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Survey of the Phosphorylation Status of the *Schizosaccharomyces pombe* Deubiquitinating Enzyme (DUB) Family

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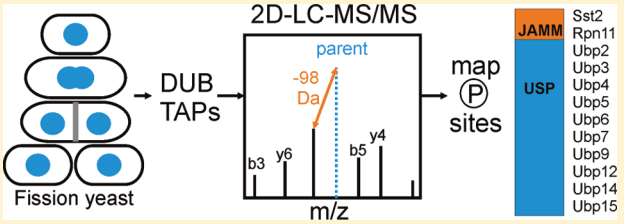
Supporting Information

ABSTRACT: Ubiquitination plays a role in virtually every cellular signaling pathway ranging from cell cycle control to DNA damage response to endocytosis and gene regulation. The bulk of our knowledge of the ubiquitination system is centered on modification of specific substrate proteins and the enzymatic cascade of ubiquitination. Our understanding of the regulation of the reversal of these modifications (deubiquitination) lags significantly behind. We recently reported a multifaceted study of the fission yeast *Schizosaccharomyces pombe* DUBs including characterization of their binding partners, *in vitro* enzymatic activity and subcellular localization.[1] Over half of the 20 fission yeast DUBs have a stable protein partner and some of those partners regulate the localization and/or activity of their cognate DUB. As a next step in understanding how DUBs might otherwise be regulated, we investigated the phosphostatus of the entire fission yeast DUB family using LC−MS/MS, and here we discuss the possible implications of phosphoregulation.



KEYWORDS: ubiquitination, mass spectrometry, LC−MS/MS, phosphorylation

INTRODUCTION

Ubiquitination of specific cellular proteins serves as a signal for protein degradation, chromatin remodeling, DNA repair, vesicular transport, and changes in protein localization and/or activity depending on the number and structure of the ubiquitin modification.[2,3] Protein ubiquitination is highly regulated and requires a cascade of enzymes that culminates in a substrate and site-specific modification. Similarly, deubiquitinating enzymes (DUBs) that remove ubiquitin (or ubiquitin-like modifiers like SUMO or Nedd8) from substrate proteins to allow recycling of ubiquitin and/or modulation of signaling pathways must be tightly controlled.

Ubiquitination and kinase cascades intersect on multiple levels and together they orchestrate key cellular events including endocytosis, cell cycle progression, and growth factor signaling.[4−9] Kinases activate E3 ubiquitin ligases (e.g., the anaphase promoting complex/cyclosome) which in turn ubiquitinate kinases (e.g., Polo) or kinase regulatory subunits (e.g., the cyclin subunit of cyclin-dependent kinases (CDK)).[10−13] Kinases also regulate protein turnover by marking substrates for phosphorylation-dependent ubiquitin-mediated degradation (e.g., by the SCF ubiquitin ligase).[14] There are many other examples of cross-regulation of ubiquitin and kinase signaling networks, including phosphorylation of deubiquitinating enzymes (e.g., CYLD).[15] Here we set the stage for understanding how DUBs might be regulated by kinases and phosphatases by cataloging phosphorylation sites of all *S. pombe* DUBs.

DUBs are a highly conserved family of proteases involved in: (1) processing of ubiquitin precursor proteins, (2) recovery of modified ubiquitin trapped in inactivatable forms, (3) cleavage of ubiquitin from target proteins, and (4) recycling of monoubiquitin from free polyubiquitin chains.[16−18] The diversity of DUB functions is reflected in the number of DUBs (95 predicted human DUBs), the variety of catalytic domains—ubiquitin C-terminal hydrolases (UCH), ubiquitin-specific proteases (USP), ovarian tumor proteases (OTU), Machado-Joseph disease proteases (MJD) and JAB1/MPN/Mov34 metalloenzymes (JAMM)[16] and DUB domain architecture.[1]

S. pombe is an amenable organism in which to conduct a global study of DUB function and regulation because of the limited number of DUBs containing the required catalytic residues (20), the diversity and conservation of catalytic domains (4 of 5 classes, see Table 1), and the genetic tractability of yeast. We recently reported the cellular localization, enzymatic activity profiles and protein interaction networks of the entire *S. pombe* DUB family.[1] A few phosphorylation sites for some *S. pombe* DUBs have been reported in large-scale phosphoproteomics studies,[19,20] but a detailed analysis of DUB phosphorylation is lacking. To begin to understand how phosphorylation impacts DUB regulation, we examined the phosphostatus of the entire *S. pombe* DUB family and their binding partners using tandem affinity purification (TAP) followed by multidimensional LC-MS/MS (MudPIT) from asynchronous and mitotic cell cultures

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Table 1. *S. pombe* Deubiquitinating Enzymes (DUBs)

