The MLLE Domain of the Ubiquitin Ligase UBR5 Binds to Its Catalytic Domain to Regulate Substrate Binding*

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E3 ubiquitin ligases catalyze the transfer of ubiquitin from an E2-conjugating enzyme to a substrate. UBR5, homologous to the E6AP C terminus (HECT)-type E3 ligase, mediates the ubiquitination of proteins involved in translation regulation, DNA damage response, and gluconeogenesis. In addition, UBR5 functions in a ligase-independent manner by prompting protein/protein interactions without ubiquitination of the binding partner. Despite recent functional studies, the mechanisms involved in substrate recognition and selective ubiquitination of its binding partners remain elusive. The C terminus of UBR5 harbors the HECT catalytic domain and an adjacent MLLE domain. MLLE domains mediate protein/protein interactions through the binding of a conserved peptide motif, termed PAM2. Here, we characterize the binding properties of the UBR5 MLLE domain to PAM2 peptides from Paip1 and GW182. The crystal structure with a Paip1 PAM2 peptide reveals the network of hydrophobic and ionic interactions that drive binding. In addition, we identify a novel interaction of the MLLE domain with the adjacent HECT domain mediated by a PAM2-like sequence. Our results confirm the role of the MLLE domain of UBR5 in substrate recruitment and suggest a potential role in regulating UBR5 ligase activity.

Ubiquitination is one of the most abundant post-translational modifications in eukaryotic cells. Catalyzed by the ubiquitin proteasome system, ubiquitination has two major roles as follows: regulation of protein degradation, essential for normal cellular function and for the removal of harmful, damaged, or misfolded proteins; and control of protein activity by regulating protein/protein interactions and subcellular localization (1, 2). The ubiquitin proteasome system targets proteins through the addition of one or more ubiquitin molecules to specific lysine residues or to the N terminus. This process is carried out by a complex cascade of reactions catalyzed by activating (E1), conjugating (E2), and ligating enzymes (2, 3). The E3 ubiquitin ligases mediate the specificity toward substrates and catalyze the final attachment of the 76-residue ubiquitin moiety to the target protein. E3 enzymes fall into two categories based on their catalytic mechanism: RING (Really Interesting New Gene) and U-box ligases promote ubiquitin transfer indirectly, whereas RBR (RING between RING) and HECT (homologous to E6-AP C terminus)-type ubiquitin ligases directly catalyze the transfer of ubiquitin to the substrate. In this latter category, the ubiquitin is transferred from the E2-conjugating enzyme to the substrate in a two-step reaction. In the first step, a catalytic cysteine in the E3 enzyme forms a thioester bond with the ubiquitin from the E2-ubiquitin intermediate. In the final step, ubiquitin is transferred from the thioester bond with the E3 to a lysine residue in the substrate (4).

