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Ubiquitin-Like Modifiers and Their Deconjugating Enzymes in Medically Important Parasitic Protozoa▽

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Protein modification by ubiquitin and ubiquitin-like proteins is one of the most complex and intensely studied mechanisms of posttranslational protein regulation in eukaryotes. Conjugation of the 76-amino-acid protein ubiquitin is first and foremost a signal for targeting proteins to the proteasome for degradation, but evidence that ubiquitin also plays diverse roles in the regulation of numerous biological pathways is building. In addition, there are many structurally related ubiquitin-like modifiers (Ubls) that utilize mechanistic pathways similar to those utilized by ubiquitin for conjugation to protein substrates and deconjugation. Despite similarities in structure between ubiquitin and other Ubls, modification by Ubls regulates such diverse cellular processes as transcriptional regulation, cell cycle control, and autophagy (see Kerscher et al. [22] for a review of Ubls and known functions). Ubiquitin has been identified in the majority of parasitic protozoa, but most Ubls in these organisms have not been characterized. Even less attention has been paid to the enzymes that regulate protein modification by ubiquitin or Ubls.

The essential roles of ubiquitin and Ubls in both protein turnover and transcriptional regulation in other organisms suggest that ubiquitin and Ubl pathways should be explored to better understand basic parasite biology. For this reason, we have compiled a comprehensive list of homologs of known Ubls and Ubl-deconjugating enzymes in medically important protozoa. We also discuss potential differences and unique characteristics of Ubls and deconjugating enzymes in parasites compared to those in mammals and yeast such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Notably absent from this review are the enzymes that conjugate ubiquitin and Ubls to their substrates. Although conjugation machinery is also important to the pathway, the essential role of deconjugating enzymes in multiple biological pathways and recent publications describing the identification of inhibitors of these enzymes indicate that they may represent a potentially important class of protease drug targets in parasites. Therefore, we have chosen to focus this review on these enzymes and the modifiers they regulate.

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REGULATING THE REGULATORS: THE UBIQUITIN MODIFICATION PATHWAY

Like ubiquitin itself, the mechanistic steps that add ubiquitin to and remove it from proteins are conserved across the Eukaryota (see Kerscher et al. [22] and Hemelaar et al. [12] for reviews of enzymatic details). Before conjugation, ubiquitin must first be proteolytically processed from its precursor form by ubiquitin-specific proteases (USPs) to reveal a C-terminal diglycine. Processed ubiquitin is then conjugated by a series of ligases to the ε-amino group of a protein lysine side chain via an isopeptide bond. Both the number of ubiquitin molecules (monoubiquitin or polyubiquitin) and the location of the modification determine the fate of the modified substrate. In addition to targeting proteins for degradation, ubiquitylation regulates protein localization and DNA damage repair (17). Ubiquitin is removed by selective proteases called deubiquitinating proteases (DUBs) that hydrolyze the isopeptide linkage. Many of these hydrolases both process ubiquitin to expose the C-terminal diglycine and cleave ubiquitin from conjugated substrates; therefore, the term DUBs is generally applied to hydrolases involved in either function. The general process of the maturation of ubiquitin, the conjugation of ubiquitin to substrates, and deconjugation is summarized in Fig. 1. It is a dynamic balance of conjugation and deconjugation that determines the fate of the protein being modified.

Although the majority of ubiquitin and Ubl pathways in mammalian and yeast cells have been studied and characterized, relatively little is known about how these systems are used by parasites. The complex life cycles and multiple disease-causing states of parasitic protozoa offer a unique context in which to study ubiquitin and Ubl modification pathways. The life cycles of most protozoan parasites within single or multiple hosts rely on strict timing of protein regulation and gene expression for both survival and virulence. The application of genomics and proteomics to numerous parasite species has confirmed that many genes and proteins are regulated in a life cycle-dependent manner (4, 6, 32). In the most striking example, the transcriptional profile of the intraerythrocytic life cycle of *Plasmodium falciparum* shows periodic waves of regulated gene expression for 80% of all genes expressed during the 48-h life cycle whereas only 15% of mammalian and yeast genes show such regulated expression patterns (4). While the regulation of gene expression and protein turnover is clearly critical for both life cycle and disease progression in medically important protozoa, the mechanisms regulating these processes are not well understood. Given the known functions of ubiquitin and Ubls in other organisms, a better understanding of these posttranslational modifiers is likely to be critical to understanding how parasites control many basic biological processes.

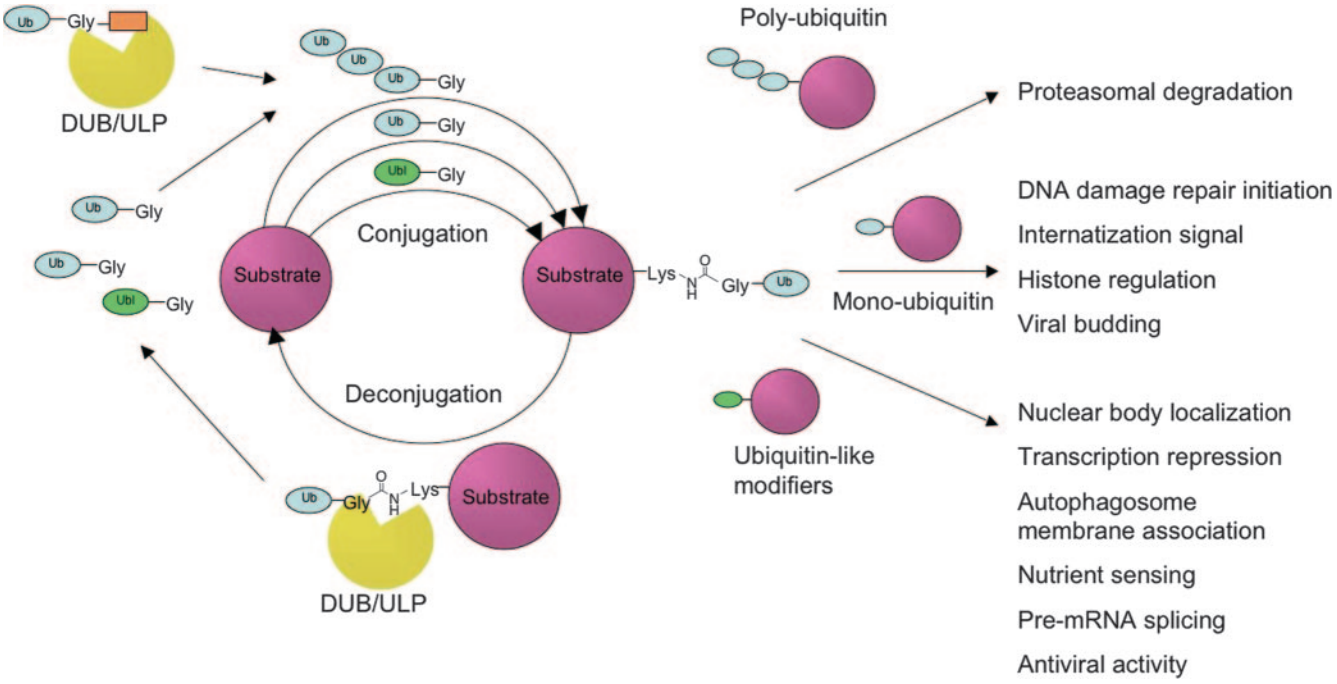

FIG. 1. Maturation, conjugation, and deconjugation of ubiquitin and Ubls. Before conjugation, ubiquitin (Ub) and Ubls are processed from a precursor form to expose their C termini. The C-terminal extension varies in both length and sequence. They are then conjugated to substrates by a series of conjugation enzymes. Modified substrates are then subjected to or stimulate a variety of biological processes, depending on the modification type. Eventually the modifier is removed and recycled by ubiquitin- or Ubl-specific proteases that cleave the isopeptide bond generated during conjugation.