DUB sub-type	*S. pombe* DUB	*S. pombe* Uniprot ID	*S. cerevisiae* DUB	*H. sapiens* DUB	Cellular Localization	Binding partners	PO_4 sites detected?
Ubiquitin-specific proteases (USP)	Ubp1	Q9USM5	Ubp12	NA	ER	—	No
	Ubp2	CU329670	Ubp2	NA	cytoplasmic	Ucp6	Yes
	Ubp3	O94269	Ubp3	Usp10	cytoplasmic	Nxt3	Yes
	Ubp4	O60139	Doa4, Ubp5	Usp8	endosomes, septa	Sfp47	Yes
	Ubp5	Q09879	Ubp15	Usp7	Golgi	Ftp105	Yes
	Ubp6	Q92353	Ubp6	Usp14	nuclear	proteasome	Yes
	Ubp7	Q9P7S5	Ubp11	Usp45	cytoplasmic	—	Yes
	Ubp8	Q09738	Ubp8	Usp22	nuclear	SAGA complex	No
	Ubp9	Q9P7V9	Ubp9, Ubp13	Usp12, Usp46	nuclear, cell tips & septa	Bun62 Bun107	Yes
	Ubp11	Q9UUD6	NA	NA	mitochondrial	Tom70	No
	Ubp12	O60079	Ubp12	Usp4, Usp15	nuclear, cytoplasmic	—	Yes
	Ubp14	Q11119	Ubp14	Usp5	nuclear	—	Yes
	Ubp15	Q9UTT1	Ubp15	Usp7	nuclear, septa, other	—	Yes
	Ubp16	O74442	Ubp10	NA	nucleolar	—	No
Ubiquitin C-terminal hydrolases (UCH)	Uch1	Q10171	Yuh1	UchL3	nuclear, cytoplasmic	—	No
	Uch2	Q9UUB6	NA	Uch37	nuclear envelope	proteasome	No
Ovarian tumor proteases (OTU)	Otu1	O13974	Otu1	YOD1	nuclear, cytoplasmic	Cdc48	No
	Otu2	Q9UUK3	Otu2	OTUD6B	cytoplasmic	—	No
JAB1/MPN/Mov34 metalloenzymes (JAMM)	Rpn11	Q9P371	Rpn11	POH1	nuclear envelope	proteasome	Yes
	Amsh (Sst2)	P41878	NA	AMSH	endosomes, septa	—	Yes

(Figure 1). Here, we present the global phosphorylation status of the *S. pombe* DUBs and their partners and discuss the implications of these modifications on DUB regulation in eukaryotes.

EXPERIMENTAL METHODS

Yeast Strains, Media, Genetic Methods, and Vector Construction

Strain construction and tetrad analysis were accomplished through standard methods. Endogenously tagged strains (Supplemental Table 1, Supporting Information) were grown in yeast extract (YE) media. For expression of N-terminally tagged proteins, strains were transformed with pREP expression vectors, containing a thiamine repressible promoter, using a standard sorbitol transformation procedure.[21] Transformed strains were first grown on minimal media containing thiamine to suppress expression and then, to induce expression, cells were grown in minimal media lacking thiamine for 18 h.[22] Cell cultures used for TAP purifications were grown in 2 L of 4× YE media (C-terminally TAP tagged proteins) or in 8 L of minimal media supplemented with the appropriate nutrients (N-terminally TAP tagged proteins). All 20 DUBs were tagged endogenously at the 3′ end with TAP or linker-TAP as previously described.[23] The linker sequence in the linker-TAP cassettes translates to ILGAPSGGGATAGAGGAGGPAGLI.[24] N-TAP cassettes for Ubp1, Ubp7, and Ubp11 were constructed as previously described.[1]

For mitotic purifications of the nuclear DUBs (Ubp6, Ubp8, Ubp9, Ubp12, Ubp14, Ubp15, Ubp16, Uch1, Uch2, Otu1, and Rpn11), log phase cells containing DUB TAP tags were blocked using a cold sensitive allele of β-tubulin (*nda3-KM11*, prometaphase) and/or released for 30 min (anaphase). Cells were snap frozen in a dry ice ethanol bath and subjected to TAP/LC-MS/MS as described below.

Protein Methods

Cell pellets were frozen in a dry ice/ethanol bath and lysed by bead disruption in NP-40 lysis buffer under native (Figure 2c) or denaturing conditions (Figure 2a/b) as previously described,[25] except with the addition of 0.1 mM diisopropyl fluorophosphate (Sigma-Aldrich). Proteins were immunoprecipitated by IgG Sepharose beads (GE Healthcare) or anti-GFP (Roche). For phosphatase collapse, immunoprecipitated proteins were incubated with lambda phosphatase (New England Biolabs) in 25 mM HEPES-NaOH pH 7.4, 150 mM NaCl, and 1 mM $MnCl_2$ for 30 min at 30 °C. Immunoblot analysis was performed as previously described[26] except that secondary antibodies were conjugated to Alexa Fluor 680 (Invitrogen) and visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences).

For the block and release experiment, a temperature sensitive strain (*cdc25−22* Ubp9-TAP) was grown overnight at 25 °C and then shifted to the nonpermissive temperature (36 °C) for 3 h to block cells in G2. The cells were then released to the permissive temperature and 20 OD pellets were collected every

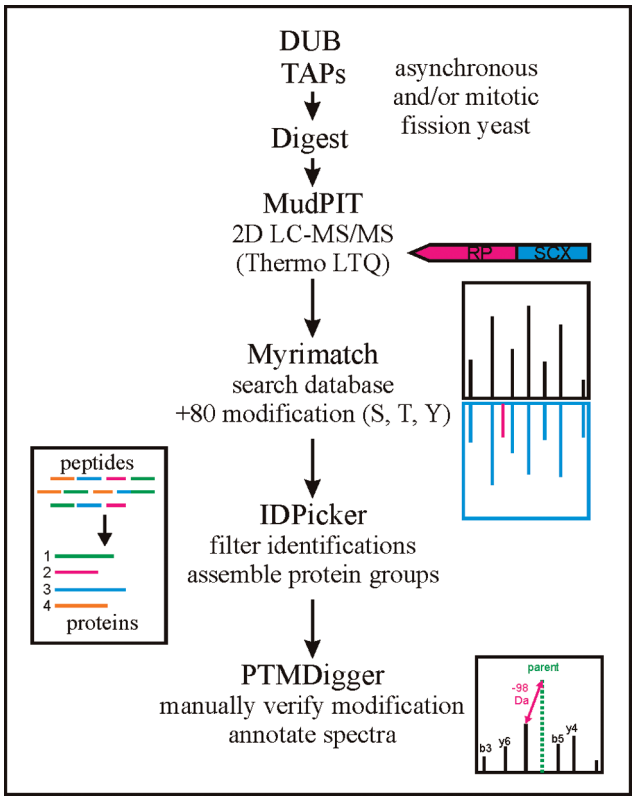

Figure 1. Experimental scheme for DUB purifications, LC−MS/MS analysis, and phosphopeptide identification and verification.

