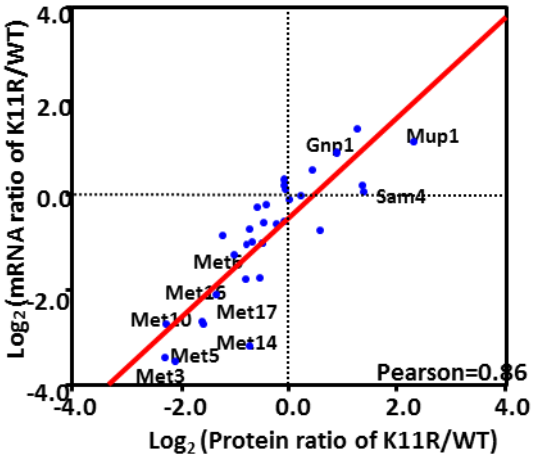
实验设计

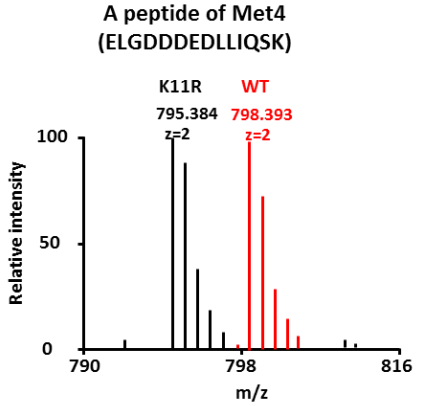


利用深度覆盖的定量蛋白质组学筛选K11修饰的底物

基于定量蛋白质组学筛选K11链调控通路探究

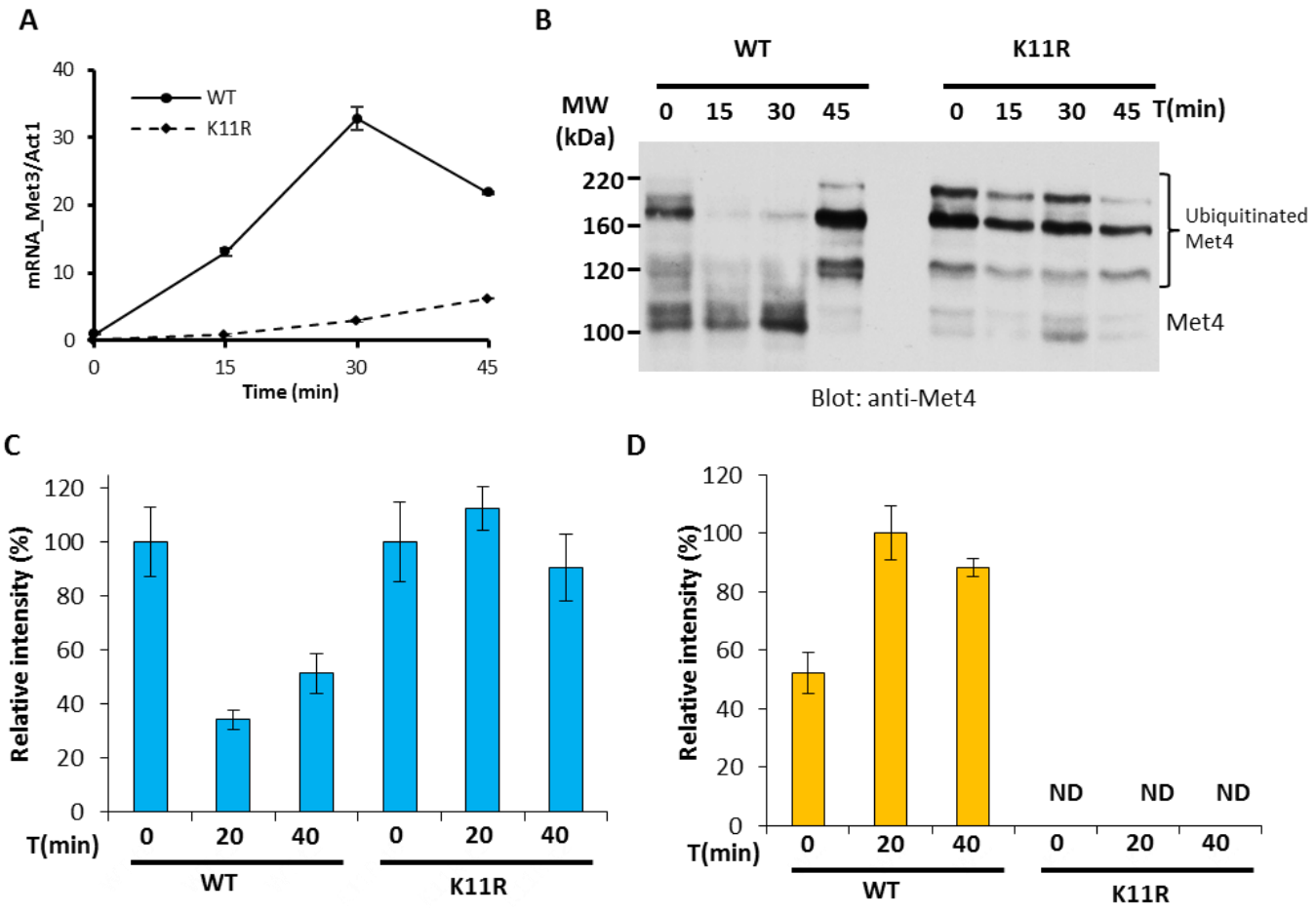






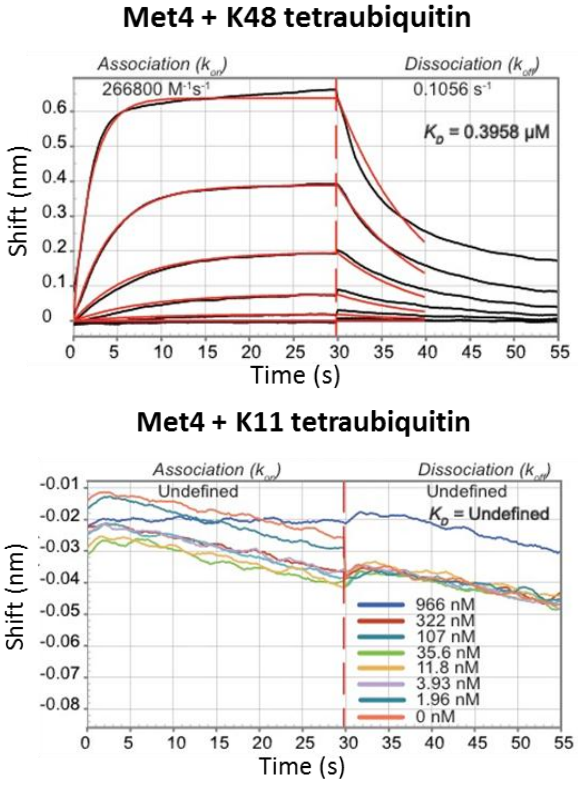
