The MID1 E3 Ligase Catalyzes the Polyubiquitination of Alpha4 (α4), a Regulatory Subunit of Protein Phosphatase 2A (PP2A)

**NOVEL INSIGHTS INTO MID1-MEDIATED REGULATION of PP2A**

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**Background:** IGBP1/α4 interacts with microtubule-associated MID1 to regulate PP2A within the mTOR pathway.

**Results:** MID1 catalyzes the polyubiquitination and degradation of α4, demonstrating for the first time a mechanism for α4 regulation.

**Conclusion:** The tandem RING-Bbox domains are required for α4 polyubiquitination and degradation.

**Significance:** Ectopic overexpression of IGBP1/α4 transforms cells. Ubiquitination of α4 impacts the stability and activity level of PP2A.

Alpha4 (α4) is a key regulator of protein phosphatase 2A (PP2A) and mTOR in steps essential for cell-cycle progression. α4 forms a complex with PP2A and MID1, a microtubule-associated ubiquitin E3 ligase that facilitates MID1-dependent regulation of PP2A and the dephosphorylation of MID1 by PP2A. Ectopic overexpression of α4 is associated with hepatocellular carcinomas, breast cancer, and invasive adenocarcinomas. Here, we provide data suggesting that α4 is regulated by ubiquitin-dependent degradation mediated by MID1. In cells stably expressing a dominant-negative form of MID1, significantly elevated levels of α4 were observed. Treatment of cells with the specific proteasome inhibitor, lactacystin, resulted in a 3-fold increase in α4 in control cells and a similar level in mutant cells.

Using in vitro assays, individual MID1 E3 domains facilitated monoubiquitination of α4, whereas full-length MID1 as well as RING-Bbox1 and RING-Bbox1-Bbox2 constructs catalyzed its polyubiquitination. In a novel non-biased functional screen, we identified a leucine to glutamine substitution at position 146 within Bbox1 that abolished MID1-α4 interaction and the subsequent polyubiquitination of α4, indicating that direct binding to Bbox1 was necessary for the polyubiquitination of α4. The mutant had little impact on the RING E3 ligase functionality of MID1. Mass spectrometry data confirmed Western blot analysis that ubiquitination of α4 occurs only within the last 105 amino acids. These novel findings identify a new role for MID1 and a mechanism of regulation of α4 that is likely to impact the stability and activity level of PP2A.

Alpha4 (α4) was first identified as an immunoglobulin binding protein (also known as IGBP1) involved in signal transduction through the B-cell antigen receptor in mammalian B and T lymphocytes (1–5). α4 functions as a novel regulatory subunit of protein phosphatase 2A (PP2A), PP4, and PP6 (6–10). With respect to PP2A, α4 binds the catalytic subunit (PP2Ac), at which time it is thought to displace the scaffolding (PP2Aα, PR65) and regulatory subunits (PP2Ab) that typically constitute the PP2A heterotrimeric complex (6, 11–13). Under growth-promoting conditions, α4 down-regulates PP2A activity within the mTOR signaling pathway, resulting in the downstream activation of eIF-4E and S6 kinase and the initiation of cell cycle progression (4, 14, 15).

Regulation of PP2A is directly linked to the strong interaction between α4 and MID1, a novel microtubule-associated E3 ligase belonging to the TRIM (tripartite motif) family of proteins (11, 14). MID1 possesses an N-terminal RING domain, two B-box domains, and a coiled-coil motif (16). The RING, Bbox1, and Bbox2 domains adopt ββα-RING folds, and each possesses ubiquitin E3 ligase activity (17–19). In their native tandem configuration, the activity of the RING domain is significantly enhanced by the B-boxes implying cooperativity in function (19). The interaction between α4 and MID1 is mediated by the C-terminal domain (residues 236–280) of α4 and the Bbox1 domain of MID1, whereas the N-terminal domain (residues 1–236) of α4 is involved in binding PP2Ac (15, 20, 21).