UBIQUITIN AND Ubls

In addition to ubiquitin, a number of Ubls exist in most organisms (Table 1). While these Ubls all share general secondary and tertiary structures with ubiquitin, they each carry out diverse functional roles when used for the posttranslational modification of proteins. In order to begin to address the roles of ubiquitin and Ubl modification pathways in parasitic protozoa, it is first necessary to identify all ubiquitin and Ubl genes from sequenced genomes (Table 2). We searched the literature and conducted BLASTP homology searches, followed by reciprocal best-hit analysis, to assemble a list of parasite homologs of the Ubls. We identified homologs for six of the nine major Ubl families, including ubiquitin, Nedd8 (neural precursor cell-expressed developmentally down-regulated 8), small ubiquitin-related modifier (SUMO), Hub1, ubiquitin-related modifier 1 (Urm1), and autophagy-8 (Atg8), but failed to identify homologs for the interferon-stimulated gene protein 15 (ISG15), FAT10, or autophagy-12 (Atg12). Of the identified

TABLE 1. Common Ubls

Ubl	Known function(s)	Protozoa with predicted homologs	Protozoa for which characterization of Ubl has been published
Ubiquitin	Protein degradation, internalization, histone regulation	*Plasmodium*, *Toxoplasma*, *Leishmania*, *Trypanosoma*, *Entamoeba*, *Giardia*, *Cryptosporidium*, and *Theileria* spp.	*Plasmodium*, *Leishmania*, *Trypanosoma*, *Entamoeba*, and *Giardia* spp.
Nedd8	Ubiquitin conjugation	*Plasmodium* spp.	None
ISG15	Interferon response	None	None
SUMO	Transcriptional regulation, protein localization	*Plasmodium*, *Toxoplasma*, *Leishmania*, *Trypanosoma*, *Entamoeba*, *Cryptosporidium*, and *Theileria* spp.	None
FAT10	Ubiquitin-independent degradation	None	None
Hub1	Pre-mRNA splicing	*Plasmodium*, *Toxoplasma*, *Cryptosporidium*, *Theileria*, and *Entamoeba* spp.	None
Urm1	Starvation response	*Plasmodium*, *Leishmania*, *Trypanosoma*, *Entamoeba*, *Cryptosporidium*, and *Giardia* spp.	None
Atg8	Autophagy	*Plasmodium*, *Toxoplasma*, *Leishmania*, *Trypanosoma*, and *Theileria* spp.	*Trypanosoma* and *Leishmania* spp.
Atg12	Autophagy	None	None

families of Ubls, only ubiquitin and Atg8 have been characterized in parasitic protozoa.

We began our search with perhaps the most ancient Ubl, Urm1. Ubiquitin and Ubls are evolutionarily related to prokaryotic sulfur carrier proteins that utilize similar enzymatic methods of conjugation. Knowledge of this evolutionary link came from the structural comparison of the *Escherichia coli* sulfur carrier protein MoaD to the yeast Ubl Urm1, a protein involved in oxidative stress response and nutrient sensing but which is apparently nonfunctional in higher eukaryotes (48). Like yeast, parasitic protozoa contain homologs of Urm1, although to date none have been characterized functionally. Urm1 may or may not be functional, but it provides evidence that parasite ubiquitin and Ubls have an origin similar to that of Ubls of other organisms. Additionally, the study of parasitic protozoa may provide information about the evolutionary origins of ubiquitin conjugation systems, since functional urmylation pathways are not known to exist in organisms other than yeast.

Unlike Urm1, ubiquitin is both highly conserved and functional in all Eukaryota, including parasitic protozoa. The best-known function of ubiquitin is the targeting of proteins modified by a chain of four or more ubiquitins to the proteasome for degradation (39). Polyubiquitin chains with two different linkages, Lys48 and Lys63, have been observed in vivo in yeast (1). Lys48 linkages are utilized in polyubiquitin that targets proteins for degradation. The function of Lys63-linked polyubiquitin is less well understood, but this chain is known to play a role in the localization of the mitosis-regulatory protein survivin to the centromere (10). Additionally, monoubiquitylation is known to regulate histones and signal internalization by membrane proteins (14).

Parasitic protozoan ubiquitin and ubiquitin modification have been most extensively studied in *Trypanosoma* spp. Ubiquitin genes were first identified in *Trypanosoma cruzi* by two independent research groups nearly two decades ago (23, 43). Unlike humans, which have two polyubiquitin and two ubiquitin fusion proteins (45), *Trypanosoma cruzi* has at least five genes encoding proteins comprising ubiquitin fused to unrelated proteins and at least five genes encoding polyubiquitin. Further study of the ubiquitin-proteasome pathway in *Trypanosoma cruzi* revealed ubiquitin-dependent degradation of cytoskeletal proteins associated with the parasite flagellum during trypomastigote-to-amastigote transformation (7) and evidence that the many ubiquitin-encoding genes of *Trypanosoma cruzi* are differentially regulated during the parasite life cycle and growth phases (31). The polyubiquitin gene of *Plasmodium falciparum* is also regulated in a life cycle-dependent manner (16), and recent analysis of *Plasmodium* targets by a yeast two-hybrid assay has linked ubiquitin-regulating proteins to mRNA stability and transcriptional regulation (27). These data suggest a role for ubiquitin in the regulation of the life cycles of *Plasmodium* and *Trypanosoma* spp. and possibly other parasites.

Ubls vary greatly in their degree of conservation across species. The Ubl most closely related to ubiquitin, Nedd8, is 49% identical to mammalian ubiquitin and is regulated by the DUB UCH-L3 in addition to its own specific deconjugating enzyme (11, 28). Interestingly, a Nedd8 homolog was identified only in *Plasmodium* spp. Nedd8 may not actually be missing from other protozoa, but the high level of sequence homology of Nedd8 to ubiquitin within a single species and the relatively low level of sequence homology of Ubls other than ubiquitin across species suggests that Nedd8 homologs in parasites may be identified as second copies of ubiquitin, thus masking them in the reciprocal best-hit analysis. The function of Nedd8 in ubiquitin conjugation and Cullin regulation suggests that it is an important Ubl and therefore requires further parasite-to-parasite comparisons and experimental study to determine if it is in fact functional in *Plasmodium falciparum*.