Ubiquitin protein ligase E3 component N-recognin 5 (UBR5) also known as EDD (E3 isolated by differential display) is a mammalian ortholog of the HYD (hyperplastic discs) protein of Drosophila melanogaster (5, 6). UBR5 belongs to the HECT-type group of 3 ubiquitin ligases. Human UBR5 mediates ubiquitination of several proteins, including β-catenin, TopBP1, TERT, RORyt, Paip2, CDK9, ATMIN, among others, highlighting its role as an important effector in cell cycle progression and DNA damage response (7–15). UBR5 has also been suggested to be a tumor suppressor. Overexpressed or mutated UBR5 has been found in solid tumors, including ovarian, breast, hepatocellular, tongue, gastric, and melanoma (16–19). In addition, UBR5 exhibits E3-independent activity as a transcriptional cofactor for the progesterone receptor and serves as a binding partner for a diverse subset of proteins such as GW182, p53, CHK2, TFIIS, and DUBA (7, 8, 12, 20–23). Despite accumulating knowledge about UBR5 function, the biochemical roles and exact mechanisms of recognition and ubiquitination by UBR5 have yet to be determined.
UBR5 is a large 309-kDa protein and consists of a N-terminal UBA domain followed by two nuclear localization signals, a zinc-finger-like UBR-box, an MLLE domain homologous to the C-terminal domain of poly(A)-binding protein (PABP), and a HECT domain at its C terminus (Fig. 1A) (24–26). Remarkably, only two proteins in eukaryotic cells contain an MLLE domain, PABP and UBR5. In PABP, MLLE is a protein/protein interaction domain that recognizes effectors of translation initiation that display a conserved peptide motif, PAM2 (PABP-interacting motif 2) (25). The term MLLE comes from a signature motif MLLEKITG in the domain and the abbreviation of Mademoiselle in French. Solution and crystal structures of the MLLE domains from human UBR5 and various PABPs have shown that the domains consist of a bundle of 4 or 5 α-helices (25, 27). The PAM2 motif was initially identified in the following three proteins associated with mRNA translation and protein synthesis: Paip1 (PABP-interacting protein 1), Paip2, and eukaryotic release factor 3 (28). A bioinformatic survey highlighted the existence of many other PAM2-containing proteins, which include ataxin-2, Tob1/2, USP10, dNF-X1, TPRD/TTC3, and dMAP 205 kDa (29). The NMR solution and crystal structures of the MLLE domain from human PABP in complex with PAM2 peptides revealed that peptides bind to the most conserved helices α2, α3, and α5 of MLLE (30–32). Recently, GW182 was shown to bind to the PABP MLLE surface largely overlapping with the PAM2-binding site (33, 34).

Accumulating evidence supports the model in which competition between UBR5 and PABP for shared binding partners is linked to translation and gene expression regulation. This has been demonstrated for UBR5-mediated proteasomal degradation of Paip2 upon PABP depletion (14) and for the recruitment of GW182 and Tob1/2 by UBR5 to Argonaute-miRNA complexes during gene silencing (23).

The MLLE domain of UBR5 was first shown to bind to a fragment of Paip1 by GST-pulldown assays (27). The peptide binding properties of the UBR5 MLLE domain were later characterized by our laboratory using NMR chemical shift mapping and isothermal titration calorimetry (32). Despite previous studies, there is no atomic structure for UBR5 MLLE bound to a PAM2 peptide. Moreover, the binding of GW182 to UBR5 in miRNA silencing has not been characterized.

A number of substrates for ubiquitination by UBR5 have been described in the last few years. In numerous cases, the C-terminal fragment of UBR5 that includes the MLLE and HECT domains mediates binding. These observations suggest a role of the MLLE domain in the substrate selectivity of UBR5. For instance, Paip2 is targeted for proteasomal degradation by UBR5. However, it is unclear whether this interaction is mediated directly by the MLLE domain. A better understanding of PAM2 recognition by UBR5 should help in the identification of novel physiological partners and provide insight into its ability to regulate ubiquitin and E3-independent activity.

In this study, we determined the crystal structure of the MLLE domain of UBR5 in complex with the PAM2 peptide from Paip1. The structure explains the overlapping binding specificity of the MLLE domains from UBR5 and PABP. We reveal a novel intramolecular interaction involving the MLLE domain and the HECT domain of UBR5. This interaction is mapped to the N-terminal lobe in the HECT domain and is mediated by a PAM2-like sequence. Our results suggest a regulatory role of the MLLE domain in the catalytic activity of UBR5 beyond binding of PAM2-containing substrates.