15 min. Lysates and immunoprecipitations were performed as described above except IgG coated dynabeads (Invitrogen) were used for immunoprecipitation.

DUB purification and LC−MS/MS analysis

Proteins were purified by TAP as described,[27] or using a one step dynabead purification as follows: tosylactivated M-280 Dynabeads were coupled to rabbit IgG (Invitrogen) and used to pull down TAP tagged proteins from native lysates (as in TAP protocol) and then the proteins were eluted using high pH. The purified proteins were then TCA precipitated and digested with trypsin (Promega), chymotrypsin (Princeton Separations), and/or GluC (Thermo) and the resulting peptides were subjected to mass spectrometric analysis on a Thermo LTQ as previously detailed.[28,29] Thermo RAW files were converted to MZML files using Scansifter (software developed in-house at the Vanderbilt University Medical Center). Spectra with less than 20 peaks were excluded from our analysis. The *S. pombe* database (http://www.sanger.ac.uk, October 2009) was searched with the Myrimatch algorithm[30] v1.6.33 on a high performance computing cluster (Advanced Computing Center for Research & Education at Vanderbilt University). We added contaminant proteins (e.g., keratin, IgG) to the complete *S. pombe* database and reversed and concatenated all sequences to allow estimation of false discovery rates (10 186 entries). Myrimatch parameters were as follows: strict tryptic cleavage; modification of methionine (oxidation, dynamic modification, +16 Da), S/T/Y (phosphorylation, dynamic modification, +80 Da) and cysteine (carboxamidomethylation, static modification, +57 Da) was allowed; precursor ions were required to be within 0.6 m/z of the peptide monoisotopic mass; fragment ions were required to fall within 0.5 m/z of the expected monoisotopic mass. IDPicker[31,32] v2.6.126.0 was used to filter peptide matches with the following parameters: max. FDR per result 0.01, max. ambiguous IDs per result 2, min peptide length per result 5, min distinct peptides per protein 3, min additional peptides per protein group 2, minimum number of spectra per protein 3, indistinct modifications M 15.994 Da, C 57.05 Da and distinct modifications S/T/Y 80 Da. IDPicker results were processed in Excel (Microsoft) to generate phosphopeptide lists for the DUBs and their binding partners. Spectra were manually inspected and annotated in SeeMS and a related program called PTMDigger, software developed by in-house (Surendra Dasari, Matthew Chambers, and David Tabb, Vanderbilt University Medical Center). Supplemental Figure 1 (Supporting Information) was generated using software developed in-house (Zeqiang Ma, Surendra Dasari, Matthew Chambers, and David Tabb, Vanderbilt University Medical Center). DUBs and partners were purified with sequence coverage (%) as follows: Otu1 − 51, Otu2 − 35, Ubp1 − 67, Ubp2 − 95, Ubp3 − 64, Ubp4 − 42, Ubp5 − 72, Ubp6 − 56, Ubp7 − 81, Ubp8 − 62, Ubp9 − 67, Ubp11 − 71, Ubp12 − 67, Ubp14 − 80, Ubp15 − 88, Ubp16 − 44, Rpn11 − 63, Sst2 − 65, Uch1 − 84, Uch2 − 82, Ucp6 − 78, Nxt3 − 71, Sfp47 − 46, Ftp105 − 58, Bun62 − 57 and Bun107 − 64. Note that mildly overexpressed N-terminal TAP fusions were used for the low abundance DUBs Ubp1, Ubp7 and Ubp11. For complete protein identification information for each TAP, see a previous publication.[1] Using the stringent filter of FDR < 1%, approximately 1500 mass spectra contained +80 Da shifts, indicative of phosphorylation. These spectra were manually inspected and filtered according to the following criteria: (1) exhibit a prominent (often base) 98 Da (H_3PO_4) neutral loss peak at the MS2 level and (2) b and y ion intensities >20% of the neutral loss peak (3) contained two or more sequential fragments (b and/or y) bracketing the phosphorylation site(s); 1242 spectra met these criteria. Phosphorylation sites were assigned based on the presence of sequential fragment ions surrounding the modification; if these ions were missing, the phosphorylation site(s) were assigned to multiple sites ambiguously.

RESULTS AND DISCUSSION

Deubiquitinating enzymes are present in nearly every cellular compartment[1] (Table 1) and participate in essential cellular processes including regulation of endocytosis, protein degradation, transcription, DNA repair, and protein localization and/or activity.[18] We and others have shown that DUBs are regulated by interaction with protein partners[1,18,33] and now we have assessed the phosphostatus of the *S. pombe* DUB family to set the stage for understanding the interplay of phosphorylation and ubiquitination.