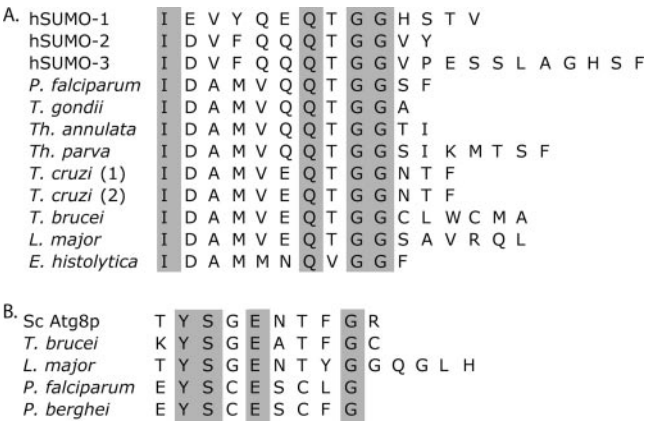

FIG. 2. C termini of aligned sequences of Ubls. (A) The alignment of parasite and human SUMO (hSUMO) homologs reveals that all species have one or more amino acids after the diglycine motif required for conjugation, thus indicating that processing is required before conjugation. *P. falciparum*, *Plasmodium falciparum*; *T. gondii*, *Toxoplasma gondii*; *Th. annulata*, *Theileria annulata*; *Th. parva*, *Theileria parva*; *T. cruzi*, *Trypanosoma cruzi*; *T. brucei*, *Trypanosoma brucei*; *L. major*, *Leishmania major*; *E. histolytica*, *Entamoeba histolytica*. (B) The alignment of parasite and yeast Atg8 homologs reveals that yeast and members of the kinetoplastid family, represented here by *Trypanosoma brucei* and *Leishmania major*, have residues beyond the conserved glycine but that members of the apicomplexan family, represented here by *Plasmodium falciparum* and *Plasmodium berghei*, have no additional residues, questioning the necessity of C-terminal processing before conjugation. Sc Atg8p, *Saccharomyces cerevisiae* Atg8p.

Like Nedd8 and all Ubls, SUMO is nearly identical to ubiquitin in overall structural fold but is divergent in both amino acid sequence and function (33). The primary function of SUMO is transcriptional regulation, usually in the form of repression, but other functions include the regulation of protein interaction and localization (20). SUMO homologs were identified in all of the organisms surveyed except *Giardia* spp. Unlike humans, which have four forms of SUMO, all of the parasite species surveyed (except *Trypanosoma cruzi*) have a single SUMO homolog, similar to yeast. The examination of alignments revealed that all parasite homologs have at least one amino acid after the final diglycine motif, confirming the necessity of a processing enzyme before SUMO can be conjugated (Fig. 2A).

The functions of SUMO are not limited to transcriptional regulation; protein interactions and localization can also be affected by SUMOylation. A yeast two-hybrid assay of *Plasmodium falciparum* provided evidence for interaction between SUMO and serine repeat antigen 4, an essential papain fold protease localized to the parasitophorous vacuole and hypothesized to play a role in erythrocyte rupture (27) (34). These

TABLE 2. Ubls and their putative parasite homologs as determined by reciprocal best-hit analysis

Ubl	Species	Ubl gene accession no. (parasite genome identifier)	BLASTP reciprocal best-hit		Reference(s)
			Accession no. (organism, protein name)	Expectation value	
Ubiquitin	*Plasmodium falciparum*	NP_701482 (PFL0585w)	AAH53371 (*Homo sapiens*, ribosomal fusion protein S27a)	$4e^{-36}$	16
	Toxoplasma gondii	38.m01076[a]	AAH08955.2 (*Homo sapiens*, ubiquitin C)	$7e^{-150}$	
	Theileria annulata	CAI73380	AAH08955.2 (*Homo sapiens*, ubiquitin C)	$4e^{-72}$	
	Theileria parva	EAN33959	AAH08955.2 (*Homo sapiens*, ubiquitin C)	$3e^{-72}$	
	Leishmania major	CAJ09316	BAA23486 (*Homo sapiens*, polyubiquitin)	0	23
	Trypanosoma brucei	XP_829056	BAA23486 (*Homo sapiens*, polyubiquitin)	0	23
	Trypanosoma cruzi	P08565	BAA23486 (*Homo sapiens*, polyubiquitin)	$4e^{-36}$	
	Cryptosporidium hominis	XP_667472	AAH08955.2 (*Homo sapiens*, ubiquitin C)	$8e^{-112}$	
	Cryptosporidium parvum	XP_626192	AAH08955.2 (*Homo sapiens*, ubiquitin C)	$2e^{-112}$	
	Entamoeba histolytica	CAA67177	AAH53371 (*Homo sapiens*, ribosomal fusion protein S27a)	$3e^{-34}$	46
	Giardia lamblia	X70050	AAH53371 (*Homo sapiens*, ribosomal fusion protein S27a)	$2e^{-33}$	26
Nedd8	*Plasmodium falciparum*	NP_705038 (MAL13P1.64)	AAI04202 (*Homo sapiens*, Nedd8)	$7e^{-17}$	
	Plasmodium chabaudi	CAH83092	AAI04202 (*Homo sapiens*, Nedd8)	$1e^{-16}$	
	Plasmodium berghei	CAH95491	AAI04202 (*Homo sapiens*, Nedd8)	$1e^{-16}$	
ISG15	None		CAI15574 (*Homo sapiens*, ISG15)		
SUMO	*Trypanosoma cruzi*	EAN92418	CAA67896 (*Homo sapiens*, SUMO3)	$3e^{-19}$	
	Trypanosoma cruzi	EAN95569	CAA67896 (*Homo sapiens*, SUMO3)	$4e^{-19}$	
	Trypanosoma brucei	AAX79561	AAH66306 (*Homo sapiens*, SUMO1)	$9e^{-19}$	
	Leishmania major	CAJ02226	CAA67896 (*Homo sapiens*, SUMO3)	$4e^{-18}$	
	Plasmodium falciparum	NP_703403 (PFE0285c)	CAA67896 (*Homo sapiens*, SUMO3)	$5e^{-18}$	
	Theileria annulata	CAI73057	CAA67896 (*Homo sapiens*, SUMO3)	$8e^{-13}$	
	Entamoeba histolytica	XP_655984	AAI07854 (*Homo sapiens*, SUMO2)	$5e^{-18}$	
	Theileria parva	EAN34278	CAA67896 (*Homo sapiens*, SUMO3)	$8e^{-13}$	
	Cryptosporidium hominis	XP_665282	AAH66306 (*Homo sapiens*, SUMO1)	$6e^{-13}$	
	Cryptosporidium parvum	XP_627315	AAH66306 (*Homo sapiens*, SUMO1)	$6e^{-13}$	
	Toxoplasma gondii	57.m01794	AAH66306 (*Homo sapiens*, SUMO1)	$2e^{-13}$	
FAT10	None		AAD52982 (*Homo sapiens*, FAT10)		
Hub1	*Plasmodium falciparum*	XP_001350772 (PFL1830w)	NP_014430 (*Saccharomyces cerevisiae*, Hub1p)	$4e^{-18}$	
	Plasmodium berghei	XP_680294	NP_014430 (*Saccharomyces cerevisiae*, Hub1p)	$1e^{-18}$	
	Plasmodium yoelii	XP_726593	NP_014430 (*Saccharomyces cerevisiae*, Hub1p)	$1e^{-18}$	
	Toxoplasma gondii	55.m04782	NP_014430 (*Saccharomyces cerevisiae*, Hub1p)	$3e^{-20}$	
	Cryptosporidium parvum	XP_001388147	NP_014430 (*Saccharomyces cerevisiae*, Hub1p)	$9e^{-18}$	
	Theileria parva	XP_762746	NP_014430 (*Saccharomyces cerevisiae*, Hub1p)	$3e^{-17}$	
	Theileria annulata	XP_955328	NP_014430 (*Saccharomyces cerevisiae*, Hub1p)	$9e^{-17}$	
	Entamoeba histolytica	XP_648708	NP_014430 (*Saccharomyces cerevisiae*, Hub1p)	$4e^{-16}$	
Urm1	*Plasmodium chabaudi*	XP_740984	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$8e^{-8}$	
	Plasmodium falciparum	NP_701252 (PF11_0393)	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$4e^{-8}$	
	Plasmodium berghei	CAH95991	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$1e^{-7}$	
	Giardia lamblia	XP_779378	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$5e^{-6}$	