**Experimental Procedures**

**Protein Expression, Purification, and Peptide Synthesis—** Human Paip2 protein and the MLLE, HECT, and MLLE-HECT domains of rat UBR5 were cloned into BamHI and Xhol restriction sites of the pGEX-6P-1 vector (Amersham Biosciences), and the construct was transformed into the *Escherichia coli* expression host BL21 Gold Magic (DE3) (Stratagene). The proteins were expressed and purified by affinity chromatography to yield a GST-fused domain or an isolated domain with a five-residue (Gly-Pro-Leu-Gly-Ser) N-terminal extension. Prior to crystallization, the MLLE protein was additionally purified using size-exclusion chromatography in gel filtration buffer (50 mM Tris, 100 mM NaCl (pH 7.5)). The final yield of purified protein was ~7 mg/liter of Luria broth culture media.

A plasmid coding for the full-length human UBR5 was kindly donated by Dr. Darren N. Saunders (Garvan Institute of Medical Research), and the protein was expressed in HEK293 cells as a His tag fusion protein.

The Paip1(123–144), Paip2(106–127), and GW182(1380–1401) peptides were synthesized by Fmoc solid-phase peptide synthesis and purified by reverse-phase chromatography on a C18 column (Vydac, Hesperia, CA). The composition and purity of the peptides were verified by electrospray ionization mass spectroscopy. The HECT peptide and its F2505A mutant were expressed as GST-fused proteins in *E. coli*, purified with affinity chromatography, and cleaved with PreScission protease leaving a five-residue (Gly-Pro-Leu-Gly-Ser) N-terminal extension. Peptides were further purified by reverse-phase chromatography. Western blot analyses were done using anti-UBR5 and anti-Paip2 antibodies (Sigma).

**Isothermal Titration Calorimetry Measurements—** Experiments were carried out on a Microcal iTC200 titration calorimeter in 50 mM Tris-HCl buffer (pH 7.6) and 150 mM NaCl at 20 °C. The reaction cell contained 200 μl of 0.1 mM HECT-N lobe and was titrated with 19 injections of 2 μl of 1.0 mM MLLE domain. The binding isotherm was fit with a binding model employing a single set of independent sites to determine the thermodynamic binding constants and stoichiometry.

**Crystallization—** Crystallization conditions for the UBR5 MLLE-Paip1(123–144) complex were identified utilizing hanging drop vapor diffusion with the JCSG+ crystallization suite (Qiagen). The best crystals were obtained by equilibrating a 1.0-μl drop of MLLE-Paip1(123–144) mixture in a 1:2 ratio (10 mg/ml) in 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, mixed with 1.0 μl of reservoir solution containing 1.0 M ammonium sulfate, 0.2 M lithium sulfate, 10% glycerol, and 0.1 M Tris-HCl (pH 8.5). Crystals grew in 3–10 days at 20 °C. The crystals contain two MLLE and two peptide molecules in the asymmetric unit corresponding to $V_m = 2.89 \text{Å}^3 \text{Da}^{-1}$ and a solvent content of
57.4%. Residue numbers used here and in the PDB deposition are 14 less than in UniProt entry Q62671.

Structure Solution and Refinement—Diffraction data from a single crystal of the MLLE-peptide complex were collected on an ADSC Quantum-210 CCD detector (Area Detector Systems Corp.) at beamline A1 at the Cornell High Energy Synchrotron Source (CHESS) (Table 1). Data processing and scaling were performed with HKL2000 (11). The structure of UBR5 MLLE/Paip1 was determined by molecular replacement with Phaser (35), using the coordinates of MLLE from human UBR5 (PDB entry 1I2T). The initial model obtained from Phaser was completed and adjusted with the program Xfit (36) and was improved by several cycles of refinement, using the program REFMAC 5.2 (37) and model refitting. At the latest stage of refinement, we also applied the translation-libration-screw (TLS) option (38). The final model has good stereochemistry according to the program PROCHECK (39) and WHAT IF (40). The refinement statistics are given in Table 1. The coordinates and structure factors have been deposited in the RCSB Protein Data Bank (accession number 3NTW).

