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Review of the Structural Basis of Human E2 Conjugating Enzymes in Complexed with RING E3 Ligases

Erin Meghan Gladu, Iman Sayed and Michael Anthony Massiah

Abstract

Protein ubiquitination is a post-translational modification that controls essential biological processes through its regulation of protein concentration, function, and cellular location. RING E3 ligases are a critical component of a three-enzyme cascade that facilitates the ubiquitination of proteins. RING-type E3 ligases represent one class of E3 ligases that function by binding the substrate protein and ubiquitin-conjugating enzymes (E2s). Proteins exhibiting RING-type E3 ligase activities do so via a domain that adopts a $\beta\beta\alpha$ -RING fold and coordinates two zinc ions. To date, structural studies show that the RING domain interacts with the catalytic domain of the E2 enzyme. The catalytic domain is approximately 150 amino acids and adopts a canonical structure consisting of four α -helices and 3–4 β -strands. Structural analyses of RING–E2 complexes reveal that RING domains interact on a similar surface of the E2 enzyme. We postulate that the mechanism of interaction between an E2 enzyme and its cognate RING E3 domain may contribute to the extent of substrate modification. In this review, we compare the primary and secondary structures of human E2 enzymes and examine their quaternary structure with RING domains. Our analyses reveal the interactions appear to be relatively conserved with similar types of amino acids involved.

Keywords: ubiquitination, ubiquitin, really interesting new gene, E2–E3 binding, protein degradation; E2 conjugating enzyme, zinc-binding proteins, protein–protein, E2 recognition

1. Introduction

Protein ubiquitination is a highly conserved process in eukaryotic cells that plays key roles in cellular functions [1–4]. Depending on the type and extent of ubiquitin (Ub) modification, the cellular fate of the protein can be defined. Proteins that are composed with a chain of Ub (polyubiquitination) are usually captured by accessory proteins, including deubiquitinating enzymes (dubs) on the proteasome [5]. The Ub are cleaved and recycled while the target protein is degraded. In other instances, proteins with a single Ub (monoubiquitination), diubiquitination or less extensively modified can have their cellular location or function altered [6–10]. As a result,

ubiquitination can regulate most, if not all, signaling processes through the temporal and spatial regulation of proteins. Dysregulation in the ubiquitination pathway is associated with several diseases, including cancers, genetic defects and neurodegenerative diseases [11–16].

Ubiquitination is a highly coordinated event that is conserved in plants and animals as well as prokaryotes. The process of protein recycling in bacterial cells is called pupylation [17–20]. In eukaryotic cells, there are several mechanisms by which a protein is covalently modified with the small and stable Ub protein (**Figure 1**). The process involving RING E3 ligases is the most common because of the abundance of proteins with RING domains. Typically, RING-mediated ubiquitination involves three classes of enzymes. The first enzyme is common to all types of protein ubiquitination mechanisms. The ubiquitin-activating enzyme (E1) prepares the C-terminal carboxylate group of Gly-76 for chemistry by first catalyzing the addition of adenine monophosphate (AMP). The AMP group serves as a good leaving group and prepares the C-terminus carboxylate group for nucleophilic attack. The activated Ub ~ AMP first becomes covalently attached to the thiol group of an active site cysteine residue via a thioester bond. To date, there are 8–10 different types of E1 enzymes [21] and of these two are associated with activation of Ub [22]. In step two, the Ub is transferred [17] to an active site cysteine residue on one of four classes of human Ub-conjugating enzymes (E2). The thioester bond between C-terminal carboxylate group and the cysteine residue preserves the bond energy associated with the phosphoester bond with the AMP and the thioester bond with the E1 enzyme. RING-type E3 ligases promote substrate ubiquitination by binding the E2 enzyme and the substrate. In this case, a lysine residue serves as the nucleophile to attack the E2 ~ Ub thioester bond to form a stable isopeptide bond with the Ub. Subsequently, Ub can be attached to other lysine residues on the chain to generate a multi-ubiquitinated protein or Ub can form a Ub-chain on a lysine. This means that Ub can form a chain via one or several of its seven lysine residues. While lysine sidechain is the most common and most stable

Figure 1. Schematics of the ubiquitination cascade associated RING E3 ligases. The process begins with the ubiquitin-activating enzyme (known as E1), adding an AMP moiety to the C-terminal carboxylate group before transferring it to an internal cysteine to form an E1 ~ Ub intermediate. The ubiquitin is then transferred to the ubiquitin conjugating enzyme (E2) via a thioester bond involving a cysteine residue and Gly76 of ubiquitin. A RING E3 ligase then interacts with the E2 enzyme, influencing, among several things, the closed conformation between the E2 and ubiquitin. The closed conformation allows for aminolysis.

covalent linkage with Ub, evidence suggests that the Ub can be transferred to the side-chains of cysteines, serine, and threonine of substrates [23, 24]. The lysineless Pexp5 protein was observed to be monoubiquitinated via one of its cysteine residue and this modification affect its translation [25]. Hydroxyester linkages with Ub were observed for MHC-1 heavy chain in the ER-associated degradation pathway [26]. It would appear that both these types of linkages should be transient because they would be very susceptible to hydrolysis.

Furthermore, it is observed with in vitro assays, which are routinely performed to confirm that proteins with RING domain possess E3 ligase activity, that RING E3 ligases also facilitate autoubiquitination [2, 27, 28]. While the function and consequences of self-ubiquitination are poorly understood, it was demonstrated that autoubiquitination of Mdm2 and Nedd4 RING E3 ligases enhance their substrate ubiquitination activity [27, 29]. In contrast, autoubiquitination can result in self-induced degradation [30, 31].

To analyze the sequence, structure and interactions of E2 and RING E3 enzymes, this review focuses on a limited number of E2 and E3's from humans, and for the most part, the analysis presented is consistent with proteins in cells from plants and animals. From the literature, there are 394 reports of E2 enzymes with their cohort E3 enzymes from animal and human origins. These complexes represent the interactions from 23 specific E2 enzymes with 247 different E3 ligases. Of these, the quaternary structures of 33 E2–E3 pairs are reported in the protein database (PDB) for which the structure of the E2 and the E3 enzymes are known individually as well. These proteins are of human origin.

This review focuses on RING-type ubiquitination that includes E2 enzymes representing 4 classes with specific RING-type E3 ligases (**Figure 2, Tables 1 and 2**).

Figure 2.
Classification of 36 current human E2 enzymes. The E2 enzymes are classified based on the differences in number of amino acids for at their termini. Class one E2's do not have any terminal extensions and is essentially approximately 150 amino acids that adopts the catalytic domain, class 2 E2's have >20 amino acids at their N-terminus, class 3 enzymes have > 20 amino acids at their C-terminus, and class 4 enzymes have extensions at their C and N termini. The bolded E2's represent the E2's that for which structural analysis of E2 and E3 interactions were discussed.