Each of the 20 *S. pombe* DUBs was purified two or more times from asynchronous cultures using an endogenous C-terminal TAP tag or an inducible N-terminal TAP tag (see Experimental Methods for details).[1] We also performed purifications of the (partially) nuclear DUBs (Ubp6, Ubp8, Ubp9, Ubp12, Ubp14, Ubp15, Ubp16, Uch1, Uch2, Rpn11, and Otu1) from cells arrested in prometaphase using the tubulin mutation, *nda3-KM11*, and released for 30 min into anaphase to enrich our data set with mitotic phosphorylation events (denoted in Tables 2, 3 and 4). Each purification was precipitated, digested, and analyzed on a Thermo LTQ using a MudPIT protocol (see Experimental Methods for details). The resultant mass spectra were processed using software developed at Vanderbilt Medical Center (Figure 1) to identify phosphorylation sites.

Over 1500 mass spectra (FDR > 0.5%) indicative of phosphorylation (+80 Da) were identified from our bioinformatic

Table 2. Phosphorylation Sites Detected for Cytoplasmic *S. pombe* DUBs

Protein	Sequence	Site(s)	# PO4	TSC	unmod	Motif	Kinase/binding motif
Ubp2	LIQDLDVFDsPSAGWNDPWSPHSSR	S25	1	5	262	sP	proline-directed (WW-IV)
	LIQDLDVFDSPSAGWNDPWsPHSSR	S35	1	5	262	sP	proline-directed (WW-IV)
	DRIPVYYEEEELPEPHVtsPTK	T112/S113	1	4	103	sPxK	proline-directed/ FHA-2 pT binding motif
	IPVYYEEEELPEPHVTsPTK	S113	1	20	143	sPxK	proline-directed, CDK
	IPVYYEEEELPEPHVTsPTKSEFATTSTCMK	S113	1	31	18	sPxK	proline-directed, CDK
	IPVYYEEEELPEPHVtSPTKSEFATTSTCMK	T112	1	20	18		
	TTKsEIEVEWR	S135	1	8	146	s[DE]xE	CKII-like
	SSSHQGSSHsSQPSLFTTFTSLELFLR	S179	1	4	19	Sxx[st]	CKI
	SGTsSVMDLSSSR	S722	1	12	86	Sxx[st]	CKI
	SGtssVMDLSSSR	T721/S722/S723	2	2	86	Sxx[st]	CKI/FHA-1 pT binding motif
	SGTSSVMDLsssRELSNLNER	S728/S729/S730	1	30	1		
	sGTsSVMDLsSsRELSNLNER	S719/S722, S728/S730	2	8	1		
	SSSVHPSSVLTYLALIPLTLDQVKsGTsSVMDLSSSR	S719, S722	2	2	0		
Ubp3	HVQGDsPVKK	S89	1	2	22	sPxK	proline-directed (WW-IV)
	SGLDSFDDQssVEASGWTEVGK	S281/S282	1	3	768		
Ubp4	sPIAPLTEDQLSAR	S343	1	5	74	sP	proline-directed
Ubp5	MVtGETLVDSQK	T3	1	6	290		
	MVTGETLVDsQK	S10	1	2	290	[st]xxx[ST]/[s	GSK3/PIKK
	MVTGETLVDsQKSLINNDTLLNEK	S10	1	15	0	[st]xxx[ST]/[s	GSK3/PIKK
	SLINNDtLLNEK	T19	1	6	127		
	CFYNLQFMNEPVstTELTK	S274/T275	1	5	72	[st]xx[ILV]	FHA-1 pT binding motif
	EALNPsIQLAELR	S554	1	3	27		
	VEsPVNELNSTMEEVK	S648	1	2	80	sP	proline-directed
	tPtQSELSTGDIICFEPCRPSALEDDIVNSGFDSALK	T763/T765	1	4	197		
Ubp7	sLQLsPCLTDDEQLSK	S333, S337	2	20	44	sP	proline-directed (WW-IV)
	sLQLSPCLTDDEQLSK	S333	1	1	44	[st]xxx[ST]	GSK3
	SLQLsPCLtDDEQLSK	S337, T341	1	7	44	sP	proline-directed (WW-IV)
	SLQLsPCLTDDEQLSK	S337	1	94	44	sP	proline-directed (WW-IV)
	SLQLSPCLtDDEQLSK	T341	1	9	44		
	ELSQSSDSSQHQHDSFLPANSsPLAASSTK	S420	1	17	50	sP	proline-directed (WW-IV)
	ELsQSSDssQHQHDSFLPANSSPLAASSTK	S401/S406/S407	2	2	50	Sxx[st]	CKI
	SLPSSELLDSssDKGQQVFK	S439/S440	1	21	23		
	RsLDILR	S493	1	2	3		
	LGELsSDDMMLDK	S503	1	3	68		
	RLsDLsVNSSGQISK	S648, S651	2	1	26	Sxx[st]	CKI
	RLsDLSVNsSGQISK	S648, S654	2	10	26	Sxx[st]	CKI
	RLsDLSVNSSGQISK	S648	1	11	26		
	RLSDLSVNsSGQISK	S654	1	2	26	Sxx[st]	CKI
	LSDLSVNsSGQISK	S654	1	2	31	Sxx[st]	CKI
	GGLTSDNDKysFNNSVYR	Y722/S723	1	3	57		
Sst2	SSSDLPQFDYPsLNssPtFNsNLPISSSR	S186/S189/S190/T192/S195	1	7	45		
	FEKTsLSDSK	S208	1	2	0		
	LVsPEPLDDNKDIQFIK	S216	1	3	74	sP	proline-directed

analysis (Figure 1) and manual validation showed 1242 spectra corresponding to phosphorylation sites (for criteria see Experimental Methods). The overall spectral quality and peptide sequence coverage is illustrated with two examples of parent and daughter spectra (Supplemental Figures 2 and 3, Supporting Information). In total, we identified over 130 phosphorylation sites in over half (12/20) of the *S. pombe* DUBs and DUB partners (Tables 2, 3, and 4). Only ca. 15% of the phosphosites we identified have been previously reported (see Supplemental Table 2 for details, Supporting Information).[19,20] We confirmed biochemically that Upb9, Bun107, Ftp105 and Sfp47 are phosphoproteins by lambda phosphatase collapse and Western blot (Figure 2a). The other DUBs exhibited no discernible gel shift after phosphatase treatment (data not shown), but gel conditions were not optimized for each protein.