NMR Spectroscopy—NMR assignments of the MLLE domain of rat UBR5 were described earlier (32). All NMR experiments were recorded at 298 K. NMR titrations were carried out by monitoring the chemical shift differences between the 15N-labeled MLLE domain and monitored by 15N-1H heteronuclear single quantum correlation spectra. NMR spectra were processed using NMRPipe (41) and analyzed with XEASY (42).

Results

GW182 Interacts with the UBR5 MLLE Domain—Human GW182, a core component of the miRNA-induced silencing complex, interacts with PABP via its MLLE domain, and this interaction is required for miRNA-mediated deadenylation (33, 34). In a similar fashion, UBR5 was recently suggested to be a key component of the miRNA-silencing pathway with the MLLE domain being essential for its silencing function (23). UBR5 regulated miRNA-mediated gene silencing in an E3 ligase-independent manner by targeting the GW182 family of Argonaute-miRNA complexes. In this study, UBR5 recruited the translation effectors GW182 and Tob1/2 without prompting their proteasomal degradation. Previous studies have characterized the binding properties of several effectors of translation initiation that interact with the PABP MLLE domain through PAM2 motifs. To understand the ability of UBR5 to bind GW182, we performed a titration of the 15N-labeled UBR5 MLLE domain with GW182(1380–1401). Addition of the peptide produced large chemical shift changes in a number of residues, including specific binding (Fig. 1B). The titration resulted in fast-intermediate exchange that suggests high micromolar binding affinity. A fit of the chemical shift changes measured a $K_d$ of 175 ± 35 μM. Although significantly weaker than the interaction with the PABP MLLE domain (6 μM), the chemical shift changes upon GW182 binding are similar to those seen upon binding the PAM2 peptide from Paip1 (Fig. 1C). The largest chemical shift changes upon GW182 peptide binding were leucine, threonine, lysine, glycine, and alanine residues in helices α2, α3, and the C terminus of helix α5 (Fig. 1D). This confirms that GW182 binds the UBR5 MLLE domain through its PAM2 motif as seen in other PAM2-containing proteins.

Structure of UBR5 MLLE Bound to a PAM2 Peptide—To further understand the binding specificity of the MLLE domain from UBR5, we attempted to crystallize the domain in complex with the GW182 peptide. However, no crystals were obtained during crystallization trials. Alternatively, we were able to obtain diffracting crystals for MLLE in complex with the Paip1 peptide. This peptide showed the highest affinity ($K_d$ of 3.4 μM) among those tested in previous isothermal titration calorimetry studies (32). The asymmetric unit contains two copies of the MLLE-peptide complex, which are very similar with an r.m.s.d. of 0.24 Å over 58 Cα atoms. The electron density was missing for three and six residues at the N and C termini of the Paip1 peptide suggesting they are disordered (Table 1).

The structure of the peptide-bound UBR5 MLLE shows a helical bundle with four α-helices folding into a right-handed superhelix. When compared with the structure of the unliganded domain from human UBR5 (27), both structures are very similar, displaying an r.m.s.d. of 0.72 Å over residues Glu-2381–Ala-2437. The only significant difference can be seen in the N-terminal helix, which slightly bends toward the peptide in the complex structure (Fig. 2A). As the structure of the MLLE domain from PABP contains the additional α-helix at the N terminus (25), the helices in the domain from UBR5 are numbered from α2 to α5 for easier comparison. In the complex, the Paip1 peptide adopts an extended conformation except for a β-turn at residues Ser-129–Ala-132 that allows it to wrap around the highly conserved helix α3.

Hydrophobic interactions make major contributions to peptide binding to MLLE domains (33). The side chain of Paip1 Phe-135 interacts with Cα of Gly-2384, the methyl group of Thr-2403, and stacks with the side chain of Tyr-2388 in a classical “fishbone” stacking arrangement (Fig. 2B). Next to it, the side chain of Pro-137 packs against the aromatic ring of Tyr-2388. The side chain of Leu-128 inserts into a hydrophobic pocket formed by the side chains of Met-2405, Leu-2406, Leu-2409, Ala-2431, Leu-2434, and the aliphatic part of Glu-2430 (Fig. 2C). An additional hydrophobic interaction involves Ala-132 of Paip1, which is invariant in PAM2 sequences. The methyl group of Ala-132 packs against Cα of Met-2405, carboxyl of Gly-2404, and the Cα of Glu-2408 (Fig. 2C).