Class I Class II Class III Class IV		Percent identity matrix of E2 enzymes in human binary reactions											
		Ube2A	Ube2B	Ube2d1	Ube2d2	Ube2d3	Ube2d4	Ube2g1	Ube2g2	Ube2i	Ube2L3	Ube2L6	Ube2N
		Ube2NL	Ube2V2	Ube2W	Ube2C	Ube2E1	Ube2e2	Ube2e3	Ube2F	Ube2M	Ube2q1	Ube2q2	Ube2v1
		Ube2H	Ube2J1	Ube2J2	Ube2K	Ube2R1	Ube2R2	Ube2S	Ube2T	Ube2U	Ube2O	Ube2z	Birc6
1	UBE2A	100 (100)	95 ^a (99) ^b	41 (78)	40 (78)	40 (77)	42 (78)	34 (73)	41 (73)	42 (73)	26 (72)	31 (70)	37 (70)
		33 (68)	22 (56)	31 (62)	42 ^c [32]	39 (63)	38 (62)	38 (57)	25 (57)	27 (61)	24 (57)	23 (56)	24 (46)
		32 (62)	24 (52)	29 (59)	29 (57)	39 (65)	39 (67)	31 (61)	32 (60)	33 (58)	26 (57)	30 (58)	30 (62)
2	UBE2B	95 (99)	100 (100)	40 (78)	38 (76)	38 (79)	40 (76)	34 (74)	41 (78)	42 (75)	26 (74)	30 (69)	37 (69)
		33 (63)	22 (56)	32 (62)	42 (64)	39 (64)	39 (63)	39 (63)	25 (55)	27 (60)	24 (56)	22 (57)	23 (46)
		32 (64)	25 (53)	29 (58)	29 (57)	39 (64)	39 (67)	32 (61)	33 (60)	32 (57)	27 (57)	30 (61)	29 (63)
3	UBE2D1	41 (78)	40 (78)	100 (100)	89 (97)	88 (97)	92 (97)	37 (77)	39 (72)	34 (76)	38 (69)	38(71)	44 (71)
		40 (65)	25 (53)	35 (61)	36 (63)	61 (80)	61 (80)	61 (80)	35 (63)	30 (66)	24 (54)	23 (53)	25 (45)
		34 (62)	31 (61)	24 (54)	41 (69)	38 (62)	35 (63)	35 (66)	43 (68)	31 (61)	22 (54)	33 (63)	31 (59)
4	UBE2D2	40 (78)	38 (76)	89 (97)	100 (100)	97 (99)	93 (99)	34 (71)	38 (75)	36 (68)	37 (67)	36 (69)	45 (71)
		41 (64)	23 (52)	33 (63)	35 (78)	64 (78)	64 (78)	65 (79)	34 (63)	31 (64)	24 (55)	24 (53)	25 (41)
		31 (63)	29 (57)	24 (56)	42 (69)	33 (60)	33 (63)	37 (67)	43 (67)	35 (64)	24 (55)	36 (62)	31 (59)
5	UBE2D3	40 (77)	38 (79)	88 (97)	97 (100)	100 (100)	92 (99)	34 (71)	38 (76)	36 (68)	36 (67)	35 (69)	45 (72)
		41 (65)	23 (52)	33 (60)	35 (64)	63 (80)	63 (80)	64 (80)	34 (63)	31 (64)	24 (53)	24 (52)	25 (41)
		30 (63)	30 (59)	24 (54)	41 (69)	33 (60)	33 (62)	35 (67)	42 (67)	35 (63)	24 (56)	36 (62)	31 (58)
6	UBE2D4	42 (78)	40 (76)	92 (97)	93 (99)	92 (99)	100 (100)	36 (79)	39 (73)	35 (69)	38 (68)	38 (70)	45 (70)
		41 (67)	25 (50)	35 (58)	38 (61)	63 (80)	63 (80)	63 (81)	36 (64)	32 (66)	25 (54)	24 (53)	26 (43)
		32 (62)	29 (59)	24 (56)	43 (67)	36 (60)	35 (63)	37 (65)	41 (66)	32 (62)	23 (55)	35 (63)	32 (59)
7	UBE2G1	34 (73)	34 (74)	37 (77)	34 (71)	34 (71)	36 (79)	100	50 (69)	34 (61)	28 (69)	24 (62)	32 (69)
		29 (63)	23 (54)	30 (50)	33 (57)	31 (55)	32 (55)	32 (55)	27 (58)	26 (55)	15 (45)	16 (46)	24 (49)
		27 (52)	23 (54)	24 (54)	27 (57)	50 (69)	54 (73)	26 (62)	29 (58)	29 (59)	22 (55)	25 (59)	25 (57)

		Percent identity matrix of E2 enzymes in human binary reactions											
Class I		Ube2A	Ube2B	Ube2d1	Ube2d2	Ube2d3	Ube2d4	Ube2g1	Ube2g2	Ube2i	Ube2L3	Ube2L6	Ube2N
Class II		Ube2NL	Ube2V2	Ube2W	Ube2C	Ube2E1	Ube2e2	Ube2e3	Ube2F	Ube2M	Ube2q1	Ube2q2	Ube2v1
Class III		Ube2H	Ube2J1	Ube2J2	Ube2K	Ube2R1	Ube2R2	Ube2S	Ube2T	Ube2U	Ube2O	Ube2z	Birc6
Class IV													
8	UBE2G2	41 (73)	41 (78)	39 (72)	38 (75)	38 (76)	39 (73)	50 (73)	100	37 (75)	26 (68)	23 (65)	30 (66)
		28 (60)	29 (48)	27 (57)	33 (56)	31 (61)	32 (61)	32 (60)	21 (56)	25 (55)	18 (53)	18 (53)	18 (47)
		27 (54)	27 (55)	27 (52)	26 (57)	48 (71)	48 (73)	30 (62)	27 (55)	30 (56)	24 (57)	29 (59)	28 (58)
9	UBE2I	42 (73)	42 (75)	34 (76)	36 (68)	36 (68)	35 (69)	34 (61)	37 (65)	100	27 (61)	27 (63)	31 (62)
		31 (61)	23 (50)	27 (58)	36 (57)	35 (59)	36 (64)	36 (63)	26 (55)	31 (58)	23 (47)	23 (49)	23 (48)
		26 (56)	22 (51)	22 (56)	27 (58)	36 (60)	35 (58)	30 (61)	30 (61)	28 (52)	21 (56)	30 (55)	30 (58)
10	UBE2L3	26 (72)	26 (74)	38 (69)	37 (67)	36 (67)	38 (68)	28 (69)	26 (68)	27 (61)	100	55 (80)	30 (62)
		28 (63)	24 (53)	20 (61)	26 (56)	35 (60)	35 (60)	35 (60)	29 (57)	32 (58)	18 (50)	19 (50)	25 (45)
		28 (56)	25 (52)	21 (54)	29 (51)	29 (62)	29 (63)	24 (51)	34 (58)	22 (56)	19 (53)	21 (53)	26 (53)
11	UBE2L6	31 (70)	30 (69)	38 (71)	36 (69)	35 (69)	38 (70)	24 (62)	23 (65)	27 (65)	55 (80)	100	29 (63)
		27 (63)	21 (52)	24 (57)	26 (56)	33 (57)	32 (57)	31 (56)	27 (52)	29 (59)	18 (50)	19 (46)	24 (49)
		24 (55)	25 (52)	23 (56)	27 (58)	27 (60)	26 (61)	25 (55)	33 (59)	25 (57)	20 (48)	23 (59)	24 (58)
12	UBE2N	37 (70)	37 (71)	44 (71)	45 (71)	45 (72)	45 (70)	32 (69)	30 (66)	31 (62)	20 (62)	29 (63)	100
		92 (97)	21 (49)	27 (57)	38 (64)	45 (64)	44 (64)	44 (64)	27 (61)	26 (62)	19 (50)	19 (53)	22 (46)
		29 (53)	25 (58)	27 (59)	44 (71)	32 (65)	30 (69)	36 (65)	44 (71)	29 (59)	20 (58)	30 (64)	25 (62)
13	UBE2NL	33 (68)	33 (69)	40 (65)	41 (64)	41 (65)	41 (69)	29 (63)	28 (60)	31 (61)	28 (63)	27 (63)	92 (97)
		100	17 (52)	24 (56)	35 (61)	42 (61)	41 (61)	40 (61)	24 (56)	23 (61)	16 (51)	17 (52)	18 (45)
		25 (50)	23 (53)	25 (55)	43 (68)	30 (59)	28 (63)	34 (63)	41 (69)	25 (52)	17 (52)	28 (62)	22 (54)
14	UBE2V2	22 (56)	22 (56)	25 (53)	23 (52)	23 (52)	25 (50)	23 (54)	19 (48)	23 (50)	24 (53)	21 (52)	21 (49)
		17 (52)	100	22 (50)	23 (57)	23 (57)	23 (55)	23 (54)	18 (54)	16 (53)	18 (58)	19 (57)	88 (94)
		21 (44)	23 (52)	18 (51)	26 (54)	22 (54)	21 (54)	19 (48)	26 (46)	18 (53)	15 (51)	20 (57)	20 (48)