Continued on following page

TABLE 2—*Continued*

Ubl	Species	Ubl gene accession no. (parasite genome identifier)	BLASTP reciprocal best-hit		Reference(s)
			Accession no. (organism, protein name)	Expectation value	
	Plasmodium yoelii	EAA18635	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$2e^{-7}$	
	Cryptosporidium hominis	XP_668249	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$2e^{-13}$	
	Cryptosporidium parvum	EAK90632	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$5e^{-13}$	
	Trypanosoma cruzi	EAN88200	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$1e^{-12}$	
	Trypanosoma brucei	AAX79740	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$2e^{-12}$	
	Leishmania major	CAJ08004	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$4e^{-11}$	
	Entamoeba histolytica	XP_657081	NP_012258 (*Saccharomyces cerevisiae*, Urm1p)	$3e^{-10}$	
Atg8	*Plasmodium falciparum*	NP_700667 (PF10_0193)	NP_009475 (*Saccharomyces cerevisiae*, Atg8p)	$1e^{-24}$	
	Plasmodium berghei	XP_678543	NP_009475 (*Saccharomyces cerevisiae*, Atg8p)	$5e^{-28}$	
	Plasmodium yoelii	EAA17180	NP_009475 (*Saccharomyces cerevisiae*, Atg8p)	$5e^{-28}$	
	Plasmodium chabaudi	XP_745350	NP_009475 (*Saccharomyces cerevisiae*, Atg8p)	$5e^{-28}$	
	Toxoplasma gondii	52.m00003	NP_009475 (*Saccharomyces cerevisiae*, Atg8p)	$4e^{-30}$	
	Theileria parva	EAN32621	NP_009475 (*Saccharomyces cerevisiae*, Atg8p)	$9e^{-18}$	
	Theileria annulata	CAI74649	NP_009475 (*Saccharomyces cerevisiae*, Atg8p)	$3e^{-17}$	
	Trypanosoma brucei	AAX78826 (Tb07.10C21.40)	NP_009475 (*Saccharomyces cerevisiae*, Atg8p)	$7e^{-33}$	13
		AAX78827		$3e^{-30}$	
		AAX70074		$3e^{-18}$	
	Trypanosoma cruzi	EAN97061 (Tc00.1047053510533.180)	NP_009475 (*Saccharomyces cerevisiae*, Atg8p)	$6e^{-20}$	13
		EAN96431		$2e^{-34}$	
	Leishmania major	CAJ07266 (LmjF19.1630)	NP_009475 (*Saccharomyces cerevisiae*, Atg8p)	$2e^{-31}$	13, 40
Atg12	None		P38316 (*Saccharomyces cerevisiae*, ATG12)		13, 40

[a] *Toxoplasma* protein sequences are not yet available in NCBI, so all accession numbers are for ToxoDB only.

data must be confirmed in vivo, but they offer another example of the potential for the regulation of proteins unique to parasitic protozoa.

Although ubiquitin and SUMO have different functions, evidence indicates that these modifiers act as competing controls of several biological pathways. During S phase in the yeast cell cycle, both ubiquitin and SUMO can modify proliferating cell nuclear antigen (PCNA) at the same lysine residue (38). Ubiquitylation of PCNA at K164 is required for DNA damage repair, while SUMOylation of PCNA at K164 prevents recombination events in replicating regions of DNA. Although the relationship between ubiquitylation and SUMOylation is not fully understood, their competing roles in essential cell cycle process controls in yeast suggest that they also play interesting roles in the unique life cycles of parasitic protozoa.

Atg8 is a unique Ubl that is conjugated to lipids rather than proteins. Autophagy is the process by which cells engulf and degrade proteins and organelles during differentiation or as a defense under starvation conditions (29). The process of autophagy is characterized by the formation of autophagosomes, membranous structures that engulf cellular matter for degradation. The formation of the autophagosome is dependent on the conjugation of Atg8 to the amine group of phosphoethanolamine (PE). In addition, Atg12, another Ubl, must be conjugated to the ε-amino lysine (L128) side chain of Atg5 (44). Parasite homologs show 30 to 50% conservation compared to yeast Atg8. Homologs in kinetoplastids, the family of parasites that includes *Trypanosoma* and *Leishmania* spp., have one or more amino acid residues after the single C-terminal diglycine that need to be processed before conjugation to PE. However, apicomplexans, the family of parasites that includes *Plasmodium* and *Toxoplasma* spp., have no additional residues (Fig. 2B). Surprisingly, Atg12 is missing from all protozoa examined despite the observation of functional autophagosomes and autophagy in *Leishmania major* (3).

Several Ubls, such as the diubiquitins FAT10 and ISG15, are noticeably absent in parasitic protozoa. As Ubls that appear to function in response to cancer and immune stimuli, respec-

TABLE 3. Ubl-deconjugating enzymes and their putative parasite homologs based on BLASTP results and active-site-residue alignment