As part of the MID1-α4-PP2Ac complex, α4 is thought to modulate the functionality of both PP2A and MID1, facilitating either dephosphorylation of MID1 and its dissociation from the microtubules or the MID1-dependent ubiquitination of PP2Ac and thus turnover of the microtubule pool of PP2A (10, 20, 22).

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2 The abbreviations used are: PP2A, protein phosphatase 2A; Ub, ubiquitin; MDCK, Madin-Darby canine kidney; EGFP, enhanced GFP; B1B2, Bbox1-Bbox2; RB1, RING-Bbox1.
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Loss of function of MID1 is associated with a range of birth defects, including cleft lip/palate, cardiac septal defects, and hypospadias (23, 24). An increase in PP2Ac levels and decreased serine/threonine phosphorylation of cytoskeletal proteins seen in patient fibroblasts supports the notion that proper regulation of the microtubule associated PP2A activity is critical for normal development (9, 10).

Recent data suggest that α4 can play both a protective and destructive role in regulating PP2A activity and that this functionality is dependent on its monoubiquitination by MID1 (25). Given the promiscuity of PP2A, the MID1–α4–PP2Ac complex likely participates in the regulation of many different cellular processes. For example, this complex has been shown to directly regulate mTORC1 signaling as well as associate with the translational apparatus to regulate translation of various mRNAs including those for MID1 itself (6, 8, 10, 16, 26). In each case, it has been concluded that this regulatory role is conferred by MID1-mediated ubiquitination of PP2Ac (6, 8, 10, 28).

**MATERIALS AND METHODS**

**Generation of Stable MDCK Cell Lines**—MDCK cells were cultured at 37 °C in DMEM (Thermo Scientific) supplemented with 10% fetal bovine serum (Thermo Scientific) and 1× GlutaMAX (Invitrogen). To generate MID1delCTD-expressing cell lines, MDCK cells were transfected with either a pEGFP-C2-MID1delCTD or pEGFP-C2 control expression construct (35) using TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI). The MID1delCTD mutant involved a construct in which the MID1 reading frame was truncated near the start of the SPRY/B30.2 domain (K491stop; Fig. 1A). Stable cell lines were selected in 0.6 μg/ml Geneticin (Invitrogen) for 10 days. Antibiotic-resistant clonal cells were obtained by diluting the cells into 96-well plates at a concentration of less than one cell per well, and the growth of clonal cells was monitored under a light microscope as well as a fluorescent microscope for GFP expression. To confirm the expression of both intact GFP and GFP-MID1delCTD in selected clonal lines, extracts from each were examined by Western blotting. Briefly, cells cultured in a 10-cm plate were lysed in radioimmune precipitation assay buffer (Thermo Scientific) with protease inhibitors (Protease Inhibitor Mixture, EDTA-free, Thermo Scientific) for 15 min at 4 °C. Lysates were collected with a cell scraper and clarified by centrifugation for 10 min at 10,000 × g. Proteins were separated by SDS-PAGE (8–16%, Precise Protein Gel, Thermo Scientific, IL) and semi-dry-transferred to PVDF membranes (Millipore). Membranes were blocked with 5% nonfat dry milk in Tris-buff ered saline with 0.05% Tween 20, probed with anti-GFP (ab290, Abcam), and developed using chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific).

**Proteasome Inhibition Studies and Analysis of α4 Protein Levels**—GFP control and MID1delCTD-expressing MDCK cell lines were cultured with or without 10 μM lactacystin (Millipore) in complete DMEM media for 3 h before cell lysates were prepared as described above. Western blot analysis was performed using a mouse anti-α4 antibody (clone 5F6, Millipore) and a goat anti-mouse peroxidase conjugated secondary antibody (catalog #A-4416, Sigma). The membranes were then stripped and probed with a rabbit anti-GAPDH antibody (cat-
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alog #IMG-5143A, IMGENEX, San Diego, CA) and a goat anti-rabbit peroxidase conjugated secondary antibody (catalog #A-0545, Sigma) as a loading control. A digital image of the Western blot bands was obtained by scanning non-saturating x-ray film exposures, and the band intensities were quantified with Image J. A two-tailed t test was used (p < 0.05) to determine the significance of differences in normalized protein levels in the control and mutant cells.