		Percent identity matrix of E2 enzymes in human binary reactions											
Class I		Ube2A	Ube2B	Ube2d1	Ube2d2	Ube2d3	Ube2d4	Ube2g1	Ube2g2	Ube2i	Ube2L3	Ube2L6	Ube2N
Class II		Ube2NL	Ube2V2	Ube2W	Ube2C	Ube2E1	Ube2e2	Ube2e3	Ube2F	Ube2M	Ube2q1	Ube2q2	Ube2v1
Class III		Ube2H	Ube2J1	Ube2J2	Ube2K	Ube2R1	Ube2R2	Ube2S	Ube2T	Ube2U	Ube2O	Ube2z	Birc6
Class IV													
15	UBE2W	31 (62)	32 (62)	35 (61)	33 (59)	33 (60)	35 (58)	30 (60)	27 (57)	27 (58)	20 (61)	24 (57)	27 (57)
		24 (56)	22 (50)	100	27 (54)	34 (57)	35 (55)	36 (54)	20 (49)	20 (53)	23 (50)	23 (52)	24 (44)
		19 (44)	23 (52)	22 (51)	27 (54)	31 (54)	33 (54)	23 (48)	28 (46)	24 (53)	17 (51)	26 (57)	22 (48)
16	UBE2C	42 (65)	42 (64)	36 (63)	35 (63)	35 (61)	38 (64)	33 (57)	33 (56)	36 (57)	26 (56)	26 (56)	38 (64)
		35 (61)	23 (57)	27 (54)	100	32 (57)	33 (61)	34 (55)	25 (60)	26 (64)	20 (56)	19 (57)	25 (57)
		29 (56)	22 (50)	20 (51)	31 (57)	33 (57)	33 (57)	28 (54)	34 (55)	30 (54)	20 (59)	21 (55)	24 (56)
17	UBE2E1	39 (63)	39 (64)	61 (80)	64 (78)	63 (80)	63 (80)	31 (55)	31 (61)	35 (59)	35 (60)	33 (57)	45 (64)
		42 (61)	23 (57)	34 (58)	32 (57)	100	88 (89)	84 (88)	27 (59)	30 (65)	23 (63)	21 (59)	24 (57)
		28 (50)	30 (46)	25 (51)	40 (54)	29 (44)	28 (47)	31 (52)	41 (53)	25 (44)	18 (53)	26 (56)	23 (56)
18	UBE2E2	38 (62)	39 (63)	61 (80)	64 (78)	63 (80)	63 (80)	32 (55)	32 (61)	36 (64)	35 (60)	32 (57)	44 (64)
		41 (61)	23 (55)	35 (58)	33 (61)	88 (89)	100	87 (93)	28 (62)	30 (64)	21 (61)	20 (60)	24 (54)
		28 (50)	29 (43)	25 (48)	41 (52)	29 (42)	28 (44)	31 (49)	41 (52)	23 (41)	19 (52)	26 (57)	24 (53)
19	UBE2E3	38 (57)	39 (63)	61 (80)	65 (79)	64 (80)	63 (81)	32 (55)	32 (60)	36 (63)	35 (60)	31 (56)	44 (64)
		40 (61)	23 (54)	36 (58)	34 (55)	84 (88)	87 (93)	100	29 (63)	29 (67)	20 (62)	19 (60)	24 (53)
		28 (50)	28 (42)	25 (46)	40 (52)	29 (42)	28 (42)	32 (48)	41 (52)	25 (40)	18 (50)	25 (56)	23 (58)
20	UBE2F	25 (57)	25 (55)	35 (63)	34 (63)	34 (63)	36 (64)	27 (58)	21 (56)	26 (55)	29 (57)	27 (52)	27 (61)
		24 (56)	18 (54)	20 (49)	25 (60)	27 (59)	28 (62)	29 (63)	100	38 (69)	15 (52)	15 (51)	17 (52)
		27 (46)	21 (38)	15 (43)	32 (55)	27 (51)	23 (53)	28 (49)	26 (44)	23 (45)	17 (56)	24 (54)	20 (47)
21	UBE2M	27 (61)	27 (60)	30 (66)	31 (64)	31 (64)	32 (66)	26 (55)	25 (55)	31 (58)	32 (58)	29 (59)	26 (62)
		23 (51)	16 (53)	20 (53)	26 (64)	30 (65)	30 (64)	29 (67)	38 (69)	100	18 (59)	18 (58)	16 (60)
		22 (46)	15 (43)	16 (48)	24 (51)	23 (56)	25 (54)	24 (52)	27 (50)	24 (52)	20 (48)	25 (51)	24 (55)

		Percent identity matrix of E2 enzymes in human binary reactions											
Class I		Ube2A	Ube2B	Ube2d1	Ube2d2	Ube2d3	Ube2d4	Ube2g1	Ube2g2	Ube2i	Ube2L3	Ube2L6	Ube2N
Class II		Ube2NL	Ube2V2	Ube2W	Ube2C	Ube2E1	Ube2e2	Ube2e3	Ube2F	Ube2M	Ube2q1	Ube2q2	Ube2v1
Class III		Ube2H	Ube2J1	Ube2J2	Ube2K	Ube2R1	Ube2R2	Ube2S	Ube2T	Ube2U	Ube2O	Ube2z	Birc6
Class IV													
22	UBE2Q1	24 (57)	24 (56)	24 (54)	24 (55)	24 (53)	25 (54)	15 (45)	18 (53)	23 (47)	18 (50)	18 (50)	19 (50)
		16 (51)	18 (58)	23 (50)	20 (56)	23 (63)	21 (61)	20 (62)	15 (52)	18 (59)	100	74 (75)	19 (59)
		13 (46)	18 (28)	14 (33)	18 (46)	16 (37)	16 (34)	15 (37)	20 (39)	17 (29)	18 (50)	17 (40)	18 (45)
23	UBE2Q2	23 (56)	22 (57)	23 (53)	24 (53)	24 (52)	24 (53)	16 (46)	18 (53)	23 (49)	19 (50)	19 (46)	19 (53)
		17 (52)	18 (57)	23 (52)	19 (57)	21 (59)	20 (60)	19 (60)	15 (51)	18 (58)	74 (75)	100	20 (56)
		14 (44)	18 (29)	17 (33)	19 (46)	16 (36)	16 (33)	16 (37)	20 (40)	16 (27)	19 (46)	17 (31)	18 (48)
24	UBE2V1	24 (46)	23 (46)	25 (45)	25 (41)	25 (41)	26 (43)	24 (49)	18 (47)	23 (48)	25 (45)	24 (49)	22 (46)
		18 (45)	88 (94)	24 (44)	25 (57)	24 (57)	24 (54)	24 (53)	17 (52)	16 (60)	19 (59)	20 (56)	100
		18 (44)	23 (48)	18 (43)	27 (50)	21 (55)	22 (57)	21 (47)	26 (47)	19 (45)	16 (54)	21 (59)	19 (50)
25	UBE2H	32 (62)	32 (64)	34 (62)	31 (63)	30 (63)	32 (62)	27 (52)	27 (54)	26 (56)	28 (56)	24 (55)	29 (53)
		25 (50)	21 (44)	19 (49)	29 (56)	28 (50)	28 (50)	28 (50)	27 (46)	22 (46)	13 (46)	14 (44)	18 (44)
		100	20 (61)	19 (59)	29 (67)	30 (65)	29 (67)	26 (65)	27 (61)	20 (59)	22 (54)	18 (50)	23 (48)
26	UBE2J1	24 (52)	25 (53)	31 (61)	29 (57)	30 (59)	29 (59)	23 (54)	27 (55)	22 (51)	25 (52)	25 (52)	25 (58)
		23 (53)	23 (52)	23 (50)	22 (50)	30 (46)	29 (43)	28 (42)	21 (38)	15 (43)	18 (28)	18 (29)	23 (48)
		20 (61)	100	31 (67)	21 (54)	23 (56)	25 (58)	21 (64)	20 (54)	16 (49)	20 (47)	21 (44)	19 (43)
27	UBE2J2	29 (59)	29 (58)	24 (54)	24 (56)	24 (54)	24 (56)	24 (54)	27 (52)	22 (56)	21 (54)	23 (56)	27 (59)
		25 (55)	18 (51)	22 (54)	20 (51)	25 (51)	25 (48)	25 (46)	15 (43)	16 (48)	14 (33)	17 (33)	18 (43)
		19 (59)	31 (67)	100	19 (57)	23 (50)	24 (53)	19 (53)	20 (63)	18 (50)	16 (57)	23 (48)	18 (44)
28	UBE2K	29 (57)	29 (57)	41 (69)	42 (69)	41 (69)	43 (67)	27 (57)	26 (57)	27 (58)	29 (51)	27 (58)	44 (71)
		43 (68)	26 (54)	27 (58)	31 (57)	40 (54)	41 (52)	40 (52)	32 (55)	24 (51)	18 (46)	19 (46)	27 (50)
		29 (67)	21 (54)	19 (57)	100	27 (62)	27 (64)	29 (61)	36 (62)	24 (62)	21 (52)	21 (55)	22 (53)