Cell Cycle Regulation of DUB Phosphorylation

The mitotic purifications revealed upregulated [st]P proline-directed kinase consensus sites, as one might expect for modification by mitotic CDK. Phosphorylation sites detected in Ubp6, Ubp9 and its partner Bun107 were much more abundant in the mitotic purifications (denoted in Tables 3 and 4), suggesting that these DUBs are cell cycle regulated. Ubp6 is recruited to the proteasome under conditions of ubiquitin stress[34] which was not the case for our experiments, but it is possible that mitotic phosphorylation plays some role in localization or activity of Ubp6.

Phosphorylation of Ubp9 is clearly cell cycle dependent based on block and release experiments (Figure 2c) and enrichment of S11 phosphopeptides identified from mitotic cells; thus, phosphorylation may alter the affinity of Ubp9 for its substrates and/or enhance its catalytic activity rather than affect its cellular localization (which is regulated by its WD partners, see discussion below). All components of the Ubp9 complex are phosphorylated (this study) and conserved throughout eukaryotes.[35] The larger WD partner, Bun107, contains multiple phosphorylation sites consistent with CDK phosphorylation based on amino acid sequence and increased abundance in mitotic purifications (Table 4).

Phosphorylation of DUB Complexes

Cross-regulation between ubiquitination and phosphorylation appears to be a common theme for DUB complexes. Over half of the DUBs interact with protein partners near stoichiometric ratios[1] and most of these DUBs *and* their partners are phosphorylated (Tables 1−4 and Figure 3), signifying that kinases and phosphosphatases regulate DUBs. Ubp9, a DUB present in the nucleus and at cell tips and septa, is part of a complex

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Table 3. Phosphorylation Sites Detected for (Partially) Nuclear *S. pombe* DUBs

Protein	Sequence	Site(s)	# PO4	TSC	unmod	Motif	Kinase/binding motif	Mitotic?
Ubp6	AIPELSDAVsQFNSSGGLVAEYR	S129	1	6	1381	[st]Q	PIKK	X
	AIPELSDAVSQFNsSGGLVAEYR	S133	1	2	1381	[st]xxx[ST]	GSK3	X
	TLLNSMQSNAPVtPMR	T143	1	25	747	tP	proline-directed	X
	IQPsEDEKEAEAECR	S359	1	7	200	s[DE]xE	CKII-like	
Ubp9	WMGMNsPGSTDR	S11	1	47	75	sP	proline-directed	X
	KSsLYGSNGINSCGCVDISNVGSESGTK	S110	1	12	14			X
	ESIKPMSIPSQLKQESVEVSNLSStPR	T462	1	3	6	tP	proline-directed	X
	QESVEVSNLSStPR	T462	1	12	43	tP	proline-directed	X
	SYFDREPsLDAER	S505	1	5	52	[KR]xxs	PKA/PKC	X
	SKFFGSSQSNsPK	S550	1	6	24	sPK	proline-directed/ Histone H1 kinase	X
	FFGSSQSNsPK	S550	1	9	49	sPK	proline-directed/ Histone H1 kinase	X
	FFGSSQSNsPKDSPLR	S550	1	2	2	sPK	proline-directed/ Histone H1 kinase	X
	FFGSSQsNsPKDSPLR	S548, S550	2	2	2	sPK	proline-directed/ Histone H1 kinase	X
	FFGSSQsNSPKDsPLRDTHK	S548, S554	2	1	0	[st]xx[ST]/sP	CKI/proline-directed	X
	FFGSSQSNsPKDsPLRDTHK	S550, S554	2	1	0	sPK, sP	proline-directed/ Histone H1 kinase	X
Ubp12	sLsEEsQssSNMDDISQK	S19/S21/S24/S26/S27	2	6	14	Sxx[st]/ sxxE	CKI/CKII-like	
	sLSEESQSSSNMDDISQK	S19	1	5	14	s[DE]xE	CKII-like	
	SLSEESQSSSNMDDISQKsISLGDASEISK	S37	1	11	0	Sxx[st]	CKI	
	sISLGDASEISK	S37	1	17	84	Sxx[st]	CKI	X
	SIsLGDASEISK	S39	1	9	84	[KR]xxs	PKA/PKC	X
	IPTGWNMsVSNLPLLTER	S708	1	5	157			
	DLEsTVDPLDAHSIEEEDDSEFK	S725	1	13	33			
	DLESTVDPLDAHsIEEEDDSEFK	S734	1	21	33			
	DLEstVDPLDAHsIEEEDDSEFK	S725/T726, S734	2	5	33			
	DLEsTVDPLDAHSIEEEDDSEFKDVAPGSYPEPSK	S725	1	14	91			
	DLESTVDPLDAHsIEEEDDSEFKDVAPGSYPEPSK	S734	1	23	91			
	TILSDKKDDsEDSR	S819	1	4	437	[KR]xxs	PKA/PKC	
Ubp14	YssFSSQGLTLTVPR	S453/S454	1	19	381	Sxx[st]/ Rx[st]/ RxRxx[st]	CKI/PKA/PKB	X
Ubp15	IPTDNDDSRDsVAYALQR	S271	1	8	189	Sxx[st]	CKI	X
	LDHILsPVTAEDVPFHVR	S553	1	4	502	sP	proline-directed (WW-IV)	
	YHGFDMtDFSASDDDPVLITTK	T608	1	13	568			
Rpn11	VQNEVTLsPEQLR	S265	1	7	50	sP	proline-directed	X