The peptide binding is reinforced by ionic interactions with the UBR5 MLLE domain. The carbonyls of Val-130 and Ala-132 form hydrogen bonds with the side chain of Lys-2401 (Fig. 2C). The amide of Phe-135 forms a hydrogen bond with the carbonyl and the side chain of Ser-2400 (Fig. 2B), which also makes hydrogen bonds with the carbonyl of Phe-135. The carbonyl of Tyr-136 makes a hydrogen bond with the side chain of Gln-2381. The side chain of Glu-2385 makes a salt bridge with side chain and amide of Ser-138 (Fig. 2B). The side chain of Ser-129 makes a salt bridge with the side chain of Glu-2408. Carbonyl of this serine makes an intramolecular hydrogen bond with the side chain of Ala-132, which stabilizes the bound conformation of the peptide (Fig. 2C).

UBR5 Binds Paip2—Conservation in the binding properties of the MLLE domains from PABP and UBR5 suggests that the
E3 ubiquitin ligase activity of UBR5 may play a role in translation. For instance, UBR5 targets the translation inhibitor Paip2 for ubiquitination and proteasomal degradation when PABP is depleted (14). To confirm this is due to a direct interaction, we tested binding of Paip2 to full-length UBR5 and the UBR5 MLLE-HECT fragment (Fig. 1A). We performed a series of pulldown assays using GST-fused full-length Paip2 as bait for binding to the full-length UBR5 (Fig. 3A) and GST-MLLE or GST-MLLE-HECT fragments of UBR5 as bait for Paip2 binding (Fig. 3B). In all cases binding of Paip2 to either the full-length UBR5 or the MLLE-containing fragments was observed. The presence of a phenylalanine residue in PAM2 motifs is conserved throughout PAM2-containing proteins and is required for their interactions with MLLE domains (30). We tested whether the Phe-118 of Paip2 was required for the interaction with UBR5 in our binding assays. The Paip2 F118A mutation abrogated binding to both full-length UBR5 and its MLLE domain confirming that the interaction was direct and specific to the MLLE domain of UBR5 (Fig. 3, A and B).

MLLE Interacts with the HECT Domain of UBR5—The ability of UBR5 to regulate its activity throughout the many pathways it is involved in remains elusive. In E3 ligases, often sequences or domains located in proximity to the HECT domain are involved in intra- and/or intermolecular interactions that modulate the catalytic activity (4, 43–45). The MLLE domain in UBR5 is located at the N-terminal side of the catalytic HECT domain with a 50-residue separation. Thus, we asked whether the MLLE domain might interact with the HECT domain. To test this, we performed an NMR titration of 15N-labeled MLLE (Fig. 4A) with the unlabeled GST-fused HECT domain (residues 2520–2799). Stepwise addition of the GST-HECT domain resulted in severe line broadening and the loss of most of the peaks in the NMR spectrum (Fig. 4B) suggesting formation of a high molecular weight complex. As controls, titrations of MLLE with GST, with the UBA domain (residues 180–230), or with the UBR box (residues 1177–1244) of UBR5 showed no spectral changes, indicating no binding (data not shown). An additional control with the MLLE domain of PABP
showed that HECT binding was limited to the MLLE domain of UBR5 (data not shown). Together, these data demonstrate that the MLLE domain of UBR5 specifically binds to its HECT domain.