		Percent identity matrix of E2 enzymes in human binary reactions											
Class I		Ube2A	Ube2B	Ube2d1	Ube2d2	Ube2d3	Ube2d4	Ube2g1	Ube2g2	Ube2i	Ube2L3	Ube2L6	Ube2N
Class II		Ube2NL	Ube2V2	Ube2W	Ube2C	Ube2E1	Ube2e2	Ube2e3	Ube2F	Ube2M	Ube2q1	Ube2q2	Ube2v1
Class III		Ube2H	Ube2J1	Ube2J2	Ube2K	Ube2R1	Ube2R2	Ube2S	Ube2T	Ube2U	Ube2O	Ube2z	Birc6
Class IV													
29	UBE2R1	39 (65)	39 (64)	38 (62)	33 (60)	33 (60)	36 (60)	50 (69)	48 (71)	36 (60)	29 (62)	27 (60)	32 (65)
		30 (59)	22 (54)	31 (53)	33 (57)	29 (44)	29 (42)	29 (42)	27 (51)	23 (56)	16 (37)	16 (36)	21 (55)
		30 (65)	23 (56)	23 (58)	27 (62)	100	82 (94)	31 (59)	30 (61)	25 (56)	23 (52)	25 (53)	27 (52)
30	UBE2R2	39 (67)	39 (67)	35 (63)	33 (63)	33 (62)	35 (63)	54 (73)	48 (73)	35 (58)	29 (63)	26 (61)	30 (69)
		28 (63)	21 (54)	33 (55)	33 (57)	28 (47)	28 (44)	28 (42)	23 (53)	25 (54)	16 (34)	16 (33)	22 (57)
		29 (67)	25 (58)	24 (53)	27 (64)	82 (94)	100	27 (63)	29 (60)	24 (58)	21 (53)	23 (49)	24 (55)
31	UBE2S	31 (61)	32 (61)	35 (66)	37 (67)	35 (67)	37 (65)	26 (62)	30 (62)	30 (61)	24 (51)	25 (55)	36 (65)
		34 (63)	19 (48)	23 (58)	28 (54)	31 (52)	31 (49)	32 (48)	28 (49)	24 (52)	15 (37)	16 (37)	21 (47)
		26 (65)	21 (64)	19 (53)	29 (61)	31 (59)	29 (63)	100	30 (62)	22 (56)	25 (61)	23 (55)	22 (53)
32	UBE2T	32 (60)	33 (60)	43 (68)	43 (67)	42 (67)	41 (66)	29 (58)	27 (55)	30 (61)	34 (58)	33 (59)	44 (71)
		41 (69)	26 (46)	28 (53)	34 (55)	41 (53)	41 (52)	41 (52)	26 (44)	27 (50)	20 (39)	20 (40)	26 (47)
		27 (61)	20 (54)	20 (63)	36 (64)	30 (61)	29 (60)	30 (62)	100	21 (59)	25 (54)	28 (56)	27 (53)
33	UBE2U	33 (58)	32 (57)	31 (61)	35 (64)	35 (63)	32 (62)	29 (59)	30 (56)	28 (52)	22 (56)	25 (57)	29 (59)
		25 (52)	18 (53)	24 (54)	30 (54)	25 (44)	25 (41)	25 (40)	23 (45)	24 (52)	17 (29)	16 (27)	19 (45)
		20 (59)	16 (49)	18 (50)	24 (62)	25 (56)	24 (58)	22 (56)	21 (59)	100	16 (45)	22 (41)	18 (44)
34	UBE2O	26 (57)	27 (57)	22 (54)	24 (55)	24 (56)	23 (55)	22 (55)	24 (57)	21 (56)	19 (53)	20 (48)	20 (58)
		17 (52)	15 (51)	17 (51)	20 (59)	18 (53)	19 (52)	18 (50)	17 (56)	20 (48)	18 (50)	19 (46)	16 (54)
		22 (54)	20 (47)	16 (57)	21 (52)	23 (52)	21 (53)	25 (61)	25 (54)	16 (45)	100	25 (27)	21 (47)
35	UBE2Z	30 (58)	30 (61)	33 (63)	36 (62)	36 (62)	35 (63)	25 (59)	29 (59)	30 (55)	21 (53)	23 (59)	30 (64)
		28 (62)	20 (57)	26 (57)	21 (55)	26 (56)	26 (57)	25 (56)	24 (54)	25 (51)	17 (40)	17 (31)	21 (59)
		18 (50)	21 (44)	23 (48)	21 (55)	25 (53)	23 (49)	23 (56)	28 (56)	22 (41)	25 (27)	100	26 (63)

Class I Class II Class III Class IV		Percent identity matrix of E2 enzymes in human binary reactions											
		Ube2A	Ube2B	Ube2d1	Ube2d2	Ube2d3	Ube2d4	Ube2g1	Ube2g2	Ube2i	Ube2L3	Ube2L6	Ube2N
		Ube2NL	Ube2V2	Ube2W	Ube2C	Ube2E1	Ube2e2	Ube2e3	Ube2F	Ube2M	Ube2q1	Ube2q2	Ube2v1
		Ube2H	Ube2J1	Ube2J2	Ube2K	Ube2R1	Ube2R2	Ube2S	Ube2T	Ube2U	Ube2O	Ube2z	Birc6
36	BIRC6	30 (62)	29 (63)	31 (59)	31 (59)	31 (58)	32 (59)	25 (57)	28 (58)	30 (58)	26 (53)	24 (58)	25 (62)
		22 (54)	20 (48)	22 (53)	24 (56)	23 (56)	24 (53)	23 (58)	20 (47)	24 (55)	18 (45)	18 (48)	19 (50)
		23 (48)	19 (43)	18 (44)	22 (53)	27 (52)	24 (55)	22 (50)	27 (53)	18 (44)	21 (47)	26 (63)	100
^a Pairwise percent identity between E2 enzymes between the classes. Comparison made using ClustalW.													
^b Pairwise percent homology, in parenthesis, between E2 enzymes between the classes. Comparison made using ClustalW.													
^c The names and %identity and %homology are color coded to corresponds to class 1 (blue), class2 (red), class3 (orange), and class4 (green).													

Table 1.
Matrix of pairwise sequence comparisons of 36 human E2 conjugating enzymes representing four classes.

For Class 1, there are 20 structures between Ube2D1–3 and RING E3 domains, class 2 involves Ube2e1–2 with 2 structures bound to RING E3 domains, class 3 involves Ube2g2 with 2 structures with RING E3 domains, class 4 consists of 2 structures with Ube2l3 bound with RING E3 domains, and class 5 consists of seven structures of Ube2n bound with RING E3 domains. This review discusses the structures of RING domains and the E2 catalytic domains, their interactions, and evaluating patterns of key amino acids and the role they may play in how E2 and RING E3 domains interact and facilitate substrate ubiquitination.

2. Ub E2 conjugating enzymes are categorized into four classes

To date, there are between 40 and 75 different Ub E2 enzymes identified in plants and animals [33, 34]. In mammals and humans, there are currently 36 known Ub E2 enzymes [35]. It is expected that this number should increase as more research in this central area continues. All E2 enzymes consist of a catalytic domain of approximately 150-amino acids. Given this commonality, the human E2 enzymes are categorized into four classes based on the number of amino acids that precede or follow the catalytic domain (**Figure 2**). Class 1 E2 enzymes are essentially just the catalytic domain and are approximately 145–160 amino acids. Class 1 has the most members, consisting of 15 of the 36 E2 enzymes. The most studied, and possibly the most promiscuous when it comes to binding different RING E3 domains, are the three isoforms, Ube2D1–3. Class 2 E2 enzymes have a considerably larger number of residues (20–45 amino acids) N-terminus to the catalytic domain and include the enzymes Ube2e1, Ube2e2, Ube2e3, and Ube2f. Class 3 E2 enzymes have between 10 and 50 amino acids at the C-terminus and includes Ube2r1, Ube2r2, Ube2s, Ube2t, and Ube2u, which represent 5 of the 9 E2 members [35]. Both classes 2 and 3 consist of nine E2 enzymes. Class 4 E2s have both N- and C-terminal extensions and consists of three members, Ube2Z, Ube2O and Birc6. While the exact role the N- or C-terminal residues have on impacting specificity with specific E3 ligases is unclear, they bind is unclear but they have been shown to contribute to substrate binding and the linkages of Ub chain elongation [36–38].

3. Primary sequence alignments show high identity and homology

To understand how the E2 enzymes of the four classes are related and whether the classifications using N- and C-terminal extensions are appropriate criteria, the sequences of the catalytic domains are compared (**Table 1**). The matrix shows the percent identity and homology (in parenthesis) between pairs of E2 enzymes within and between classes. Within class 1, the identities of the primary sequences range between 25% and 45% and the similarity/homology between 70% and 78%. The Ube2A enzyme has sequence identities between 40% and 42% with Ube2D1–4, Ube2g2, and Ube2i. The Ube2A and Ube2B enzymes may be isoforms because they are 95% identical and 99% homologous. The Ube2D [1–4] family has the highest percent identities and homology among each other with values of 88–92% and 97%, respectively. Interestingly, Ube2v2 shows the lowest percent identity among its class with an average identity of $21 \pm 4\%$. The E2 with the next lowest identity is Ube2L3 with an average value of 26%; however Ube2D1–4 shows $\sim 38\%$ identity with Ube2L3. The Ube2L3 E2 enzyme only reacts with cysteine, indicating it would interact with the

other classes of E3 enzymes; only two HECT-type E3 ligases that interact with Ube2L3 have been identified. In general, the remaining E2 show an average identity of $42 \pm 8\%$. When comparing the class 1 and 2 enzymes, Ube2V2 not only has the lowest percent identity within class 1, but also with almost all of the E2 enzymes with an average value of $20 \pm 3\%$. In contrast, it has 88% identity with Ube2V1. Many of the class 1 E2s show considerably higher identity and homology with Ube2C and Ube2E1–3 of $\sim 32\%$ and 70% , respectively. Ube2D1–4 show higher identities with these four enzymes, which is $\sim 63\%$. The identities between class 1 E2s with Ube2F, Ube2M, Ube2Q1–2, and Ube2V1 are considerably lower. The sequences between classes 1 and 3 have identities between 30 and 45% with homologies in the 55–70% range. Again, Ube2D1–4 sequences show the highest sequence identity with class 3 sequences. Class 1 shows high sequence identities with the Class 4 E2 enzymes. Specifically, Ube2A, Ube2B, Ube2D1, Ube2D2, Ube2D3, Ube2D4, and Ube2N show sequence identities and homologies of approximately 30% and 60%, respectively, with the class 4 Ube2Z. Ube2O shows the lowest sequence identity with the class 1 sequences.