containing two WD proteins (Bun62 and Bun107). Both WD partners are required for Ubp9's DUB activity and regulate its cellular localization.[1] The Ubp9 complex shuttles between the nucleus and cytoplasm, but at steady state, accumulates at active sites of endocytosis (cell tips and septa). When Bun62 is deleted, Ubp9 localizes to cell tips and septa, but not the nucleus, whereas deletion of Bun107 causes retention of Ubp9 in the nucleus.[1] We have discovered that Ubp9 and both of its partners are phosphorylated (Tables 3 and 4 and Figures 2 and 3). To investigate how phosphorylation might impact Ubp9 localization or function, we examined the phosphostatus of Ubp9 in strains where each partner had been deleted individually and in combination (Figure 2b). When either WD partner is lost, Ubp9 is no longer efficiently phosphorylated (Figure 2b), suggesting that Ubp9 is not competent for phosphorylation unless it is in complex with its partners. Both partners of four other DUB complexes are phosphorylated, including two cytoplasmic DUBs Ubp2 and Ubp3 and their partners Ucp6 and Nxt3, respectively, and two endocytic DUBs Ubp4 and Ubp5 and their partners Sfp47 and Ftp105, respectively (Tables 2 and 4, Figures 2 and 3). Sfp47, an SH3 domain protein, and Ftp105, a putative transmembrane protein, recruit their respective DUB partners to specific cellular locations (endosomes for Ubp4 and the Golgi for Ubp5).[1] Phosphorylation and dephosphorylation cycles may modulate complex formation, cellular localization, DUB activity and/or substrate specificity.

Location of Phosphosites within DUBs and Their Partners

Surprisingly, most of the DUB phosphorylation sites map to the catalytic DUB domains (Figure 3). In fact, all detected sites for Ubp7 are within its extended USP domain, suggesting that its catalytic activity and/or structure could be regulated by phosphorylation. Ubp2 and Ubp12 each have two clusters of phosphosites—one within their USP domain and one near the N-terminus; perhaps this arrangement allows tuning of DUB cellular localization, substrate binding or catalytic activity by kinases and phosphatases. Finally, two endocytic DUBs Ubp5 and Ubp9 have two clusters of sites at their N- and C- termini, respectively, predominately outside the USP domains. As discussed above, the cellular localization of these two DUBs is regulated by protein partners[1] and so phosphorylation may add another layer of regulation for substrate binding and/or catalytic activity. The phosphosites detected for the DUB partners also cluster within or very near domains (e.g., Ubcp6 and Ftp105) or in regions predicted to be intrinsically disordered (e.g., Sfp47 and Bun107) (Figure 3). These sites may regulate the availability of specific protein domains for interaction with other partners or the catalytic activity of the holo DUB complex.

DUB Phosphorylation Consensus Motifs

Given the diversity of DUB cellular localization and function, it is not surprising that the DUB phosphosites match consensus sequences for multiple protein kinases. The majority of DUB phosphopeptides are products of proline-directed