The HECT domain of E3 ligases consists of a bilobal structure with a C-terminal lobe containing the catalytic cysteine residue and an N-terminal lobe that binds the E2 enzyme. The lobes are linked by a flexible region, which presumably facilitates proper positioning of the catalytic cysteine toward the ubiquitin-E2 thioester bond (4). Our next question involved the determination of whether the MLLE/N-lobe interaction required the peptide-binding surface of the MLLE domain by adding a PAM2 peptide to the complex of MLLE and N-lobe domains. If the MLLE/N-lobe interaction was dependent on the PAM2-recognition surface of MLLE, then the peptide would compete with the N-lobe for MLLE, and the NMR signals would reappear, which is what we observed. Addition of a peptide corresponding to residues 106–127 of Paip2 resulted in the reappearance of signals for MLLE at the positions consistent with MLLE binding the PAM2 peptide (Fig. 4E). Isothermal titration calorimetry measurements of the MLLE/N-lobe interaction measured a $K_d$ of $50 \pm 2.0 \mu M$ (Fig. 4F). These results show that the MLLE domain from UBR5 interacts with the HECT domain in a PAM2-dependent manner.

**Discussion**

The specificity of the ubiquitination process relies on the E3 ubiquitin ligases and their ability to directly interact with substrates. Over 600 different E3s have been identified in the human genome, and 28 belong to the HECT-type E3 family. In all cases, the HECT domain is located at the C terminus of the protein, and the substrate binding is mediated by various domains located N-terminal to the HECT domain (4). The activity by HECT E3s can be regulated at two levels. In the first level, substrate binding is mediated through protein/protein interactions by domains/motifs located N-terminal to the HECT domain. Some HECT proteins also interact with regulatory/auxiliary proteins that facilitate or interfere with substrate binding (46–48). In the second level, regulation occurs through intramolecular interactions that inhibit ubiquitin-thioester formation or E2 binding (43–45, 49). Despite accumulating functional knowledge about the regulatory mechanisms that govern E3 ligases, our structural understanding of the inter- and intramolecular interactions that modulate the catalytic activity of HECT-type enzymes has lagged. The
HECT-type ligases in the Nedd4 family are the most studied to date. In SMURF2, the C2 domain interacts with the HECT domain rendering the full-length protein inactive. The N-terminal lobe of the HECT domain interacts with the C2 domain and with ubiquitin. Both interacting surfaces overlap, affecting transthiolation and noncovalent binding of ubiquitin to the N-lobe (44). In the case of Itch, the autoinhibitory mechanism involves an intramolecular interaction between the WW domains and the HECT domain. Phosphorylation of the PRR regions of Itch causes a conformational change that weakens the WW/HECT interaction increasing its catalytic activity (43). A similar regulatory mechanism is seen in the non-Nedd4 HECT-type ligase HUWE1. An N-terminal helical element was shown to affect the catalytic activity of the HECT domain in HUWE1. In the absence of this N-terminal helix, the isolated HECT domain gained activity relative to the helix-extended counterpart; the authors hypothesize that this could be due to an increase in the inner flexibility of the HECT domain that allows the enzyme to acquire a favorable orientation for ubiquitin transfer or product release (49).

In the case of UBR5, we have identified an intramolecular interaction between the HECT domain and the adjacent MLLE domain. This interaction has the potential to regulate the catalytic activity of HECT in a manner similar to that seen in other E3 ligases. We measured the affinity of the interaction between the isolated domains to be 50 \text{M}, which is relatively strong considering that, in the intact UBR5 protein, the two domains are separated by only 50 amino acids (Fig. 3A). Previous phosphoproteomic studies have reported UBR5 to be heavily phosphorylated (50). It is possible that specific phosphorylation sites in the protein lead to conformational changes that regulate protein orientation. Both surfaces overlap, affecting transthiolation and noncovalent binding of ubiquitin to the N-lobe (44).
ubiquitin activity. The MLLE/HECT interaction might regulate HECT domain activity by preventing proper E2 binding or positioning of the C-lobe to receive the ubiquitin. In parallel, the MLLE domain also acts as a substrate-binding domain so that substrate binding might be correlated with activation of ligase activity.