The class 2 enzymes appear to fall in two groups based on their sequence identities. Six of the nine show identities between 25 and 35, while conversely showing identities ranging between 15 and 25% with Ube2Q1, Ube2Q2, Ube2V1. Ube2Q1 and Ube2Q2 are 75% identical but show 20% identities with Ube2V1. Similarly to Ube2D1–4, the Ube2E1, Ube2E2, and Ube2E3 enzymes have the highest identities at $>85\%$ among each other. Class 3 E2 enzymes show identities in the 16–30% range, with an average of $26 \pm 11\%$ among each other. The similarities in sequence are in the 60–70% range. Incidentally, this range and average values are lower than those values when comparing with class 1 sequences. The enzymes Ube2R1 and Ube2R2 are 82% identical. Given that the other E2 enzymes in this class are not as related sequentially with each other, Ube2K and Ube2T stand out with showing the next highest identity of 36%. These enzymes show the lowest sequence identities with class 4 enzymes, with an average percentage of $22 \pm 3\%$. Lastly, the class 4 enzymes show the average percent identities of $24 \pm 2\%$. Based on sequences, the Ube2Z enzyme has 25% and 26% identities with BIRC6 and Ube2O, respectively, while BIRC6 and Ube2O have 21% with each other. As noted above, the three class 4 enzymes have the highest sequence identities with class 1 enzymes than with the other two classes.

Identities compare identical amino acids in the same location between proteins. Sequence homology, which includes identical and amino acids with similar properties, provides another perspective on how these enzymes preserve their structure and function. For class 1 enzymes, the Ube2d1, Ube2d2, Ube2d3, and Ube2d4 are the most related and commonly used in ubiquitination assays, sharing 97–100% homology with each other. In fact, these four enzymes will most likely interact with the same RING E3 ligase if one is shown to be a cohort. Similarly, Ube2A and Ube2B share 99% homology and Ube2NL share 97% homology with Ube2n. Ube2V2 has the lowest percent homologies when compared with other E2's within class 1, and those values range between 48–56%. In summary, class 1 enzymes have an average pairwise homology of 56% with each other. In class 2, Ube2E1, Ube2E2, Ube2E3 share 88–93% homologies with another, and the average pairwise homology among members is 54%. For Class 3, the E2's share greater than 50% homology with one another (average of 54%), with the exception of Ube2U and Ube2J1, which share 49% homology. Ube2R1 and Ube2R2 share 94% homology. For Class 4, Ube2O and Ube2Z share only 27% homology with each other.

Comparing the homologies between classes, class 1 Ube2V2 shares 94% homology with class 2 Ube2V1, and class 1 Ube2D1–4 share 78–81% homologies with class 2

Ube2E1, Ube2E2, and Ube2E3. 93% of class 1 E2 enzymes shared the lowest percent homologies with Ube2V2 (class 2), ranging between 41 to 49%. Most of class 1 E2's shows the least percent homology with class 3 Ube2J1 and Ube2J2, ranging from 52 to 56%. Class 1 Ube2V2 and Ube2W share 44% homology with Ube2H, the lowest homology between these two classes. Class 2 Ube2Q1 and Ube2Q2 share 27–29% homologies with class 3 Ube2U. 93% of class 1 E2 enzymes have the lowest percent homology with class 4 Ube2O, compared with Ube2Z and Birc6, however, the values are greater than 48% homology.

Based on these sequence alignments, it appears that proteins with sequence identities as low as 16% and sequence homologies at 50% with similar function will adopt a very similar tertiary structure. It would be important to analyze the role of the amino acids that are not conserved among those proteins to determine their role in catalytic rates, substrate binding, mechanism of Ub binding, and specificity for RING domains. Furthermore, given that class 1 enzymes show the highest sequence identity among each other than those of the other classes, it would be interesting to determine how residues on the N- and C-termini may compensate for the lower sequence identities within the catalytic region.

4. The tertiary structure of the catalytic subunit of E2's is conserved

The structure of the catalytic subunit of E2 enzymes consists of four-antiparallel β strands that forms one surface (**Figure 3**). On one end of the surface is the C-terminal helix-turn-helix region and on the other side is the N-terminal helix. Across the inner surface of the β -sheet sits the fourth helix, along with a helical turn located adjacent to

Figure 3. Ribbon representation of E2 structures. A. Tertiary structure of Ube2D2 class1 E2 enzyme showing the structure of the catalytic domain. The catalytic cysteine that forms the thioester bond with Ub is shown. B. Overlay of the catalytic subunits of an E2 enzyme from each of the four classes. Class1 (Ube2A, 6cyo) is colored red, class2 (Ube2C, 1ik7) is in green, class3 (Ube2T,1yh2) is in blue and class4 (Ube2Z, 5a4p) is in yellow-orange. C. Same superposition as B but with the loop containing the catalytic cysteine highlighted. Ube2C is solved with a serine instead of the cysteine and it adopts a very similar orientation. D. Table of the pairwise RMSD values for the superposition of the backbone atoms between structures.

the catalytic cysteine. This helical turn is important for interaction with the RING E3 domain and activation for nucleophilic attack of the thioester linkage with Ub [37]. There are several loops connecting the β -strands that the quaternary structures of E2 enzymes in complex with RING domains show which are important for contacts with the RING E3 domains.

Not surprisingly, structures of the catalytic domains of E2 enzymes belonging to the four classes show remarkable similarity (**Figure 3B**). For the most part, all four proteins have the same number of amino acids that contribute to the four β -strands. The number of amino acids associated with the helices are very similar. The N-terminal and central helices as well as the 1-turn helix appears to have the same number of amino acids in their composition. Helix 1 is formed by ~ 15 amino acids of the first residues that form the start of the secondary and tertiary structure. The last 25 amino acids of the catalytic core adopt a 4-turn α -helix, followed by what appears to be a reverse turn and a 2-turn terminal α -helix.

Interestingly, the significant difference between an overlay of four structures belonging to each class is observed within the C-terminal helix-turn-helix region. The lengths of these helices differ, with the helices of Ube2Z (class4) being the longest with two additional 2-helical turns. There are also greater variations in the position from of the C-terminal helices, which is surprising given the fact that its location is on the opposite surface to where the RING domains typically bind E2 enzymes. The residues that precedes the C-terminal helix for Ube2Z did not superimpose well with the other structures. The relative positions of the loops are very similar among the four structures, however, Ube2Z has two additional loops that are not present in the other classes [39]. One of these loops is absent in all Class1–3 E2 enzymes and only present in class 4 E2 BIRC6.

To measure the overall similarity and consistency in the structures, the backbone $C\alpha, C, N$ atoms are superimposed. The root mean square deviation (RMSD) values of the superposition of the backbone atoms between the four structures range from 0.8 to 1.3 Å, confirming the similarity in structures. The lower the RMSD between pairs of structures, the more closely aligned the structures. RMSD values ranging from 0.8–1.3 Å indicate that while there are small differences in the structures, overall they are very similar. The RMSD values between Ube2A (class1) and Ube2C (class2) is 0.8 Å, and with Ube2T (class3) and Ube2Z (class4) are 1.1 and 1.2 Å, respectively. The RMSD values of the superposition of Ube2Z with Ube2A and Ube2T are 1.26 Å and 1.34 Å. The similarity in structures indicates a common mechanism of function.

The catalytic cysteine that forms the thioester bond with the C-terminal carboxylate group of Gly76 is located on a 11-amino acid structured loop that precedes the single helical turn. The sequences of this loop show a 74% sequence homology with a consensus sequence of H-P-N-h-D/Y-x-x-G-p-I/V-C-L, where h, p and x indicate a hydrophobic, a polar residue, and any residue, respectively. The proline residue is important because it introduces a bend in the loop that positions the backbone carbonyl of the adjacent Asn (N) residue to form a hydrogen bond with the backbone NH of the Cys residue, which is located between two aliphatic hydrophobic residues. The side-chain amine (NH_2) hydrogens form two hydrogen bonds with the backbone carbonyl groups of a conserved N-x-x-S motif on the structured loop that precedes the C-terminal helix-turn-helix region. Residues D/Y-x-x-G are involved in forming a tight type-I turn in the loop. The positions of the loops between the four structures superimposed well (< 0.6 Å). The conserved sequence and structure indicate their importance in chemistry associated with the covalent binding of an Ub molecule. The structure shown for Ube2C E2 enzyme is that of a mutant that consists of a serine

instead of a cysteine (**Figure 3C**). The structure of the serine side-chain is positioned very similar to those of the cysteine residues of the other structures. Interestingly, the C/S mutant Ube2C was able to form ester linkages with the Ub in vitro [40]. This observation suggests that the native Ube2NL (Ube2N-linked) should function as an E2 ligase given that it has a serine in place of the catalytic cysteine.

Furthermore, sequence alignment of all 36 E2 enzymes revealed several motifs, conserved residues, and conserved properties in certain parts of their sequences (**Figure 4A**). Starting from the N-terminus, there are conserved I-h-P-G and P-a-Q/E-GG motifs, where ‘h’ represents a hydrophobic residue and ‘a’ indicates an aromatic residue, at around position 37. Near the 60th position, there is a PF motif (Y-P-F) followed by a conserved proline three amino acids downstream. There are also several additional conserved regions that include K/R-I-Y/a, H-P-N, I-C-L-D-I-L, a conserved tryptophan around position 93, followed by S-P-A-L and S-L-L motifs, and a hydrophobic residue at positions near 105 and 109.