**Functional Screening Using a Bacterial Two-hybrid System**—Because of the much faster generation time of bacteria over yeast and the fact that plasmid DNA is more readily recovered from bacteria, we chose the BacterioMatch® II Two Hybrid system as a means to efficiently and functionally screen randomly generated mutations in MID1 B-box1 for loss of ability to bind α4. The ability of two proteins of interest to interact in this system is assessed by growth of co-transformants on selective medium containing 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of histidine synthesis, and confirmed by growth of transformants on dual-selective medium containing both 3-AT and streptomycin. Assessment of self-activation of fusion constructs and testing of the expected interaction pairs was performed essentially as described (BacterioMatch® II Two Hybrid System Vector kit manual, Stratagene).

To generate bait and prey constructs, full-length α4 was digested with BamHI and EcoRI, gel-purified, and cloned into similarly digested pTRG vector (BacterioMatch® II, Stratagene). The MID1 B-box1 domain was amplified from full-length MID1 using the M1BB1-F (5'-GTG AAT TCT ACT AGT GCC GAG AAG GTC CTC TGC CAG TT-3') and M1BB1-R (5'-GTG TCT CGA GTC AGT CCG GAA TTG GCC CGG TCT CAG TGC CAG CAG TTG CTA TCA GAC-3') primers, and the product was digested with EcoRI and XhoI and directionally ligated into the bacterial host that already contained the pTRG-expression vector. The MID1 B-box1 domain was amplified from full-length MID1 using the M1BB1-F (5'-GTG AAT TCT ACT AGT GCC GAG AAG GTC CTC TGC CAG TT-3') and M1BB1-R (5'-GTG TCT CGA GTC AGT CCG GAA TTG GCC CGG TCT CAG TGC CAG CAG TTG CTA TCA GAC-3') primers, and the product was digested with EcoRI and XhoI and directionally ligated into the bacterial host that already contained the pTRG-expression vector. The His6-tagged α4 C-terminal construct was made by amplifying the DNA encoding residues Glu-236—Gly-339 using the forward primer 5′-CAC CAT GGA CGG GCT CAA GCC CAG GTG CCT ACA G-3' and the reverse primer 5′-TCA CTC ACT CAA AGC TGC CAC CTG ATG ATC-3'. The His6-tagged α4 N-terminal domain was generated using the forward primer 5′-CCCA AGG TTG ATC TTA AGC GAA GCC GTC CAG TGA AAC CCT T-3' and the reverse primer 5′-CCAG TTA AGC TTT CAG CCC ATG TTC TGT CGG TTC CC-3'. The PCR product was cloned into the pSKB3 vector, a derivative of pET28, for expression as an N-terminal His6-tag fusion. DNA sequencing confirmed the orientation and integrity of all vectors before they were transformed into BL21 (DE3) cells. Changing leucine 146 to glutamine within the MID1 constructs was performed by a standard mutagenesis protocol.

**Expression and Purification of Recombinant Proteins**—Cells expressing full-length α4 (His6-α4), the N-terminal domain (His6-α4N, residues 1–235), and the C-terminal domain (His6-α4C, residues 236–339) were grown in LB media at 37 °C to an A600 of ~0.6 and induced with 1 mM isopropyl-1-thio-D-galactopyranoside for 18 h at 16 °C (15). Harvested cells were resuspended in buffer 50 mM Tris, 200 mM NaCl, 10% glycerol (pH 8.0) with protease inhibitors and lysed by lysozyme. The lysates were clarified by centrifugation at 4 °C for 30 min at 20,000 × g. The supernatant was purified with Ni2+-NTA resin (Qiagen) and ion-exchange chromatography by FPLC (Bio-Rad). The MID1 RING-Bbox1-Bbox2 domains (His6-RB1B2), MID1 RING-Bbox1 domains (His6-RB1), MID1 Bbox1-Bbox2 domains (His6-B1B2, residues 71–214), and His6-RING92 (MID1 residues 1–92 that includes the RING domain) were expressed and purified as previously described (19, 31).