K11链缺失引起甲硫氨酸合成通路下调，暗示其在转录活性调控的“新功能”？

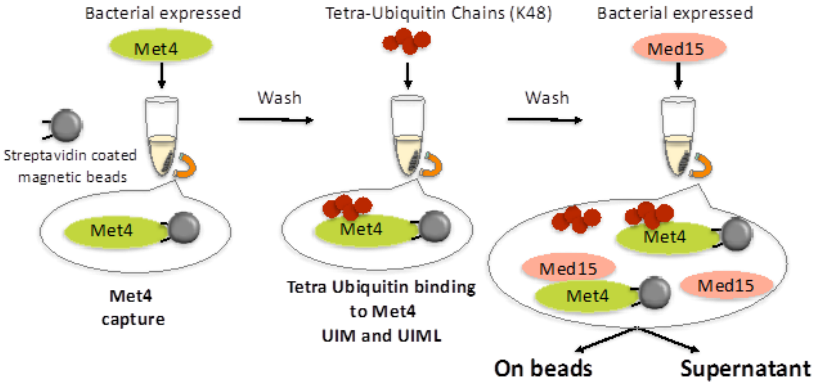
泛素链及其修饰位点靶向鉴定与定量

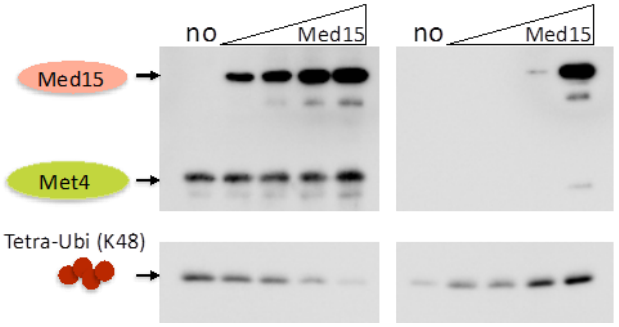


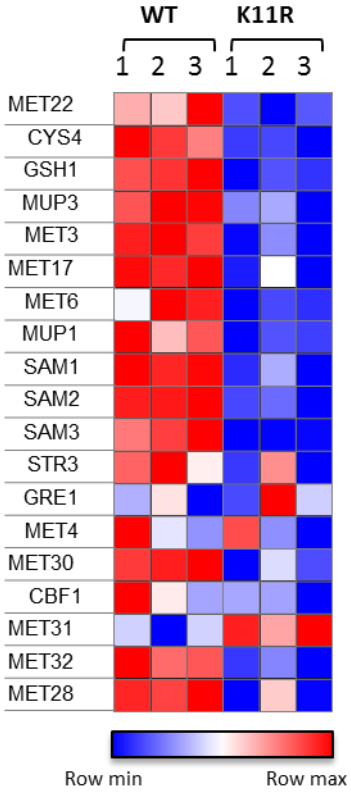
- 转录因子Met4发生K11链修饰；
- K11泛素链缺失导致Met4转录激活受阻.