Enzyme (organism, accession no.)	Parasite species with homolog	Parasite homolog gene NCBI accession no. (parasite genome identifier)	Expectation value for BLASTP vs genome	Reference(s)
Ubiquitin-deconjugating enzymes				
UCHs				
UCH-L3 (*Homo sapiens*, P15374)	*Plasmodium falciparum*	AAN37189 (PF14_0576)	$6e^{-20}$	9, 47
	Plasmodium yoelii	EAA21121	$6e^{-15}$	
	Toxoplasma gondii	55.m05062	$8e^{-36}$	
	Cryptosporidium hominis	XP_668440	$6e^{-18}$	
	Cryptosporidium parvum	XP_627961	$4e^{-19}$	
	Trypanosoma cruzi	EAN94987	$8e^{-38}$	
	Trypanosoma brucei	XP_828117	$6e^{-42}$	
	Leishmania major	CAJ04230	$4e^{-27}$	
UCH-L5 (*Homo sapiens*, Q9Y5K5)	*Plasmodium falciparum*	NP_701037 (PF11_0177)	$1e^{-29}$	47
	Plasmodium berghei	CAH95599	$1e^{-30}$	
	Plasmodium yoelii	XP_724692	$1e^{-33}$	
	Plasmodium chabaudi	XP_740948	$6e^{-30}$	
	Toxoplasma gondii	50.m00034	$3e^{-49}$	
	Cryptosporidium hominis	XP_668440	$3e^{-52}$	
	Cryptosporidium parvum	XP_627961	$9e^{-52}$	
	Trypanosoma cruzi	EAN86456	$1e^{-52}$	
		EAN81045	$2e^{-33}$	
	Trypanosoma brucei	XP_828589	$3e^{-51}$	
	Leishmania major	CAJ04230	$4e^{-50}$	
	Entamoeba histolytica	XP_654194	$2e^{-42}$	
USPs				
USP7 (*Mus musculus*, AAI0067)	*Plasmodium falciparum*	NP_704193 (MAL7P1.147)	$5e^{-52}$	47
	Plasmodium yoelii	XP_729206	$2e^{-52}$	
	Toxoplasma gondii	80.m00082	$7e^{-61}$	
	Theileria annulata	CAI75715	$6e^{-58}$	
	Cryptosporidium hominis	XP_666360	$4e^{-61}$	
	Cryptosporidium parvum	XP_627060	$4e^{-61}$	
	Trypanosoma cruzi	EAN91491	$2e^{-60}$	
		EAN98443	$1e^{-51}$	
		EAN95845	$1e^{-32}$	
	Trypanosoma brucei	EAN77302	$4e^{-66}$	
		EAN76617	$5e^{-51}$	
	Leishmania major	AAZ14396	$1e^{-78}$	
		CAJ03358	$4e^{-35}$	
		CAJ08130	$3e^{-25}$	
	Entamoeba histolytica	EAL48197	$3e^{-25}$	
Other USPs	*Plasmodium falciparum*	PFA0220w		47
		PFD0165w		
		PFD0608c		
		PFE1355c		
		PFE0835w		
		PFI0225w		
		PF13_0096		
		PF14_0145		
MJD Ataxin-3(*Mus musculus*, NP_08391)	*Plasmodium falciparum*	NP_701621 (PFL1295w)	$4e^{-16}$	42
	Plasmodium berghei	XP_670958	$6e^{-15}$	
	Plasmodium yoelii	EAA19332	$1e^{-14}$	
	Toxoplasma gondii	44.m02555	$2e^{-35}$	
	Cryptosporidium hominis	XP_667276	$3e^{-20}$	
	Cryptosporidium parvum	XP_627894	$3e^{-20}$	
Otubain proteases				
A20	None			
VCIP135	None			
JAB1/MPN/Mov34 metalloenzyme POH1 (*Homo sapiens*, NP_005796)	*Plasmodium falciparum*	NP_705563 (MAL13P1.343)	$5e^{-105}$	
	Plasmodium berghei	XP_676818	$6e^{-103}$	
	Plasmodium yoelii	EAA22608	$1e^{-103}$	
	Toxoplasma gondii	59.m00030	$2e^{-112}$	
	Theileria parva	EAN32483	$5e^{-108}$	
	Theileria annulata	CAI74788	$5e^{-108}$	
	Cryptosporidium parvum	CAD98369	$3e^{-104}$	
	Cryptosporidium hominis	XP_667262	$3e^{-103}$	
	Trypanosoma brucei	AAL72634	$9e^{-78}$	
	Trypanosoma cruzi	EAN85253	$8e^{-76}$	
		EAN93016	$5e^{-70}$	
	Leishmania major	CAJ07770	$1e^{-77}$	
	Entamoeba histolytica	XP_650487	$1e^{-93}$	
	Giardia intestinalis	CAB97491	$2e^{-48}$	
	Giardia lamblia	XP_778570	$2e^{-48}$	

Continued on following page

TABLE 3—*Continued*

Enzyme (organism, accession no.)	Parasite species with homolog	Parasite homolog gene NCBI accession no. (parasite genome identifier)	Expectation value for BLASTP vs genome	Reference(s)
PPPDE[a] (*Cryptosporidium parvum*, XP_627971)	*Plasmodium falciparum*	NP_701537 (PFL0865w)	$8e^{-27}$	19
	Plasmodium berghei	XP_679861	$8e^{-27}$	
	Plasmodium yoelii	XP_725065	$6e^{-19}$	19
	Plasmodium chabaudi	XP_741893	$2e^{-27}$	
	Cryptosporidium hominis	XP_668431	0	
	Toxoplasma gondii	50.m03185	$1e^{-43}$	
	Trypanosoma cruzi	EAN94109	$1e^{-7}$	
		EAN87232	$1e^{-7}$	
	Trypanosoma brucei	EAN80399	$5e^{-5}$	
	Leishmania major	CAJ08653	$8e^{-8}$	
	Entamoeba histolytica	EAL51330	$2e^{-4}$	
	Giardia lamblia	XP_768551	$1e^{-24}$	19
Nedd8-specific deconjugating enzyme NEDP1 (*Homo sapiens*, Q96LD8)	None			
SUMO-deconjugating enzymes				
Ubiquitin-like proteases				
Ulp1 (*Saccharomyces cerevisiae*, Q02724)	*Plasmodium falciparum*	NP_701689 (PFL1635w)	$1e^{-23}$	47
		NP_704529 (MAL8P1.157)	$1e^{-5}$	
	Plasmodium berghei	XP_671926	$2e^{-18}$	
		XP_677733	$3e^{-5}$	
	Plasmodium yoelii	EAA21830	$5e^{-22}$	
		EAA23028	$9e^{-5}$	
	Plasmodium chabaudi	XP_736612	$1e^{-19}$	
		XP_743639	$4e^{-5}$	
		XP_741227	$4e^{-5}$	
	Theileria parva	EAN31525	$4e^{-18}$	
		EAN32232	$2e^{-6}$	
	Theileria annulata	CAI76227	$2e^{-12}$	
		CAI76877	$1e^{-7}$	
	Toxoplasma gondii	33.m01285	$9e^{-23}$	
		57.m01727	$5e^{-15}$	
	Trypanosoma cruzi	EAN82253	$6e^{-11}$	
		EAN90516	$8e^{-11}$	
	Trypanosoma brucei	EAN76330	$3e^{-10}$	
	Entamoeba histolytica	XP_657158	$9e^{-16}$	
	Cryptosporidium parvum	XP_626217	$3e^{-27}$	
	Cryptosporidium hominis	XP_665558	$1e^{-15}$	
Ulp2 (*Schizosaccharomyces pombe*, O13769)	*Plasmodium falciparum*	MAL8P1.157	n/a	47
Wss1p metalloprotease (*Saccharomyces cerevisiae*, NP_012002)	*Plasmodium falciparum*	NP_700566 (PF10_0092)	$4e^{-12}$	19
	Plasmodium berghei	XP_676977	$2e^{-8}$	
	Trypanosoma brucei	EAN80397	$5e^{-5}$	19
	Trypanosoma cruzi	EAN87230	$3e^{-12}$	
		EAN80397	$5e^{-12}$	
	Leishmania major	CAJ08651	$9e^{-12}$	
Autophagy-related deconjugating enzyme Atg4 (*Saccharomyces cerevisiae*, P53867)	*Plasmodium falciparum*	NP_702059 (PF14_0171)	$4e^{-4}$	
	Plasmodium yoelii	EAA22584	$1e^{-5}$	
	Cryptosporidium parvum	XP_626849	$3e^{-15}$	
	Theileria annulata	CAI74479	$2e^{-8}$	
	Trypanosoma cruzi	EAN87801 (Tc00.1047053509443.30)	$7e^{-25}$	13
		EAN84153		
		EAN91243	$7e^{-9}$	
			$2e^{-8}$	
	Trypanosoma brucei	EAN80574 (Tb11.01.7979)	$5e^{-12}$	13
		AAX79730 (Tb06.28P18.550)	$5e^{-7}$	
	Leishmania major	CAJ08920 (Lmj32.3890)	$3e^{-17}$	13, 40
		CAJ05824 (Lmj30.0270)	$2e^{-11}$	
	Entamoeba histolytica	XP_656724	$7e^{-10}$	
		XP_653798	$6e^{-9}$	
		XP_652043	$2e^{-5}$	

[a] Predicted protease family.

tively, it is not surprising that these Ubls identified in multicellular organisms are not found in unicellular organisms. Although parasites have homologs of many, but not all, of the conserved Ubls, further study will be required to determine if protozoa have their own unique Ubls.