**In Vitro Ubiquitination Assay Using Purified Recombinant Proteins**—Human Ub-activating enzyme E1, human Ub-conjugating enzymes (UbcH5c), and ubiquitin were purchased from R&D Systems Inc. (Minneapolis, MN). Ubiquitination assays of the MID1 E3 ligase domains in the presence of α4, α4C, and α4N were performed in a total volume of 30 μL. The mixture contained 1 unit of inorganic pyrophosphatase, 1 mM DTT, 5 mM ATP-Mg2+, 0.125 μM E1, 2.5 μM E2 (UbcH5c), and 30–50 μM Ub in 20 mM Tris-HCl (pH 7.5) and different amounts of purified recombinant proteins. The RB1B2-L146Q auto ubiquitination assay was performed as previously described (32–
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34. The mixtures were incubated at 30 °C, and the reaction was terminated with 2× SDS-PAGE non-reducing sample loading buffer. The reaction products were subsequently analyzed by SDS-PAGE and chemiluminescent Western blot using the following antibodies: α4 N-terminal (catalog #α4 V-20) and C-terminal antibodies (catalog #α4 D-20, Santa Cruz Biotechnology). Control reactions were performed by omitting individual components from the assay.

Mass Spectrometry Studies of Ubiquitinated Products—The ubiquitinated protein bands were excised from the SDS-PAGE gels and treated with mass spectrometry grade trypsin. The cysteine residues were reduced and alkylated with methymethanethiosulfonate. The peptides were extracted from each gel piece, dried by vacuum centrifugation, resuspended in 0.1% TFA and desalted using a C18 ZipTip column. The samples were then analyzed using a 4700 MALDI TOF-TOF mass spectrometer equipped with a Nd:YAG 200 Hz laser. Raw data files were subjected to a database search using Gly-Gly modifications of Lys and Cys modifications that represented ubiquitin stubs generated by trypsinolysis. Putative Gly-Gly modified peptides were reviewed for statistically significant Mascot and X'Tandem peptide identification scores and for contiguous b-fragment and y-fragment ions.

RESULTS

MD1 Facilitates in Vivo Proteasomal Regulation of α4—To investigate the function of MD1 in epithelia, we attempted to generate permanent Madin-Darby canine kidney epithelial cells expressing an EGFP-tagged wild-type MD1. Unfortunately, repeated attempts to generate stable MDCK cells overexpressing full-length MD1 were unsuccessful as the cells exhibited cell division defects and increased cell death. We suspected this was due to reduced PP2A activity because of increased MD1-mediated polyubiquitination. Instead, we generated permanent MDCK epithelial cells expressing an EGFP-tagged mutant form of MD1 that is missing the C-terminal SPRY/B30.2 domain. Our previous studies as well as those of others had established that this mutant form of MD1, which is the most common type of mutation found in patients with X-linked Opitz syndrome, forms cytoplasmic aggregates (35) that in patient fibroblasts leads to reduced polyubiquitination and increased levels of PP2Ac (10). Importantly, this mutant MD1 retains the ability to dimerize with wild-type MD1 and thereby sequesters it away from its usual location, reducing overall endogenous MD1 activity (35, 36). Thus, the mutant MD1 (MD1delCTD) functions as a dominant negative (35, 37). In all cases cell lines expressing low levels of the GFP or GFP-MID1delCTD were maintained. For the MD1delCTD lines, this low level expression correlated with relatively few and smaller cytoplasmic aggregates of the mutant MD1 protein than normally seen in transient transfection experiments. This also correlated with a cell size similar to that of control cells, unlike cells that express high levels of the mutant.3 Multiple clonal cell lines were used for all experiments to serve as biological replicates.