体内外结合实验探究K11链与Met4结合关系









K11链帮助打开转录因子的自抑制结构域，启动转录激活

K11泛素链介导转录因子Met4激活

Molecular Cell

CellPress

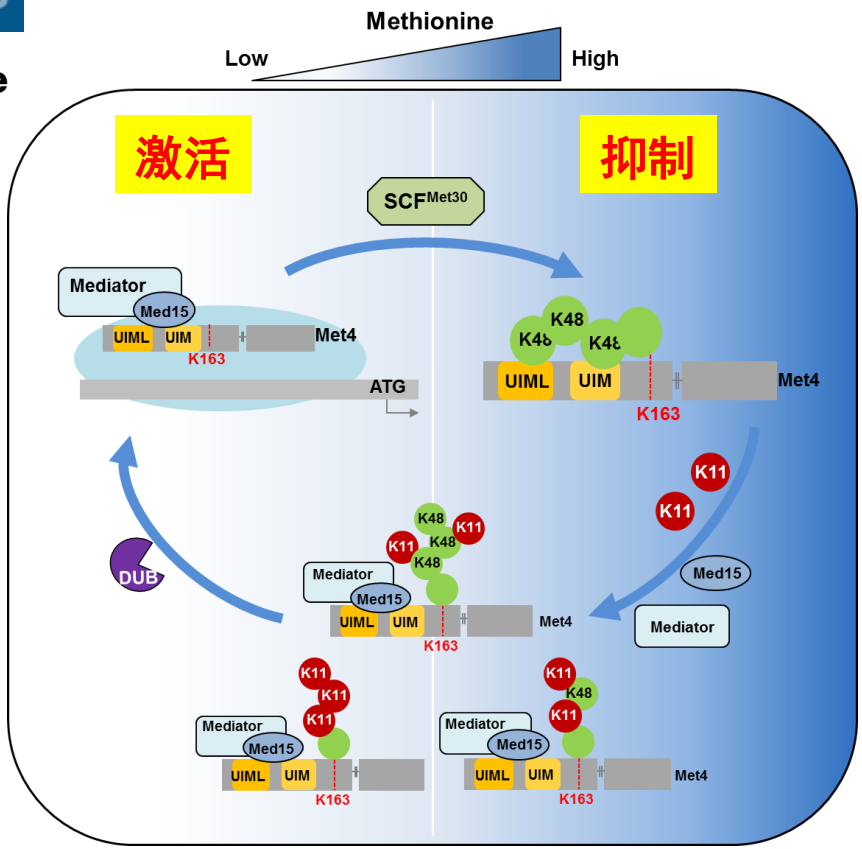
Proteomics Links Ubiquitin Chain Topology Change to Transcription Factor Activation