In **Figure 4B**, the conserved motifs are mapped to their locations on the 3-dimensional structure of an E2 enzyme with respect to the active cysteine site. The ICLDIL motif consists of the active site cysteine and encompass the single helical turn, and the HPN and SPAL motifs are spatially located to the left and top right, respectively, to this cysteine. The LLS motif is located at the center of the α -helix that lays across the β -sheet. The I-h-P-G and P-a-Q/E-GG motifs, in which h and a represent hydrophobic and aromatic residues, respectively, are located on the loops preceding the C-terminal helices on the opposite surface of the cysteine. The conserved

Figure 4. Sequence alignment of 36 human E2 enzymes. A. The sequences of the catalytic domain of the E2 enzymes from the 4 classes are aligned. motifs that are conserved among the enzymes are highlighted; these residues mostly surround the catalytic cysteine and maybe important for allosteric communication between the E2 and RING domains. The alignment is performed using ClustalW [41, 42]. B. Location of the sidechains of several conserved sequence motifs on the E2 catalytic domain, noted in the text and identified from the sequence alignment. The catalytic cysteine residue is shown as yellow spheres.

tryptophan and proline residues at positions near 93 and 65, respectively, are spatially located in the region above the N-terminal α -helix, adjacent to that SPAL motif. The side-chains of two conserved hydrophobic residues located on the α -helix point towards the cysteine while another on a β -strand appears to make hydrophobic contacts with those on the α -helix. Based on the alignment of these residues along the pathway between with the RING domain binds the E2 enzyme and the catalytic side, it appears that some of these conserved residues are involved in RING domain binding (on the right), while most may be important for allosteric effects to activate the reactivity of the cysteine and its thioester bond with the Ub (**Figure 4B**).

5. Proteins with RING domains exhibit E3 ligase activities

E3 ligases include the HECT (homologous to the E6AP carboxyl terminus)-type, SCF (Skp1–Cullin–F-box-protein), and RING (really interesting new gene) protein families [43, 44]. The RING-type represents the largest class with several hundred members in animals, including humans [45], and which includes the RBR (RING-inbetween-RING), U-box and B-box families [46–52]. Unlike the prototypical RING zinc-finger domain, U-box domains adopt the same $\beta\beta\alpha$ -RING fold but are not cysteine and histidine rich and do not bind any zinc ions. It is believed that RING E3 ligases specify themselves and specific proteins for ubiquitination. Some RING E3 ligases can target several substrates [16, 53, 54].

Proteins with a RING domain are typically hypothesized to possess E3 ligase activity. Commonly, RING proteins are demonstrated to have E3 ligase activity by performing in vitro autoubiquitination assays. In these assays, the reaction mixture consists of the E1 and E2, enzymes, Ub, the RING E3 protein, and ATP. Often, the isolated RING domain is used to confirm that it is the RING domain that confer E3 ligase activity to the RING containing protein [3, 28]. Western blot analysis of the reaction mixture is probed with either an antibody specific for the E3 protein or domain, but more often specific for a modified Ub, such as with biotin, His₆- or HA tag [3, 7, 28]. RING E3 ligase activity is confirmed by the presence of mono-ubiquitinated Ub (mono-Ub), di-Ub, and/or poly-Ub; the polyubiquitinated products with various amounts of Ub appear as a smear of high-molecular weight bands [28].

While most RING E3 ligases possess a single RING domain, the TRIM (tripartite motif) family possesses two or three domains with RING folds, with the RING domain found at the N-terminus [46, 55–60]. RING domains are 50 to 90-amino acid regions that typically consist of at least eight cysteine and histidine residues uniquely spaced along the primary sequence (**Figure 5**). RING domains are classified as zinc-fingers. As with most zinc-finger domains, the zinc ion (Zn^{2+}) is tetrahedrally coordinated. RING domains bind two zinc ions in a unique cross-braced arrangement (**Figure 5A**) [40, 57]. The cross-braced mechanism involves the first and third pair of zinc-ligands binding one zinc ion, and the second and fourth pair of zinc-binding residues coordinating the other zinc ion. The consensus sequences for RING domains (C-X₍₂₎-C-X_(9–39)-C-X_(1–3)-H-X_(2–3)-C/H-X₍₂₎-C-X_(4–48)-C-X₍₂₎C) [61] indicate that one zinc ion is normally bound by the sulfur atom of three cysteines and the imidazole nitrogen of a histidine residue while the other is usually bound by the sulfur of four cysteine residues. Other common consensus are C3H2C3, C4HC3 and C8. The number of amino acids between the Cys/His residues varies considerably in two regions, designated loops 1 and 2; the lengths are between 3 and 39 and 4–48 amino acids, respectively.

Figure 5. Sequences and structures of RING domains. **A.** Cross-braced arrangement of the zinc-binding mechanism in which the first and third pair of zinc-ligands bind one zinc ion, and the second and fourth pair binds the other zinc. **B.** Prototypical structure of the RING $\beta\alpha$ -fold adopted by RING domains exhibiting E3 ligase activity. The spheres present the two zinc ions bound by key cysteine and histidine residues. It should be noted that some RING domains do not have well-defined β -strands in their loop1. **C.** Sequence alignment of several RING domains for which the structures of E2–E3 complexes are known (Table 2).

Despite having eight covalent bonds to two zinc ions to stabilize the tertiary structure, RING domains can be susceptible to unfolding or aggregation when produced at the concentration to perform structural studies. Mutation of any of its eight zinc-binding ligands to a non-zinc binding amino acid, for example cysteine to serine, results in loss of binding to both zinc ions and unfolding of the domain [62].

As noted, RING domains can vary in the lengths of their sequences. In addition, with the exception of the conserved cysteine and histidine residues, RING domains do not share high sequence homology, which is normally in the 20% range. Despite these differences, RING domains adopt an overall beta-beta-alpha ($\beta\beta\alpha$) canonical RING fold (Figure 5B). RING fold is characterized by two large loops (L1, L2) in which L1 precedes the 2- to 3-turn α -helix, and L2 follows the helix. Loop 1 is typically more structured and consists of two short β -strands separated by a type-2 turn. Loop 1 consists of the first and second pairs of zinc-binding residues, while the first helical turn of the α -helix has another pair. Loop 2 consists of the last pair of zinc-binding residues and tends to exhibit more dynamic properties than L1. The relative locations of the two zinc ions are similar for all RING domains.

Despite the low sequence homologies among RING domains, there are several amino acids that are fairly well conserved at specific locations in the sequence. There

is a conserved hydrophobic residue (I/V/L) located between the first pair of cysteine residues and an acidic residue located two residues after the cysteine (**Figure 5C**). In addition, there are an additional 2–3 acidic (D/E) residues located between the first and second pair on L1. Just preceding and following the third pair of ligands are hydrophobic residues, in which the preceding residue is usually aromatic. For the last pair of zinc-binding residues on L2, there is a conserved proline and a hydrophobic residue located between them, while an arginine immediately follows this pair. The similarity in structures of RING E3 domains suggests a common mechanism of action.

While the RING domain confer E3 ligase activity, this function is generally in concert with it binding the substrate in order for the substrate to be ubiquitinated [63–65]. For instance, loss of interaction between the RING domain and its substrate results in loss of substrate ubiquitination [16, 65]. However, since the RING domain is only part of a larger protein, often, other domains or regions of the protein play key roles in substrate recognition and binding [16, 66, 67]. For example, mutations of the B-box domains in the MID1 protein that disrupted binding to a substrate protein prevents ubiquitination of the substrate despite MID1 possessing wild-type level ligase activity [16, 32]. Unfortunately, while the number of RING E3 ligases are prevalent, their substrates are not always known or fully characterized; so how RING E3 ligases recognize their substrates is still a work in progress.

6. Mapping E2-RING domain interactions

Unlike the other classes of E3 ligases (HECT, RBR, SCF) that first transfer the ubiquitin from the E2 enzyme to themselves, RING E3 ligases facilitate the concerted Ub transfer to the substrate protein by interacting with both the E2 and substrate, placing the substrate in close proximity to the E2 ~ Ub thioester bond. Thus, the interactions between RING E3 ligase, its cognate E2s and its substrate protein are essential for Ub transfer [63–65]. The inability of the RING domain to interact with either the E2 or substrate will result in loss of ubiquitination of either the RING protein (autoubiquitination) or the substrate (ubiquitination) [16, 32]. The ability of Ub to transfer from the E2 enzyme to the substrate is based on the reactivity towards the amino group of a lysine (aminolysis) or cysteine (transthiolation). Aside from lysine, its surrounding residues also play a crucial role in the determination of whether the protein will be ubiquitinated. The level of ubiquitination is determined by how the E2 enzyme accommodates the preferred lysine [8, 68].

7. RING domains are positioned far from the active site

Given that the interactions of RING E3 ligases and E2 enzymes impact auto- and substrate-ubiquitination, the structures of several RING domains and their cognate E2 enzymes are examined [69–74].