UBR5 plays an essential role in cellular processes such as DNA damage response, translation initiation, and cell cycle progression. However, the mechanistic details of how UBR5 interacts with substrates are poorly understood. To date, the only PAM2-containing protein identified as a substrate for ubiquitination and proteasomal degradation by UBR5 is Paip2. Yoshida et al. (14) proposed a homeostatic mechanism where PABP and UBR5 compete for binding to Paip2, an inhibitor of PABP function. A decrease in PABP levels augments the concentration of Paip2 that is available to interact with UBR5, leading to Paip2 proteasomal degradation. As Paip2 levels decrease, the relative amount of PABP increases, and the overall activity of PABP is restored in a positive feedback. In contrast, UBR5 plays an essential role in microRNA-mediated gene silencing independent of its ubiquitin ligase activity (23). To date, there are two suggested roles of the MLLE domain in miRNA silencing. First, GW182 proteins recruit UBR5 into Ago/PAM2 miRNA complexes through its MLLE domain. Second, UBR5 MLLE interacts with PAM2-containing proteins in a similar fashion to PABP thus sharing binding partners such as Paip1/2 and Tob1/2. Through protein interactions with these proteins, the extended protein network includes different deadenylase complexes, all of which play key roles in regulating translation and mRNA stability. In this study, we characterized the binding of the UBR5 MLLE domain to the GW182 PAM2 peptide and solved the crystal structure of the MLLE/Paip1 complex. Comparison of the MLLE domains of UBR5 and PABP shows that the major intermolecular interactions that mediate peptide binding are preserved in both proteins. However, in general, the affinity of the UBR5 MLLE domain for PAM2 peptides is lower.
than that of the PABP MLLE domain. The complex with the GW182 peptide is no exception. The GW182 PAM2 peptide binds to UBR5 MLLE with 30-fold lower affinity than to the PABP MLLE domain (33). This likely reflects the unique C-terminal sequence of the GW182 PAM2 motif, which contains a tryptophan residue that inserts between the helices \( \alpha_2 \) and \( \alpha_3 \) of PABP MLLE (32, 34). The biological significance of the wide range of PAM2 affinities measured in vitro is unclear. It would be interesting to investigate the functional significance of the differences in affinity for GW182 in the Ago2/miRNA complex formation.

The binding of the PAM2 peptides to UBR5 shows surprising contrasts in function. PAM2 motifs from GW182 and Paip2 have the ability to bind the MLLE domains from both UBR5 and PABP, but with a higher affinity for the latter. However, the proteins interact with UBR5 for different purposes. Paip2’s fate is to be targeted for proteasomal degradation, whereas GW182 promotes gene silencing. In contrast, the interaction of the PAM2 peptide from the HECT domain of UBR5 suggests a role in regulating UBR5 activity. Despite the fact that all of these interactions involve recognition of PAM2-like sequences, each of them seems to have a unique effect in the response of UBR5. It remains to be discovered whether the differences in affinity among PAM2 proteins are essential in determining the role of UBR5 or whether other events are key in controlling the different activities of UBR5.

In conclusion, we have characterized the PAM2 peptide binding to the MLLE domain of UBR5 by x-ray crystallography and NMR spectroscopy. Future functional and structural studies are required to address the role of the newly discovered MLLE/HECT interaction in the E3 ligase activity of UBR5.

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**Author Contributions**—K. G. coordinated the study. J. M. E., E. M. C., G. K., and K. G. wrote the paper. G. K. designed, performed, and analyzed the experiment shown in Figs. 1 and 2. E. M. C. designed, performed, and analyzed experiments seen in Figs. 3 and 4. J. M. E. designed, performed, and analyzed experiments seen in Fig. 5 and assisted E. M. C. in the experiments shown in Fig. 4. All authors reviewed the results and approved the final version of the manuscript.

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