UBIQUITIN- AND Ubl-DECONJUGATING ENZYMES

The deconjugating enzymes of ubiquitin and Ubls are mainly cysteine proteases but include representatives of multiple cysteine protease clans, as well as metalloproteases. To identify parasite homologs, BLASTP homology searches were performed with representative proteases from each class of deconjugating enzymes. Representative proteases were chosen based on available crystallographic or experimental data identifying relevant catalytic residues for that enzyme. This method allowed for subsequent ClustalW alignments. Only those homologs that were identified by both BLASTP homology searches and active-site-residue alignment are included.

DUBs. DUBs can carry out a number of processing events, including the maturation of the C termini of ubiquitin precursors, the removal of a single ubiquitin from a polyubiquitin chain, and the removal of ubiquitin from conjugated substrates. There are close to 90 DUBs in humans, and these DUBs fall into five subclasses: ubiquitin C-terminal hydrolases (UCHs), USPs, otubain proteases, Machado-Joseph disease (MJD) proteases, and JAB1/MPN/Mov34 metalloenzymes (see Nijman et al. [35)] for structural and functional comparisons). Parasitic protozoa have homologs of four out of the five major classes of DUBs as well as homologs of a predicted class, that of permuted papain fold peptidases of double-stranded RNA viruses and eukaryotes (PPPDEs) (Table 3).

The first proteolytically active DUBs to be found in protozoan parasites were recently identified in *Plasmodium falciparum* and *Toxoplasma gondii*. Using an activity-based probe, which contains full-length human ubiquitin and had the C-terminal glycine residue replaced with a reactive functional group that irreversibly binds the active-site cysteine of deconjuating enzymes, Artavanis-Tsakonas et al. (2) and Frickel et al. (9) selectively labeled and identified UCH-54 (corresponding to accession no. PF11_0177) in *Plasmodium falciparum* and UCH-L3 (corresponding to accession no. 55.m050682) in *Toxoplasma gondii*. These DUBs also showed cross-reactivity with a similar probe for human Nedd8, suggesting that the same deconjugating enzyme may regulate both ubiquitin and Nedd8 homologs. This possibility may explain why *Plasmodium falciparum* has a Nedd8 homolog but no Nedd8-specific protease homolog (Tables 2 and 3). Since the human Nedd8 used to make the probe is more closely related to human and *Plasmodium falciparum* ubiquitin (58% conserved) than to *Plasmodium falciparum* Nedd8 (52.6% conserved), parasite-derived Nedd8 probes will be required to confirm cross-reactivity.

Recent genetic analysis of variants of the rodent malaria parasite *Plasmodium chabaudi* resistant to the antimalarial drugs artesunate and chloroquine identified mutations in a ubiquitin-deconjugating enzyme with strong genetic linkage to drug resistance (18). This DUB was found to be most similar to the *Plasmodium falciparum* MAL7P1.147 DUB described as a USP7 homolog in Table 3. Although subsequent analysis of drug-resistant *Plasmodium falciparum* did not identify mutations in the MAL7P1.147 enzyme, the authors speculate that this result was due to the transient nature of the *Plasmodium falciparum* resistance compared to the stable resistance found in *Plasmodium chabaudi*. Further work to characterize this DUB in stably artemisinin-resistant *Plasmodium falciparum* is necessary to determine what, if any, role this enzyme may play in parasite drug resistance.

Interestingly, *Plasmodium*, *Toxoplasma*, and *Cryptosporidium* spp. all have homologs of the MJD subclass protease Ataxin-3, a ubiquitin-deconjugating enzyme that has been linked to neurodegenerative disease in mammals (5). The parasite homologs are 19.9 to 29.4% conserved compared to human Ataxin-3, but the catalytic triad consists of conserved cysteine, histidine, and aspartate rather than asparagine. This aspartate-for-asparagine substitution has been observed in previous Ataxin-3 homolog sequence alignments (42). Both aspartate and asparagines are found in the catalytic triads of cysteine proteases, but the functional significance of these residue substitutions in parasite homologs remains to be explored. Surprisingly, no homolog of Ataxin-3 in yeast has been identified. The parasite homologs identified do not appear to have an expanded glutamine repeat region, the hallmark of the disease-causing form of Ataxin-3. Parasitic protozoa offer a potential model system in which to study the normal function of Ataxin-3, which is still not well understood.

The parasite homologs of Ataxin-3 do not have a glutamine-rich region, but the recently identified DUB *Plasmodium falciparum* UCH-54 has an asparagine repeat region in the predicted protein sequence. The predominant protein identified by mass spectrometry was nearly double the predicted size (100 kDa compared to the predicted 54 kDa), possibly as a result of protein aggregation that was stable under sodium dodecyl sulfate-polyacrylamide gel electrophoresis conditions (2). Our own alignments revealed asparagine repeats in the *Plasmodium falciparum* USP7 and Ulp1 homologs and unusual glutamine-glutamate (QEEQ) and glutamine-glutamate-lysine (QEKK) repeats in the Ulp1 homolog in regions not homologous to any other protozoan sequences aligned (E.L. Ponder, unpublished data). In agreement with this assessment, asparagine- and glutamine-asparagine-rich regions of yeast prion-forming proteins are sufficient to form self-seeding protein aggregates similar to those that cause Alzheimer's and Huntington's diseases (37). Further study of both parasites and other eukaryotes is required to determine the significance of these repeat regions and their potential role in protein aggregation.

An additional subclass of predicted DUBs included in this survey was the PPPDEs. Using a bioinformatics approach, Iyer et al. (19) identified the PPPDE class of DUBs, whose prototype is a hypothetical protein from the apicomplexan *Cryptosporidium parvum*. Although this study did not provide confirmation of DUB activity for any members of this class, bioinformatics approaches did identify the majority of accepted classes of DUBs (35). As exemplified by the identification of PPPDEs, the study of ubiquitin and Ubls in protozoa has the potential to identify new players in these pathways as well as novel functions.

Ubl proteases. Like ubiquitin-deconjugating enzymes, SUMO-deconjugating enzymes cleave precursor SUMO to the active form containing the required C-terminal diglycine motif

and cleave SUMO from substrates (20). Although SUMO and SUMOylation pathways have not been characterized in any parasite, the conservation of SUMO across yeast and mammals suggests that SUMO is a candidate for the regulation of transcription in parasite development. Of the parasites surveyed in this study, the majority have only one homolog of the essential yeast de-SUMOylation enzyme Ulp1 (19.9 to 30.4% conserved) (Table 3). *Plasmodium* spp. and *Theileria* spp. parasites, however, have two homologs. Although *Plasmodium* was previously predicted to have homologs of both yeast Ulp1 and Ulp2 (a nonessential second homolog of Ulp1) (47), we found that both of these homologs aligned better with Ulp1 in our own searches for the alignment of active-site residues (Ponder, unpublished). Further genomic and functional characterization is needed to understand the evolutionary origins of the corresponding genes and their functions in parasites.

Autophagy-related proteases. Autophagy is the only proven example of a classic Ubl pathway with a novel function and importance in protozoa. Autophagy-related protein 4 (Atg4) is a papain fold cysteine protease that processes Atg8 to expose a C-terminal glycine for conjugation and cleaves Atg8 from its conjugated PE on the outer layer of the autophagosome (24, 25). Homologs of Atg4 in both apicomplexans and kinetoplastids (16.5 to 21.8% conserved) have been identified (Table 3). The apicomplexans have one homolog, while the kinetoplastids and *Entamoeba histolytica* have multiple copies. The disruption of Atg4 leads to defects in autophagosome trafficking in *Leishmania major* (3). Additionally, parasites expressing a mutant ATPase that results in the accumulation of autophagosomes and increased susceptibility to starvation (i.e., an autophagy defect) are unable to transition from the promastigote to the infective metacyclic stage (3). Atg8 and Atg4 are highly conserved across protozoa, while all parasitic protozoa lack Atg12, a finding that is in agreement with the results of previous bioinformatics searches for Atg12 in kinetoplastids (13). Autophagy is functional in *Leishmania major* even without an Atg12 homolog (3), confounding the hypothesis that the conjugation of both Atg8 and Atg12 is necessary for autophagy in response to starvation. More extensive experimental evaluation of the autophagy pathway in protozoa is necessary to understand this discrepancy.