Evaluation of the literature and PDB reveal that four E2 members are highly studied: Ube2D1-3, Ube2N, Ube2G2, and Ube2L3 (class 1) (**Table 2**). The Ube2D1–3 family appears to be the most promiscuous; these three enzymes are usually the first to be employed to test whether a protein with a RING domain possess E3 ligase activities. The complexes of Ube2D1 with 12 RING E3 domains (MDM2, RNF12,13,25,31,165, CNOT4, c-CBL, BIRC2,7) have been characterized [75–83]. Sequence analysis of these RING domains reveal low pairwise sequence identities. The

E2's	Interacting E3's	E2-E3 structures	E3 structures
Ube2d1	RNF147	5FER	6FLM
	2CP4	SNURF	4AP4
		ZNRF4	3OJ4
		CNOT4	5AIE
Ube2d2			2CPI
	CNOT4	1UR6	2CPI
	3L1Y	MDM2	6SQS
		RNF12	5AFG
		RNF13	6W7Z
			5ZBU
		RNF31	5ZC4
			4LJQ
		RNF165	5EDV
			4ULK
		RNF165	5D0K
			5D0I
		RNF25	5D1K
		RNF55	4A49
		RNF50	2JUJ
Ube2d3			3GT9
		cLAP1	4AUQ
			6HPR
		cLAP2	3M1D
			2UVL
	mycbp2	3EB6	5O6C
	1X23	6T7F	3KRE
		UBE4B	3L1Z
		RING1B	4S3O
		RING1B	3RPG
Ube2e1			2CKL
	TRIM21	6FGA	5OLM
	3BZH		
Ube2e2			
	RNF12	6W9A	
1Y6L			
Ube2g2	AMFR	2LXP	4G30, 2LXH(NMR)
2CYX	AMFR	3H8K	4G30, 2LXH(NMR)
Ube2l3	ARIH1	5TTE	4KC9
6XXU	ARIH1	5UDH	4KC9
Ube2n	RNF85	5VNZ	2JMD
1JBB			
	RNF85	3HCU	2JMD
	SNURF	5AIT	2EA6
	TRIM21	6S53	5OLM
	ZNRF1	5YWR	
	RNF8	4WHV	4AYC
	RNF8	4ORH	4AYC

Table 2.
PDB IDs corresponding E3, cognate E2, and E2-E3 structures.

highest sequence identity is between RNF165 and RNF12 at 25%, while the remaining RING domains have identities of $15 \pm 4\%$. The structures of Ube2D1 were solved in complexes with ZNRF7, CNOT4, TRIM25, and RNF4. NOT4 and ZNRF4 have the lowest sequence identity of 13%, while the highest pairwise identity is between TRIM25 and RNF4 at 30%. The average is $\sim 20\%$. The Ube2D3 was solved in complexes with Ube4B, Mycb2 and RING2. Structural studies of Ube2N with ZNRF1, TRAF6, TRIM21, RNF4 and 8 RING E3 ligases are also reported [84–88]. The average pairwise identity among these RING domains is $23 \pm 6\%$, while the highest is 36% between RNF4 and TRIM21; the lowest is between RNF8 and ZNRF1 at 16%.

Interestingly, despite the low sequence identities between the RING domains, the structures of the E2-RING complexes reveal a common mode of interaction (**Figure 6**). All the RING domains are located on a similar site of the E2 enzyme and oriented in a similar manner. The face of the RING domain that interacts with the E2 enzymes involves residues of the structured loop1 and its two β -strands and loop2, and incorporates both zinc ions. The zinc ions do not provide any stabilizing interaction with the E2 amino acids but may contribute an electronic effect that adds to the allosteric effects. The RING domains interact with the surface opposite to the β -sheet of the E2 structure, and contacts residues at the N-terminal end of helix 1 the loops connecting the helix traversing the β -sheet, the helical turn adjacent to the catalytic

Figure 6.

Interactions of RING E3 domains with Ube2D2. A. Structures of Ube2D2 bound to MDM2 (purple), RNF12 (salmon), RNF165 (orange), and RNF50 (yellow). This mode of binding is prototypical for all known E2-RING interactions. The closest residue of the RING domain from the catalytic cysteine is ~ 15 Å. The binding interface averages $\sim 550 \pm 30$ Å² [2]. B. Structure of an E2-RING (pdb: 4ap5) with key pairs of residues highlighted at the interface. Some of these residues are identified in 'D'. C. the tertiary structures of E2-RING pairs from each of the four E2 classes were analyzed by the program ConSurf to identify the locations of conserved residues. Residues/regions on the protein that are more conserved are colored bright pink/red and regions with variable residues are colored blue. Bright pink/red regions are located at the interface with the RING domains for all four types of Ub E2 enzymes. D. Summary of the types of interactions associated at the interface. There is usually one salt-bridge, four hydrogen bonds and ~ 40 non-covalent contacts.

cysteine, and a loop connecting two of the β -strands. The area of the interface averages to $\sim 550 \text{ \AA}^2$ [2] for the various structures, as calculated by PDBsum [89, 90].

Interestingly, the location of the RING domains is found to be at least $\sim 15 \text{ \AA}$ from the thioester linkage catalytic site (**Figure 6B**), which would suggest that RING domains are not directly involved in the chemistry of the Ub transfer. In fact, the distal location indicates that RING domains exert an allosteric effect as the key factor in influencing Ub transfer to the substrate. Based on the sequence alignment, and the structures of the complexes, it appears that E2 members of each family have uniquely conserved residues involved with their interactions with different RING domains and these residues can contribute to the long range of structural communication. Analyzing the structures and sequences of these complexes with the program ConSurf [91, 92], confirmed conserved residues at the interface. The structures of Ube2D2, Ube2N, Ube2G2 and Ube2L3 all show a large patch of conserved residues at the interface where the RING domains interact (**Figure 6B**). Most of these residues are found on the N-terminal half of helix 1, the central helix that traverse the β -sheet, on the loops connecting the β -strands and on the helical turn. Residues that are less conserved are located away from this E2-RING interface (**Figure 6C**).

Among the class 1 enzymes, for which the most structural information is available, there are four key sets of interactions with RING domains. The amino group of an arginine near position 5 (helix 1) forms a hydrogen bond with the carbonyl group of a hydrophobic residue (valine, leucine, or isoleucine) on the RING domain. The hydrophobic property of the hydrocarbon chain contributes to the arginine interaction with the hydrophobic residue. For many of E2s, a glutamine (Gln) or asparagine (at position 92 of Ube2D2), located on the helical turn, forms a hydrogen bond with an arginine side-chain found on loop2 of the RING domains. This interaction appears to be essential for the activation of the thioester bond for nucleophilic attack. Mutation of this glutamine dramatically affects Ub transfer [63, 64]. Interestingly, other members of the E2 (ex: Ube2G2, Ube2N) have a basic residue (R/K) at this position, indicating that these E2 enzyme may interact with a RING domain that instead of having an arginine on loop2 may have a complementary Asn/Gln residue. Such complementarity may provide some clue about specificity. For example, Ube2D2 with a Gln may not bind the same RING domain as Ube2G2, which has a Lys in place of the Gln. It would seem logical then that the cohort RING domain will have a Gln or Asn to form a hydrogen bond with the E2 enzyme.

The class 1 E2's have several fairly conserved proline residues, but the proline (position 95 of Ube2D2) makes hydrophobic contacts with a proline or isoleucine residue on the RING domain. Multiple sequence alignment of the RING domains confirmed these conserved residues (**Figure 6C**). The hydrophobic residue that interacts with Arg5 of Ube2D2 and the arginine that interacts with Gln92 are conserved in RING E3 domains. Adjacent to Pro95 (Ube2D2) is Ser94 that forms a hydrogen bond between its sidechain OH and backbone carbonyl group of the proline/isoleucine of the RING domain that interact with Pro95 (**Figure 6B, D**).

The Ube2N family is also found to have four conserved interactions with three RING domains. The Serine and Proline interaction is conserved, however in the Ube2N family it corresponds to Ser96. The arginine interaction with a hydrophobic residue is also conserved for this family, however it is Arg7 instead of Arg5. An interaction between Lys10 and Leucine is also conserved in almost all catalog entries, with hydrocarbons in their side chains interacting. Lastly, Arg6 is found to be involved in a conserved interaction with a negatively charged residue (Asp or Glu). It is possible that the slight differences in positions of the various RING domains with

the Ube2D2 enzyme may reflect the RING domains adjusting to make these conserved interactions with the E2.

Although the Ube2G2 and Ube2L3 families had significantly less cataloged entries, a few conserved interactions with their RING families are noted. Despite Ube2G2 being solved in a complex with only one RING domain (RNF45), the serine and proline interaction is conserved, and in Ube2G2's case it is Ser111 interacting. An interaction between Ser67 of Ube2G2 and a Gln of RING domain is conserved, as well as an electrostatic interaction between Glu108 and an arginine of RNF45. Ube2L3 has conserved interactions between its Pro95 and the RING domain proline or isoleucine, and between its Arg5 and an isoleucine of RING E3 ligase. Notably absent from conserved residues in Ube2G2 is the interaction of serine and proline. Ube2L3 is also missing the conserved interaction of Arg5 and a hydrophobic residue.