Table 4. Phosphorylation Sites Detected for Partners of *S. pombe* DUBs

Protein	Sequence	Site(s)	# PO4	TSC	unmod	Motif	Kinase/binding motif	Mitotic?
Ucp6	NMGVSEsDAKDSLER	S19	1	3	61			
	LVPIMsstsIASSLPSyQDTFFLPSPR	S54/S55/T56/S57/Y65	1	19	19	Sxx[st]	CKI	
	LVPIMsstsIASSLPSyQDTFFLPSPR	S54/S55/T56/S57/Y65	2	5	19	Sxx[st]	CKI	
	GLLFAEDNASFHRPFTDVSAHLssssLsK	S143/S144/S145/S146/S148	1	7	31	Sxx[st]	CKI	
	GLLFAEDNASFHRPFTDVSAHLssssLsK	S143/S144/S145/S146/S148	2	2	31	Sxx[st]	CKI	
	PFTDVSAHLSSSsLSK	S146	1	4	10	Sxx[st]	CKI	
	NVsPSANHNEQWYLVR	S463	1	3	117	sP	proline-directed (WW-IV)	
	NFIAEDNEYFDDELAGIIHsPtVSTR	S555/T557	1	31	10	sP	proline-directed	
	NFIAEDNEYFDDELAGIIHsPtVsTR	S555/T557, S559	2	4	10	sP	proline-directed	
	SRNsACEFPESMHVEHSG	S598	1	45	3	Sxx[st]/ s[DE]xE	CKI/CKII	
Nxt3	FLREDVEEEEEsPDAVEK	S145	1	9	556	sP	proline-directed (WW-IV)	
	FLREDVEEEEEsPDAVEKEK	S145	1	2	174	sP	proline-directed (WW-IV)	
	DVASEPYVNGVQsQEHLPSAK	S167	1	6	29			
	KDVASEPYVNGVQsQEHLPSAK	S167	1	5	55			
	SQASVSSTAsTTGQTVK	S285	1	3	816	Sxx[st]	CKI	
Sfp47	GVVLSVDTVTsPISQSPK	S221	1	17	3	sP	proline-directed	
	KLtPtTSPINSTSLSFVDAK	T231, T233	2	2	0	tP/tPxxS	proline-directed (WW-IV)/ SCF-FBW7 ligand	
Ftp105	NLPKENsELDLSNFQDDLDFENSISQK	S136	1	12	32	s[DE]xE	CKII-like	
	ENsELDLSNFQDDLDFENSISQK	S136	1	12	41	s[DE]xE	CKII-like	
	ENSELDLSNFQDDLDFENsISQKNEFSQK	S152	1	2	0	[DE]x[ST][HΦ]	Polo kinase	
	SPSVPLsPVSTFPASSISLDASSDVSAADVSVGGSSTIK	S169	1	2	5	sP	proline-directed	
	LNLsPGAAIENQYR	S374	1	3	28	sP	proline-directed (WW-IV)	
	SQPLVALNSEGssDFESK	S652/S653	1	8	64	sxxF/s[DE]xE	BRCT binding motif/ CKII-like	
	SQPLVALNsEGsSDFESK	S649, S652	2	4	64	sxxF	BRCT binding motif	
	SSDNTsLDGTPLQNTDFK	S664	1	5	11	Sxx[st]	CKI	
	SSDNTsLDGTPLQNTDFKK	S664	1	16	64	Sxx[st]	CKI	
	KVAtVEDDSPFDELDK	T680	1	4	16	txxE	FHA-2 pT binding motif	
	VAtVEDDSPFDELDKFSSPFSSSSSR	T680	1	5	99	txxE	FHA-2 pT binding motif	
	VATVEDDSPFDELDKFSsPFSSSSSR	S695	1	1	99	sP	proline-directed	
	VATVEDDSPFDELDKFsSPFSSSSSR	S694	1	6	99			
	VATVEDDSPFDELDKFSSPFssssssR	S698/S699/S700/S701/S702	1	10	99	Sxx[st]	CKI	
	NVsIsVPTVLQDVFSDSPLVLSR	S715/S717	1	16	39			
	GKIPENVsssELIK	S746/S747/S748	1	13	185			
	GKIPENVsssELIKK	S746/S747/S748	1	1	97			
	IPENVsSSELIK	S746	1	4	22			
	IPENVsSSELIKK	S746	1	3	57			
	VASPENSSNsMENATK	S893	1	4	25	Sxx[st]	CKI	
Bun107	KGNVNMPSALsPLR	S568	1	9	15	sP	proline-directed (WW-IV)	X
	GNVNMPSALsPLR	S568	1	45	37	sP	proline-directed (WW-IV)	X
	NRKPHTEVVGsPTVVR	S655	1	3	14	sP	proline-directed	X
	KPHTEVVGsPTVVR	S655	1	2	26	sP	proline-directed	X
	VFsTGTSVTSPQALSK	S710	1	15	34			X
	VFSTGTSVTsPQALSK	S717	1	73	34	sP	proline-directed	X
	SKsSKsLQTDFMK	S790, S793	2	8	0			X
	SSKsLQTDFMK	S793	1	1	0	Sxx[st]	CKI	X
	SVsEIVDKTQSLNI	S951	1	3	42			X
	SVSEIVDKTQsLNI	S959	1	1	42	[KR]xxs	PKA/PKC	X
Bun62	StPQITGSPLDPNTPVK	T37	1	1	19	tP	proline-directed (WW-IV)/ FHA-1 pT binding motif	X
	STPQITGsPLDPNTPVK	S43	1	16	19	sP	proline-directed (WW-IV)	X
	SHNFSSSNtPYLK	T116	1	3	57	tP	proline-directed/ FHA-1 pT binding motif	X
Rpn1	DISSKsPSGNDALNDK	S10	1	8	0	sP	proline-directed	X
	DISSKsPSGNDALNDKK	S10	1	6	0	sP	proline-directed	X
	DISSKsPSGNDALNDKKGTK	S10	1	2	0	sP	proline-directed	X
Rpn2	TTEEKEAtPMEMDEEK	T856	1	3	30	tP	proline-directed	X
	DAVPASADTEPGEQEAsPPEDFEYPFDDDD	S952	1	3	3	sP	proline-directed	
Rpn1301	DQDLLDPARsDVATVSDMMEVDTVEQSEPIAQPTESSK	S122	1	4	0			

kinases (e.g., MAP kinases or CDK) and many others match consensus sites for casein-type kinases (CKI, CKII, see Tables 1−4 and Supplemental Table 2, Supporting Information). Phosphosites detected in the exclusively cytoplasmic DUBs also include sequences consistent with PIKK and GSK3 consensus sites and the cellular localization of these kinases (Table 2). While the nuclear DUB sites are primarily proline-directed sites, the partially nuclear DUBs, Ubp9 and Ubp12, have phosphopeptides consistent with phosphorylation by PKA/PKC (Table 3, Supplemental Table 2, Supporting Information).