Autophagy may also be linked to the effects of chloroquine on mammalian cells and intraerythrocytic *Plasmodium falciparum*. In the early 1980s, it was reported that chloroquine induces the formation of autophagic vacuoles in treated lymphocytes (21) and the accumulation of endocytic vesicles in treated *Plasmodium falciparum* parasites (49). Although significant efforts have been made to understand chloroquine-mediated killing, its mechanism of action still remains unclear (see Olliaro and Goldberg [36] for a review of chloroquine-mediated killing). The vacuolarization of *Plasmodium falciparum* upon treatment with antimalarial agents has also been postulated to be an early sign of apoptotic blebbing. However, the potential role of apoptosis in a unicellular organism remains the subject of debate (see Deponte and Becker [8] for a review of apoptosis in protozoa). Further characterization of these vacuoles using a marker for the autophagosome, such as Atg8, may help clarify the mode of killing by antimalarial drugs. Analysis of autophagy in other protozoa will likely provide additional information to explain how parasites use autophagy in their normal development as well as to combat drug-induced starvation.

CONCLUSIONS AND FUTURE DIRECTIONS

Medically important parasitic protozoa have homologs of many key Ubls, ranging from Urm1, a minimally understood relative of a bacterial sulfur carrier protein, to ubiquitin, one of the most well-conserved proteins in all Eukaryota. Although these Ubls may have similar functions in both parasites and other eukaryotes, evidence of life cycle-dependent ubiquitin gene regulation in *Trypanosoma cruzi* and *Plasmodium falciparum*, the potential interaction of SUMO and serine repeat antigen 4 in *Plasmodium falciparum*, and the identification of a genetic linkage between a DUB and artesunate resistance in *Plasmodium chabaudi* demonstrate the need to identify unique parasite targets of these and other Ubls. Evidence of the essential roles of the ubiquitin-proteasome and autophagy pathways in the development of *Trypanosoma cruzi* and *Leishmania major*, respectively, also suggest that further study of Ubl pathways will lead to a better understanding of parasite life cycle regulation.

Proteases have generally been identified as potential drug targets in parasites including *Plasmodium falciparum* and *Trypansoma cruzi* (41). Therefore, further characterization of DUBs may help validate proteases as a new class of drug targets while also providing insight into the regulation of basic parasite biology. This characterization may be further facilitated as more inhibitors of these classes of enzymes are identified (12, 15, 30). As additional deconjugating enzymes are further characterized functionally, we hope our compilation of homologs will allow easier extrapolation of findings to other medically relevant parasites.

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REFERENCES

1. **Arnason, T., and M. J. Ellison.** 1994. Stress resistance in *Saccharomyces cerevisiae* is strongly correlated with assembly of a novel type of multiubiquitin chain. Mol. Cell. Biol. **14:**7876–7883.
2. **Artavanis-Tsakonas, K., S. Misaghi, C. A. Comeaux, A. Catic, E. Spooner, M. T. Duraisingh, and H. L. Ploegh.** 2006. Identification by functional proteomics of a deubiquitinating/deNeddylating enzyme in Plasmodium falciparum. Mol. Microbiol. **61:**1187–1195.
3. **Besteiro, S., R. A. Williams, L. S. Morrison, G. H. Coombs, and J. C. Mottram.** 2006. Endosome sorting and autophagy are essential for differentiation and virulence of Leishmania major. J. Biol. Chem. **281:**11384–11396.
4. **Bozdech, Z., M. Llinas, B. L. Pulliam, E. D. Wong, J. Zhu, and J. L. DeRisi.** 2003. The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. PLoS Biol. **1:**E5.
5. **Burnett, B. G., and R. N. Pittman.** 2005. The polyglutamine neurodegenerative protein ataxin 3 regulates aggresome formation. Proc. Natl. Acad. Sci. USA **102:**4330–4335.
6. **Cleary, M. D., U. Singh, I. J. Blader, J. L. Brewer, and J. C. Boothroyd.** 2002. *Toxoplasma gondii* asexual development: identification of developmentally regulated genes and distinct patterns of gene expression. Eukaryot. Cell **1:**329–340.
7. **de Diego, J. L., J. M. Katz, P. Marshall, B. Gutierrez, J. E. Manning, V. Nussenzweig, and J. Gonzalez.** 2001. The ubiquitin-proteasome pathway plays an essential role in proteolysis during Trypanosoma cruzi remodeling. Biochemistry **40:**1053–1062.
8. **Deponte, M., and K. Becker.** 2004. Plasmodium falciparum: do killers commit suicide? Trends Parasitol. **20:**165–169.
9. **Frickel, E. M., V. Quesada, L. Muething, M. J. Gubbels, E. Spooner, H. Ploegh, and K. Artavanis-Tsakonas.** 2007. Apicomplexan UCHL3 retains