The conserved interactions among the E2s and RING domains, noted above, are confirmed by analyzing the various structures using PDBsum. The analyses reveal an average of one salt-bridge, four hydrogen bonds and ~ 40 non-covalent interactions. The surface area of the interface is relatively small, usually less than 600 Å² [2]. These observations would indicate a rather labile interaction between RING and E2s. NMR studies probing the interactions of RING and E2 enzymes confirm fast exchange in binding by the observation of very small chemical shift changes in the protein NH NMR signals when these proteins are titrated with respect to each other [69, 93–95]. NMR, isothermal calorimetry, and SPR binding studies of 23 pairs of RING E3 and E2 proteins exhibit dissociation constants (K_d) are in the sub-millimolar (60–200 μM) range [64].

Despite this common binding mechanism, it is not exactly clear how specificity is established between different E2 enzymes and their cognate RING E3 ligases. It is possible that although the catalytic domain of these proteins have a high level of sequence and structural similarity, minor differences of amino acids may dictate binding specificity to different RING E3 ligases. Interestingly, yeast two-hybrid screening studies reveal that some E2s interact specifically with one RING protein, while others can interact with over a hundred different RING E3s [36]. For instance, Ube2U has 52 interactors, UBE2D1–4 have 29–35 interactors, and UBE2N has 28 [36]. These observations of E2s promiscuity for many RING E3s is not unsurprising given that human cells have only a few dozen E2s and hundreds of RING E3s that must associate to promote the ubiquitination of protein substrate. There are also RING E3 ligase that can interact with multiple E2s [28, 36]. However, there are still RING E3s that only bind specific E2s [36]. It is possible that a RING domain interacts with several E2s and that some of these interactions may dictate different levels of substrate ubiquitination, i.e. mono- vs. di- vs. polyubiquitination [28].

8. RING domains as activators for aminolysis of E2 ~ Ub linkage

As noted, the distal binding of RING domain from the catalytic cysteine exerts allosteric effects on the reactivity of the active site [71, 72]. Structures of a RING domain interacting with an E2 enzyme with an Ub reveal that the RING domain binds in the same position compared with when it binds the free E2 enzyme [48, 71, 80, 96, 97]. NMR and computational studies have shown that the covalently attached Ub is highly mobile making transient interactions with the E2 enzyme, but in the presence of the bound RING domain the Ub exhibits more interactions with the E2 enzyme [35, 71]. This confirmation is referred to as the closed conformation. In the absence of the RING

domain, the Ub is substantially more flexible. Thus, the RING domain promotes a more closed E2 ~ Ub conformation (more E2:Ub contacts). Mutations that destabilize the closed conformation of the E2 and Ub are shown to disrupt Ub transfer activity [71, 98]. The closed E2 ~ Ub conformation is important for activation of the thioester bond for nucleophilic attack from a lysine side chain amino group [64]. Examination of the several E2–RING structures reveals that the central helix moves slightly outwards and the N-terminal helix becomes longer by a helical turn for several E2 enzymes. Some of the β -strands also move positions ever so slightly. Despite the small interface, the binding of the RING is sufficient to induce electronic and conformational changes as part of its allosteric effect.

While this review focuses on the interaction of E2 enzymes and monomeric RING domains, there is now studies that suggest that RING homo- and hetero-dimers and multimers are important for increasing the rate of aminolysis and Ub transfer [15, 76, 85, 97, 99–106]. BRCA1 is shown to have enhanced ligase activity when in a RING-RING complex with BARD1 [101, 105, 107], and MID1 RING domain exhibits increased activity in complex with the B-box domain [16, 28, 53]. The enhanced effects of RING dimerization is observed in vivo and in vitro and cannot be rationalized structurally [63, 64, 65, 107, 108]. The structures of RING dimers reveal that they are symmetrical and that the interface involves the surface opposite to the one involved in the interaction with E2 enzymes. How this interaction enhances the allosteric and electronic effects that RING has on the reactivity of the thioester linkage is not clear. Structures of RING dimers with E2 enzymes are also dimeric structures, in which each RING binds its own E2 enzyme [80, 106, 109]. It is possible that the increased activity for some RING proteins may be due to an increased apparent concentration effect due to their dimerization or multimerization.

9. Conclusion

Ubiquitination is an essential process that serves to regulate many cellular processes, most notably in regulating the cellular concentrations of proteins (homeostasis) through cellular degradation. The pathway to covalently attach a Ub to a substrate protein is highly coordinated. Errors in this pathway have significant consequences to cell function and contribute to the pathogenesis of several human illnesses including cancers, genetic disorders, and brain disorders [13, 110–114]. Many of these defects are associated with a dysfunctional RING protein that obviously leads to an increase in concentration of their target protein.

Though the eukaryotic system have created redundancies in how it labels proteins for proteasomal degradation, RING E3 ligases are overwhelmingly the most prevalent with currently over 700 members in humans. It is expected that this number will increase to also include proteins with domains that have $\beta\beta\alpha$ -RING folds. For instance, the U-box domain is shown to have the same RING fold despite not being cysteine and histidine rich and not binding any zinc ions [47, 115, 116]. The U-box domain is shown to interact on the same interface and manner on Ube2D2 as the zinc-binding RING domains [71, 72], suggesting that function preserves structure. Furthermore, the MID1 B-box1 and B-box2 domains are shown to bind two zinc ions and adopt a similar RING fold as monomers and a RING dimers in tandem, despite having less than 25% sequence homology with RING domains [46, 58, 59]. The B-box domains are similar to RING domains in the manner in which they bind the zinc ions but the sizes of their L1 and L2 regions are on the smaller ranges of RING domains.

In contrast, there are some zinc-binding domains that adopt the same $\beta\beta\alpha$ -RING fold but function as E3 ligase enhancers or E4 ligases. The BARD1 RING domain dimerizes with BRCA1 to enhance the E3 ligase activity of BRCA1 [101, 105, 107]; similarly, MdmX dimerizes with Mdm2 to target p53 [68, 76]. It is unclear why these E4 RING domains do not facilitate ubiquitination but it is possible that their interaction with the E2 enzyme is considerably different than that of RING E3 domains. There are currently no structures of an E4 enhancer with an E2, possibly because the interaction is so weak. The structures of BRCA1:BARD1 and Mdm2:MdmX are essentially very similar to homo-RING dimers suggesting that the mechanism of enhanced activities observed for RING homo-dimers may be the same for RING hetero-dimers.

It has long been postulated that the RING E3 ligases target a specific protein for ubiquitination and therefore how a large number of proteins interact with a little over three dozen E2 enzymes is not fully understood. As noted, RING domains share very low sequence homology with each other yet they all adopt a very similar structure, indicating that their mechanism of function is very similar. To try and provide insights into how RING domains are recognized, the sequences and structures of RING domains and E2 enzymes are evaluated. And while this review does not answer all the questions about the interaction, many of which are being investigated, it does provide some clues. As noted, E2 enzymes have a central catalytic domain that adopt a common structure. Despite variations in sequence identities and similarities, it appears that the catalytic domain preserves conserved residues at the interface that bind RING domains and residues that will transmit the allosteric effect of the bound RING domain (**Figures 3 and 4**). Similarly, the RING domain with far less sequence homologies also maintain key amino acids in specific locations to interact with the E2 catalytic domain in a similar location.

RING domains are relatively small (50–70 amino acids) in the context of their roles as enzymes and in facilitating the ubiquitination of substrate proteins. It would seem quite daunting for such a small protein domain to bind large proteins (E2, substrate) while maintaining their role to influence Ub transfer [16, 28, 53]. Despite the importance of substrate ubiquitination via RING E3 ligases, the mechanism is still not completely understood. Furthermore, it appears that the mechanism of interaction helps to determine which lysine residue on a substrate gets ubiquitinated. It should be noted that the RING domain exists as part of a larger protein and that the binding of substrates is not always relegated to the RING domain; more often substrates are proteins that bind other domains within the RING-containing E3 ligase protein. Mutations in the B-box1 domain of the MID1 RING protein, which binds alpha4 and the catalytic subunit of protein phosphatase 2A (PP2Ac), prevented polyubiquitination of these proteins despite MID1 maintaining full autoubiquitination activity [53]. Similarly, the SH2 domain of the c-CBL E3 ligase functions to bind the substrate while the RING domain interacts with the E2 enzyme and facilitates ubiquitination [45, 65]. In fact, it is possible to create chimeras with a RING domain followed with a domain that specifically binds any protein. Such substrate trapping strategy can be useful in regulating proteins whose upregulation is associated with cellular dysfunction and human diseases.

Finally, while there has been considerable research in this exciting area of protein ubiquitination, specifically in understanding how E2 and E3 proteins interact, there are still many questions left unanswered, such as how does specific E2-RING interaction influences the level and type of ubiquitination, and the lysine that becomes covalently modified.

Authors contribution

EMG and IS performed literature review, data collection (sequences and structures) and performed analysis. They also drafted the manuscript. MM oversaw the whole project, guiding the analysis and writing the manuscript.

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