There are also many phosphorylation-dependent WW class IV ligand motifs present in both the cytoplasmic and nuclear DUBs (Tables 2 and 3), suggesting that DUB interactions with WW domain-containing proteins could be controlled by phosphorylation. For instance, the HECT-type E3 Ub-ligases, Pub1, Pub2 and Pub3 and multiple components of the spliceosome contain WW domains and are likely regulated by a combination

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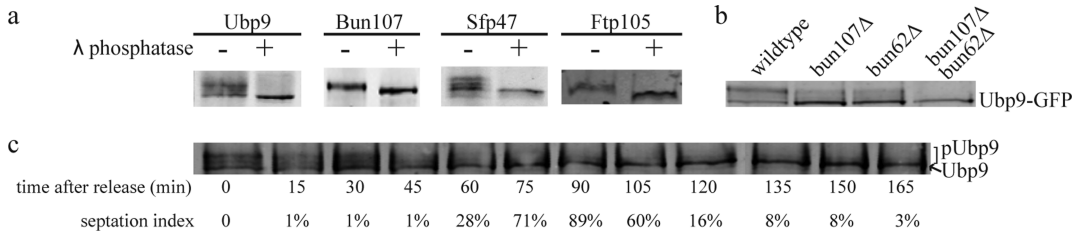

Figure 2. Biochemical analysis of DUB phosphorylation a) Lambda phosphatase collapse for Ubp9, Bun107, Sfp47, and Ftp105 b) phosphorylation status of Ubp9 in the presence or absence of its WD partners and c) block and release experiment illustrating the cell cycle dependency of Ubp9 phosphorylation (see Experimenal Methods for details).

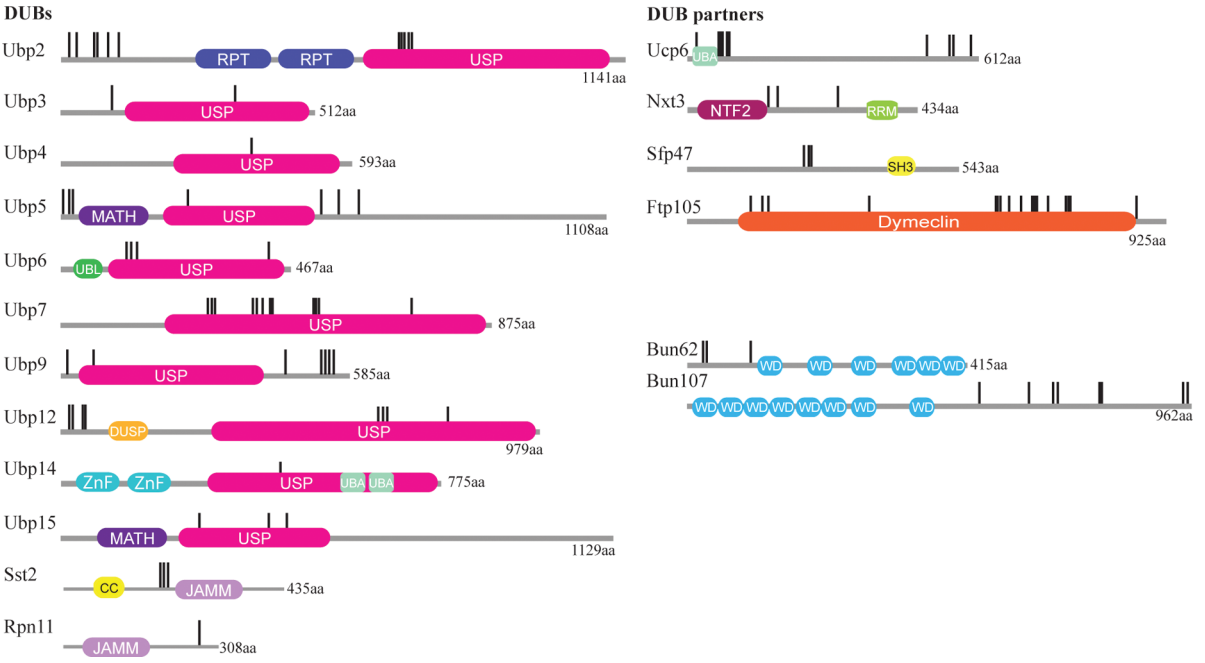

Figure 3. Domain architecture and mapping of detected phosphorylation sites within the *S. pombe* DUBs and their partners. Domain architectures were retrieved using the SMART and Pfam databases. The following domains were found: USP (ubiquitin-specific proteases) JAMM (JAB1/MPN/Mov34 metalloenzymes), DUSP (Domain in Ubiquitin-specific proteases), MATH (Meprin and TRAF homology), UBL (Ubiquitin-like), ZnF (Ubiquitin Carboxyl-terminal hydrolase-like zinc finger), UBA (Ubiquitin-associated). RPT are internal repeats. Phosphosites are denoted by vertical black lines.

of kinases and DUBs. The cytoplasmic DUB Ubp2 and endocytic DUBs Ubp5 and Ubp9 are phosphorylated on sites that match the FHA domain consensus binding motif that may function in localization and/or substrate recognition.

Concluding Remarks

Our results show that the majority of DUBs and most DUB partners are phosphorylated, some in a cell cycle-dependent manner. The phosphosites identified for *S. pombe* DUBs and their partners provide a foundation for understanding the interplay of ubiquitination and phosphorylation in this enzyme class in higher eukaryotes because sites identified in conserved proteins may be conserved or mimicked in higher eukaryotes. Future studies aimed at understanding the intersection of ubiquitination and phosphorylation will be useful for understanding DUB regulation and, more broadly, the cross-regulation of kinase and ubiquitin signaling networks.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

DUB, deubiquitinating enzyme; JAMM, JAB1/MPN/Mov34 metalloenzymes; LC−MS/MS, liquid chromatography−mass

spectrometry/mass spectrometry; MJD, Machado-Joseph disease proteases; MS, mass spectrometry; OTU, ovarian tumor proteases; Ub, ubiquitin; UCH, ubiquitin C-terminal hydrolases; USP, ubiquitin-specific proteases; TSC, total spectral counts

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