dual specificity for ubiquitin and Nedd8 throughout evolution. Cell. Microbiol. **9:**1601–1610.
10. **Gutierrez, G. J., and Z. Ronai.** 2006. Ubiquitin and SUMO systems in the regulation of mitotic checkpoints. Trends Biochem. Sci. **31:**324–332.
11. **Hemelaar, J., A. Borodovsky, B. M. Kessler, D. Reverter, J. Cook, N. Kolli, T. Gan-Erdene, K. D. Wilkinson, G. Gill, C. D. Lima, H. L. Ploegh, and H. Ovaa.** 2004. Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. Mol. Cell. Biol. **24:**84–95.
12. **Hemelaar, J., P. J. Galardy, A. Borodovsky, B. M. Kessler, H. L. Ploegh, and H. Ovaa.** 2004. Chemistry-based functional proteomics: mechanism-based activity-profiling tools for ubiquitin and ubiquitin-like specific proteases. J. Proteome Res. **3:**268–276.
13. **Herman, M., S. Gillies, P. A. Michels, and D. J. Rigden.** 2006. Autophagy and related processes in trypanosomatids: insights from genomic and bioinformatic analyses. Autophagy **2:**107–118.
14. **Hicke, L.** 2001. Protein regulation by monoubiquitin. Nat. Rev. Mol. Cell Biol. **2:**195–201.
15. **Hirayama, K., S. Aoki, K. Nishikawa, T. Matsumoto, and K. Wada.** 2007. Identification of novel chemical inhibitors for ubiquitin C-terminal hydrolase-L3 by virtual screening. Bioorg. Med. Chem. **15:**6810–6818.
16. **Horrocks, P., and C. I. Newbold.** 2000. Intraerythrocytic polyubiquitin expression in Plasmodium falciparum is subjected to developmental and heat-shock control. Mol. Biochem. Parasitol. **105:**115–125.
17. **Huang, T. T., S. M. Nijman, K. D. Mirchandani, P. J. Galardy, M. A. Cohn, W. Haas, S. P. Gygi, H. L. Ploegh, R. Bernards, and A. D. D'Andrea.** 2006. Regulation of monoubiquitinated PCNA by DUB autocleavage. Nat. Cell Biol. **8:**339–347.
18. **Hunt, P., A. Afonso, A. Creasey, R. Culleton, A. B. Sidhu, J. Logan, S. G. Valderramos, I. McNae, S. Cheesman, V. D. Rosario, R. Carter, D. A. Fidock, and P. Cravo.** 2007. Gene encoding a deubiquitinating enzyme is mutated in artesunate- and chloroquine-resistant rodent malaria parasites. Mol. Microbiol. **65:**27–40.
19. **Iyer, L. M., E. V. Koonin, and L. Aravind.** 2004. Novel predicted peptidases with a potential role in the ubiquitin signaling pathway. Cell Cycle **3:**1440–1450.
20. **Johnson, E. S.** 2004. Protein modification by SUMO. Annu. Rev. Biochem. **73:**355–382.
21. **Jones, C. J., and M. I. Jayson.** 1984. Chloroquine: its effect on leucocyte auto- and heterophagocytosis. Ann. Rheum. Dis. **43:**205–212.
22. **Kerscher, O., R. Felberbaum, and M. Hochstrasser.** 2006. Modification of proteins by ubiquitin and ubiquitin-like proteins. Annu. Rev. Cell Dev. Biol.
23. **Kirchhoff, L. V., K. S. Kim, D. M. Engman, and J. E. Donelson.** 1988. Ubiquitin genes in trypanosomatidae. J. Biol. Chem. **263:**12698–12704.
24. **Kirisako, T., Y. Ichimura, H. Okada, Y. Kabeya, N. Mizushima, T. Yoshimori, M. Ohsumi, T. Takao, T. Noda, and Y. Ohsumi.** 2000. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J. Cell Biol. **151:**263–276.
25. **Klionsky, D. J.** 2004. Cell biology: regulated self-cannibalism. Nature **431:** 31–32.
26. **Krebber, H., C. Wostmann, and T. Bakker-Grunwald.** 1994. Evidence for the existence of a single ubiquitin gene in Giardia lamblia. FEBS Lett. **343:**234–236.
27. **LaCount, D. J., M. Vignali, R. Chettier, A. Phansalkar, R. Bell, J. R. Hesselberth, L. W. Schoenfeld, I. Ota, S. Sahasrabudhe, C. Kurschner, S. Fields, and R. E. Hughes.** 2005. A protein interaction network of the malaria parasite Plasmodium falciparum. Nature **438:**103–107.
28. **Larsen, C. N., and H. Wang.** 2002. The ubiquitin superfamily: members, features, and phylogenies. J. Proteome Res. **1:**411–419.
29. **Levine, B., and D. J. Klionsky.** 2004. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev. Cell **6:**463–477.
30. **Liu, Y., H. A. Lashuel, S. Choi, X. Xing, A. Case, J. Ni, L. A. Yeh, G. D. Cuny, R. L. Stein, and P. T. Lansbury, Jr.** 2003. Discovery of inhibitors that elucidate the role of UCH-L1 activity in the H1299 lung cancer cell line. Chem. Biol. **10:**837–846.
31. **Manning-Cela, R., S. Jaishankar, and J. Swindle.** 2006. Life-cycle and growth-phase-dependent regulation of the ubiquitin genes of Trypanosoma cruzi. Arch. Med. Res. **37:**593–601.
32. **McNicoll, F., J. Drummelsmith, M. Muller, E. Madore, N. Boilard, M. Ouellette, and B. Papadopoulou.** 2006. A combined proteomic and transcriptomic approach to the study of stage differentiation in Leishmania infantum. Proteomics **6:**3567–3581.
33. **Melchior, F.** 2000. SUMO: nonclassical ubiquitin. Annu. Rev. Cell Dev. Biol. **16:**591–626.
34. **Miller, S. K., R. T. Good, D. R. Drew, M. Delorenzi, P. R. Sanders, A. N. Hodder, T. P. Speed, A. F. Cowman, T. F. de Koning-Ward, and B. S. Crabb.** 2002. A subset of Plasmodium falciparum SERA genes are expressed and appear to play an important role in the erythrocytic cycle. J. Biol. Chem. **277:**47524–47532.
35. **Nijman, S. M., M. P. Luna-Vargas, A. Velds, T. R. Brummelkamp, A. M. Dirac, T. K. Sixma, and R. Bernards.** 2005. A genomic and functional inventory of deubiquitinating enzymes. Cell **123:**773–786.
36. **Olliaro, P. L., and D. E. Goldberg.** 1995. The plasmodium digestive vacuole: metabolic headquarters and choice drug target. Parasitol. Today **11:**294–297.
37. **Osherovich, L. Z., B. S. Cox, M. F. Tuite, and J. S. Weissman.** 2004. Dissection and design of yeast prions. PLoS Biol. **2:**E86.
38. **Pfander, B., G. L. Moldovan, M. Sacher, C. Hoege, and S. Jentsch.** 2005. SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. Nature **436:**428–433.
39. **Pickart, C. M., and D. Fushman.** 2004. Polyubiquitin chains: polymeric protein signals. Curr. Opin. Chem. Biol. **8:**610–616.
40. **Rigden, D. J., M. Herman, S. Gillies, and P. A. Michels.** 2005. Implications of a genomic search for autophagy-related genes in trypanosomatids. Biochem. Soc. Trans. **33:**972–974.
41. **Rosenthal, P. J.** 1999. Proteases of protozoan parasites. Adv. Parasitol. **43:**105–159.
42. **Scheel, H., S. Tomiuk, and K. Hofmann.** 2003. Elucidation of ataxin-3 and ataxin-7 function by integrative bioinformatics. Hum. Mol. Genet. **12:**2845–2852.
43. **Swindle, J., J. Ajioka, H. Eisen, B. Sanwal, C. Jacquemot, Z. Browder, and G. Buck.** 1988. The genomic organization and transcription of the ubiquitin genes of Trypanosoma cruzi. EMBO J. **7:**1121–1127.
44. **Thompson, A. R., J. H. Doelling, A. Suttangkakul, and R. D. Vierstra.** 2005. Autophagic nutrient recycling in Arabidopsis directed by the ATG8 and ATG12 conjugation pathways. Plant Physiol. **138:**2097–2110.
45. **Webb, G. C., R. T. Baker, M. Coggan, and P. G. Board.** 1994. Localization of the human UBA52 ubiquitin fusion gene to chromosome band 19p13.1-p12. Genomics **19:**567–569.
46. **Wostmann, C., D. Liakopoulos, A. Ciechanover, and T. Bakker-Grunwald.** 1996. Characterization of ubiquitin genes and -transcripts and demonstration of a ubiquitin-conjugating system in Entamoeba histolytica. Mol. Biochem. Parasitol. **82:**81–90.
47. **Wu, Y., X. Wang, X. Liu, and Y. Wang.** 2003. Data-mining approaches reveal hidden families of proteases in the genome of malaria parasite. Genome Res. **13:**601–616.
48. **Xu, J., J. Zhang, L. Wang, J. Zhou, H. Huang, J. Wu, Y. Zhong, and Y. Shi.** 2006. Solution structure of Urm1 and its implications for the origin of protein modifiers. Proc. Natl. Acad. Sci. USA **103:**11625–11630.
49. **Yayon, A., and H. Ginsburg.** 1983. Chloroquine inhibits the degradation of endocytic vesicles in human malaria parasites. Cell Biol. Int. Rep. **7:**895.