MDCK cells expressing the dominant negative MID1delCTD showed significantly higher levels of α4 than the control GFP-expressing cells (p = 0.0135; Fig. 1B). To determine the basis for this increased level of α4, we treated the cells with the proteasome inhibitor lactacystin. We observed that inhibition of the proteasome resulted in increased α4 protein levels ~3-fold in the control cells, which only expressed endogenous MD1 (Fig. 1C) supporting the notion that α4 is regulated by the proteasome. Furthermore, when α4 levels in lactacystin-treated control and MID1delCTD-expressing cells were compared, there was no significant difference in levels (p = 0.3846; Fig. 1D), suggesting that the elevated α4 levels in untreated MID1delCTD cells is due to disruption of MID1-dependent proteasome-mediated degradation.

MD1 Facilitates Polyubiquitination of α4 in Vitro—We recently characterized the interaction between the MID1 Bbox1 domain and α4 (6, 8, 21) using a 45-amino acid peptide derived from the C-terminal region of α4 (α443, residues 236–280) that was shown to bind strongly to MID1 (6). We observed that α443 interacted with the Bbox1 domain at a site that was close to Lys-153, the lysine that is autoubiquitinated (19, 21). α443 was observed to be monoubiquitinated at one of the three lysine residues (19, 21).

In light of the results from the MDCK cells, we sought to determine whether the full-length α4 is directly targeted for polyubiquitination by MID1. To test this using a well established in vitro ubiquitination assay, we used the first three domains (RING, Bbox1, Bbox2) of MID1 that we previously showed possessed E3 ligase activity. Using full-length α4 in the presence of a MID1 construct consisting of the RING-Bbox1-Bbox2 domains (RB1B2, residues 1–214), we observed polyubiquitinated α4 products, as indicated by the high molecular weight “smear pattern” bands (Fig. 2A). For the control experiments, in which individual components of the assay were omitted, no polyubiquitination of α4 was observed. A very low level of monoubiquitination of α4 was observed when no MID1 protein was included.

RING and Bbox1 Are Necessary for α4 Polyubiquitination—In our previous studies, we showed that the RING domain possessed greater E3 ligase activity than either of the Bbox domains or the Bbox domains in tandem based on the amount of polyubiquitinated products observed in in vitro ubiquitination assays (19). When the RING and Bbox domains are linked, as observed in native MID1, the activity of the RING domain is significantly enhanced compared with the RING domain alone (19). To decipher the minimal MID1 construct that could promote the ubiquitination of α4, assays were performed with RING, B1B2, RING–Bbox1 (RB1), and RB1B2 domains.

α4 has been shown to strongly interact with the Bbox1 domain (7, 10, 21). However, in the presence of Bbox1–Bbox2 (B1B2) domains only a trace amount of monoubiquitinated full-length α4 was observed (Fig. 2B). Interestingly, using the RING domain alone (residues 1–92), a strong monoubiquitinated band and very weak higher molecular weight bands were observed (Fig. 2B), suggesting some level of polyubiquitination of α4. In contrast to the B1B2 and RING domain-only constructs, the RB1 and RB1B2 constructs resulted in polyubiquitination of α4 (Figs. 2, A and B). Testing of larger constructs of
MID1 (e.g. RING-Bbox1-Bbox2-Coiled-Coil (RBCC) and full-length MID1) resulted in similar levels of polyubiquitination of H9251 to those of the RB1 and RB1B2 constructs (Fig. 2C), consistent with the fact that domains C-terminal to the RING and Bbox domains have no impact on ubiquitination of H9251. These results also validated our use of RB1 and RB1B2 constructs instead of full-length MID1 for the ubiquitination assay of H9251.