- 利其器

泛素化检测技术体系
泛素化蛋白高效富集
泛素链高灵敏检测
修饰位点深度覆盖

- 善其事

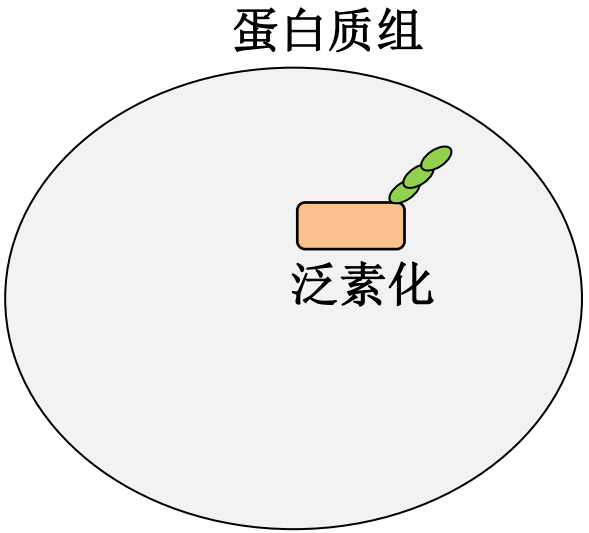
K11泛素链新功能



该工作揭示不同泛素链转换，可以作为生物学信号发挥重要的调控功能，为深刻理解“泛素密码”提供了范例。

Li Yanchang#, et al., ***Molecular Cell***, 2019.

关于泛素化研究的思考



- 丰度低，占蛋白质组的~1%
- 泛素链易被去泛素化酶移除
- 泛素化蛋白易被蛋白酶体降解

富集
- 非典型泛素链的富集技术
- 不同PTMs的共富集技术

检测
- 混合泛素链检测
- 修饰位点的高效鉴定

调控
- 特定E3/DUB与泛素链精确关系
- 特定E3/DUB与底物调控机制

功能
- 非典型泛素链的新功能
- 泛素化修饰与重要病理的关系

总　结

● 利其器

泛素化检测技术体系

- 泛素化蛋白高效富集
- 泛素链高灵敏检测
- 修饰位点深度覆盖

Anal Chem, 2020
Anal Chem, 2019
Mol Cell Proteomics, 2019
Mol Cell Proteomics, 2016

进一步发展针对特定泛素链（如M1线性链）富集技术

● 善其事

泛素功能机制研究

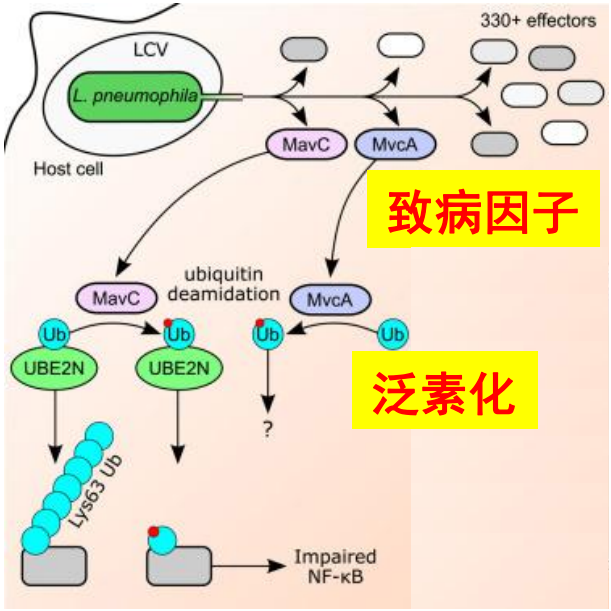
- 去泛素化酶与泛素链特异性关系
- 去泛素化酶调控抑免蛋白新功能
- K11泛素链新功能研究

Mol Cell, 2019

iScience, 2020

进一步探究重要非典型泛素链（K6）的新功能与调控机制

● 泛素化基础研究与临床医学相结合



着力探究致病因子与泛素化调控关系，基础科研紧密结合临床医学

致　谢

- 感谢翻译后修饰课题组——徐平老师给予的指导与支持，
- 感谢泛素组（兰秋艳、肖伟弟、王一豪、黄帅等）的共同的目标努力。

国家自然科学基金：
青年基金（31700723）
面上项目（32071431）

科技部：
重点研发计划
（2017YFA0505002）

BPRC
Beijing Proteome Research Center
北京蛋白质组研究中心

ProteomeSKY
State Key Laboratory of Proteomics

欢迎提问、交流

谢　谢！

liyanchang1017@163.com