Interaction of MID1 Bbox1 and H9251 Is Essential for H9251 Polyubiquitination—As stated above, the MID1 RING domain alone could monoubiquitinate H9251. However, only when the Bbox1 domain was present was H9251 polyubiquitinated. To investigate the requirement of direct binding of H9251 to Bbox1 for polyubiquitination, we used a mutant MID1 that was identified in an unbiased functional screen for mutations that disrupt the strong interaction of H9251 with Bbox1. Briefly, in this screen 850 randomly mutated clones were assessed using a bacterial two-hybrid system. Of the 850 clones, nine showed obvious reduced ability to grow on the selection plates (Fig. 3A), suggesting that these clones carried mutations that interfered with H9251 binding to Bbox1. Sequencing of these nine clones revealed a variety of mutations including a number of nonsense mutations as well as single base deletions. Three clones contained missense mutations, but two of these carried more than one mutation. The only clone with a single missense mutation encoded a Bbox1 domain with the amino acid substitution, leucine 146 to glutamine (L146Q). Sequence alignment of the Bbox1 domains of MID1 orthologs from human, rat, mouse, chick, and zebrafish together with the cognate MID2 orthologous sequences, which also bind H9251, revealed that Leu-146 is highly conserved (Fig. 3B). When the sequence of MID1 and MID2 was aligned with the other TRIM proteins of the C-I subfamily, which do not bind H9251, we observe variation at this position. Notably, TRIM67 has a glutamine in the equivalent position supporting the notion that the L146Q substitution maintains the overall Bbox1 topology and function and that the leucine residue is directly involved in H9251 binding.

To validate whether this amino acid substitution truly abolished the interaction with H9251, the mutation was introduced into full-length MID1 and expressed as a GFP fusion protein in Cos1 cells (Fig. 3C). In notable contrast to almost all Opitz syndrome-related MID1 mutations (35), the L146Q mutant did not appear to affect the ability of MID1 to associate with the microtubule network. However, when co-expressed with myc-tagged H9251, H9251 failed to associate with the microtubule cytoskeleton (Fig. 3C), as seen when expressed with wild-type MID1 (39). These data confirm the importance of leucine 146 for the interaction with H9251.
To test the effect of the L146Q mutation on the ability of MID1 to ubiquitinate \( \alpha_4 \), we expressed the RB1B2 fragment containing the mutation (RB1B2-L146Q) and used it in the same in vitro ubiquitination assays. The results showed that the mutant was unable to polyubiquitinate \( \alpha_4 \) (Fig. 4A). However, \( \alpha_4 \) did still appear to be monoubiquitinated, consistent with the result obtained with the RING domain alone. As noted, the RING domain has not previously been shown to physically interact with \( \alpha_4 \). Intriguingly, in the control experiments, RB1B2-L146Q had full auto-polyubiquitination activity (Fig. 4B) similar to wild-type RB1B2. Taken together with the lack of impact of the L146Q mutation on microtubule binding of full-length MID1, these data indicate that the structure and activity of RB1B2 is not affected by the specific mutation but that it only affects binding and polyubiquitination of \( \alpha_4 \).

**Polyubiquitination of \( \alpha_4 \) Occurs within Its C-terminal End**—To determine where on full-length \( \alpha_4 \) polyubiquitination occurs, we conducted reactions with either the N-terminal (residues 1–235) or C-terminal (residues 236–339) regions of \( \alpha_4 \). Polyubiquitination was observed within the C-terminal fragment (Fig. 5A), but no ubiquitin modification was seen within the N-terminal half (Fig. 5Aii). To identify the site of ubiquitin modification of \( \alpha_4 \), trypsin digestion and peptide mapping mass spectrometry analyses were performed on the ubiquitinated full-length form (Fig. 5B) as well as unmodified \( \alpha_4 \) and ubiquitin for comparison (Fig. 5B). The spectra for the unre-
Our data from experiments using different MID1 domain combinations suggest that the MID1 RING domain can facilitate monoubiquitination and a low level of polyubiquitination of α4. This result was surprising as there is no previous evidence that the RING domain interacts with α4 (7, 10). Although the mechanism of this action is unclear, the increased monoubiquitination and weak polyubiquitination of α4 may be due to weak interaction between the RING domain and the C-terminal domain of α4 or that the RING-E2 complex may enhance the E2 enzyme functionality and transfer of ubiquitin to α4.

In contrast, the Bbox1 domain in the absence of the RING domain is unable to facilitate ubiquitination of α4 even though it interacts strongly with α4 and has a similar fold as RING E3 ligase domains (7, 10, 18, 21). The weak monoubiquitinated band observed of α4 in the presence of B1B2 (Fig. 2B, first lane) suggest that the Bbox domains alone are not able to target α4 to the E2 enzyme. It is unclear why the Bbox domains are unable to catalyze the ubiquitination of α4. In our previous studies, the Bbox domains were shown to possess E3 ligase activity, albeit considerably less than the classic RING domain. On the other hand, the Bbox domains from TRIM16, which is technically not a TRIM protein because it lacks a RING domain but nevertheless has 28% identity to MID1 (40), were shown to facilitate polyubiquitination. We postulate that the binding of the C terminus of α4 to the Bbox1 domain alters the interface on Bbox1 that would typically be involved in E2–E3 interactions. Indeed, our NMR studies revealed that α4_k2 binds on a surface of the Bbox1 domain that overlaps the surface that would be required for interaction with the E2 enzyme (21, 41, 42). Consistent with this, the presence of α4_k2 markedly reduced the autoubiquitination activity of B1B2 (19).

It is clear from the data that the MID1 Bbox1 domain functions to bind and recruit α4 and that this interaction is essential for its polyubiquitination. Specifically, we have shown that Leu-146 is critical for the MID1–α4 interaction, and its mutation to glutamine completely disrupted the polyubiquitination of α4. These data demonstrate that the ubiquitination of α4 is directly linked to its association with MID1. To understand how the L146Q mutation might affect the structure and function of MID1, we modeled a glutamine in place of Leu-146 and observed that the side chain carbonyl and amino group of Gln-146 extend past one of the methyl groups of the leucine. These groups are positioned in close proximity to form multiple hydrogen bonds with Asp-143 and Lys-132, both of which were previously shown to be involved in binding the C-terminal region of α4 (21).

We postulate that these hydrogen bonds affect the α4 binding surface on Bbox1 but not its overall tertiary structure (Fig. 6). Although these results do not rule out other possibilities for the role of Bbox1, they do indicate that physical association of α4 with the MID1 Bbox1 is essential for regulating polyubiquitination of α4.

Mechanistically the polyubiquitination of α4 requires both the RING and Bbox1 domains. In addition to binding and possibly orienting α4 for polyubiquitination, the Bbox1 domain may function to enhance the E3 ligase functionality of the RING domain (i.e. function like an E4 enzyme). This observation suggests that the RING domains of the large TRIM/Bbox1-Box2-Coiled-Coil (RBCC) family may all require the invariantly asso-

**FIGURE 4.** Leucine 146 of Bbox1 domain is essential for MID1–α4 interaction and α4 polyubiquitination. A, a Western blot assay shows the ubiquitination of α4 in the presence of wt RB1B2 (WT) and mutant RB1B2_L146Q in which Leu-146 was mutated to glutamine. The first lane represents a control assay in which MID1 was omitted. Antibody was specific for α4 C-terminal region. B, a Western blot shows the autoubiquitination of RB1B2_L146Q and wt RB1B2 (WT). Antibody was specific for the N-terminal domain of MID1.
Associated Bbox domains, located just C-terminal to the RING domain, to enhance their E3 ligase activity as well as determine substrate specificity. For instance, TRIM5α/H9251, which consists of the RING-Bbox2 domains, requires Bbox2 for anti-retroviral activity (43). Deletion or mutation that disrupts Bbox2 structure resulted in loss of TRIM5α antiviral activity even though the protein is expressed and localized properly (43). Similar functions have been observed with the RING domains of BARD1 and MdmX, which have been shown to enhance the E3 functionality of BRCA1 and MDM2, respectively (44–48). In all these cases, these E4 enzymes possess limited E3 ligase activities but are routinely observed to heterodimerize with a RING domain and function as ubiquitin chain elongation factors. As expected, E3 ligase functionality of the RING-Bbox domains is maintained completely within the TRIM motif (Fig. 2C) and does not require either the coiled coil domain of the TRIM motif or the C-terminal half of MID1.

Our results showing polyubiquitination of α4 are in contrast to a recent report by Watkins et al. (25), which showed only monoubiquitination of full-length α4 by MID1 when using UbcH5b (UBE2D2) and UbcH5c (UBE2D3) as the E2 enzymes (25). It was reported that monoubiquitination of α4 promotes its cleavage by calpain, and that this results in increased processing of PP2Ac (25). That said, close examination of the ubiquitination data of Watkins et al. (25) suggests the presence of diubiquitinated α4; higher molecular weight products were not shown with the Western blot. In addition, it was reported that a ubiquitin interacting motif within the N-terminal region of α44 was important for directing the level of ubiquitination of α4 and PP2A (38). However, in our data we observed that the proposed ubiquitin interacting motif had no effect on the level of ubiquitination of α4; in fact, ubiquitination occurred independently of the ubiquitin interacting motif, as observed with the polyubiquitination of the C terminus. Nonetheless, taken together these data indicate that the extent of ubiquitination of α4 regulates its function and level, perhaps via a combination of classic proteasome-mediated degradation and calpain-mediated turnover. Furthermore, we suggest that this is in part determined by the specific E2 enzymes available within the cell, thus providing an additional level of regulation of α4 and PP2A activity.

Importantly, the entire C-terminal MID1 binding domain (residues 236–339), whether as a domain by itself or as part of the full-length α4 protein, is necessary for polyubiquitination. The site of polyubiquitination (lysine-287) is outside the range of the residues used in our initial studies using α445 (residues 236–280) (19), which was only observed to be monoubiquitinated. This implies that the full C terminus helps to position this key lysine residue in close proximity to the active site of the activated E2 enzyme. The N-terminal region of α4 neither

FIGURE 5. Identification of α4 ubiquitination site. Ai, a Western blot assay shows the ubiquitination of the C-terminal domain (α4C, residues 236–339) of α4. Antibody was C-terminal-specific. ii, a Western blot assay shows the ubiquitination of the N-terminal domain (α4N, residues 1–235) of α4. Antibody was specific for the N-terminal region of α4. Ub, ubiquitin. B, a portion of the tandem mass spectra shows fragmentations of unmodified α4 (i), ubiquitinated α4 (ii), and ubiquitin (iii). The peptide (GALPDQGIAAPEEFKRK) was identified showing Lys-287 as the site of ubiquitination. Tandem mass spectrometry was performed in the linear ion trap, and y-ion and b-ion series were assigned by a database search assuming potential Gly-Gly residues covalently attached to any of the Lys residues on the protein.

FIGURE 6. Model of L146Q mutation of Bbox1. A ribbon representation of MID1 Bbox1 shows a proposed model of the effect of the L146Q mutation. Labeled are the residues on one surface of Bbox1 that are shown by NMR studies to be involved with binding the C terminus of α4 (21). The position of Leu-146 overlay with Gln-146 is shown in greater magnification to depict potential hydrogen bonds that can result between Gln-146 and Asp-143 and Lys-132.
interacts with MID1 nor is it a target for MID1-mediated ubiquitination.

To summarize, our data support a new role for MID1, namely the regulation of α4 via various levels of ubiquitination that could alter α4 function and/or target it for proteasome degradation. In either case the prospect that α4 plays such a key role in cellular transformation and is linked to different types of cancer suggests that MID1 may also play an important role in tumor suppression. Further studies are needed to resolve the differential contribution of proteasome- and calpain-dependent regulation of PP2A activity in determining specific cellular responses.

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