

The autism-linked UBE3A<sup>T485A</sup> mutant E3 ubiquitin ligase activates the Wnt/ $\beta$ -catenin pathway by inhibiting the proteasome

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Running title: *UBE3A activates Wnt signaling*

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## ABSTRACT

UBE3A is a HECT domain E3 ubiquitin ligase whose dysfunction is linked to autism, Angelman syndrome, and cancer. Recently, we characterized a de novo autism-linked UBE3A mutant (UBE3A<sup>T485A</sup>) that disrupts phosphorylation control of UBE3A activity. Through quantitative proteomics and reporter assays, we found that the UBE3A<sup>T485A</sup> protein ubiquitinates multiple proteasome subunits, reduces proteasome subunit abundance and activity, stabilizes nuclear  $\beta$ -catenin, and stimulates canonical Wnt signaling more effectively than wild-type UBE3A. We also found that UBE3A<sup>T485A</sup> activates Wnt signaling to a greater extent in cells with low levels of ongoing Wnt signaling, suggesting that cells with low basal Wnt activity are particularly vulnerable to UBE3A<sup>T485A</sup> mutation. Ligase-dead UBE3A did not stimulate Wnt pathway activation. Overexpression of several proteasome subunits reversed the effect of UBE3A<sup>T485A</sup> on Wnt signaling. We also observed that subunits that interact with UBE3A and affect Wnt signaling are located along one side of the 19S regulatory particle, indicating a previously unrecognized spatial organization to the proteasome. Altogether, our findings indicate that UBE3A regulates Wnt signaling in a cell context-dependent manner and that an autism-linked mutation exacerbates these signaling effects. Our study has broad implications for human disorders associated with UBE3A gain or loss-

of-function, and suggest that dysfunctional UBE3A might affect additional proteins and pathways that are sensitive to proteasome activity.

Loss of UBE3A causes a debilitating neurodevelopmental disorder called Angelman syndrome (1-3), while excess UBE3A, through gene duplication or gain-of-function mutation, increases risk for autism (4-7). Moreover, ectopic activation of UBE3A (also known as E6AP), via the human papillomavirus E6 oncoprotein, contributes to cervical cancers (8). UBE3A is an E3 ubiquitin ligase that targets several substrate proteins, including itself, for proteasomal degradation (9). While much has been learned from studying postnatal functions for UBE3A in mature neurons (10-13), precisely how ubiquitination of these proteins, or other yet to be identified substrates, affects brain development is largely unclear. Here, we sought to gain new insights into developmental functions for UBE3A by exploring a novel connection between UBE3A, the proteasome, and the Wnt signaling pathway.

Wnt signaling is a principal determinant of cell fate and proliferation during development. Abnormal Wnt signaling is implicated in autism pathogenesis (14-17), and Wnt signaling is dysregulated in other diseases, including cancer (18,19). Induction of the canonical Wnt pathway centers on the dynamics of  $\beta$ -catenin degradation by the protea-

some. In the absence of Wnt ligand, a complex of cytosolic proteins, referred to as the destruction complex, sequentially phosphorylates and ubiquitinates  $\beta$ -catenin, resulting in its proteasomal degradation (Fig. 1) (20,21). Conversely, the presence of Wnt induces the formation of a Frizzled receptor and low-density lipoprotein receptor-related protein (LRP) co-receptor complex (22). This stabilizes  $\beta$ -catenin and allows its translocation to the nucleus, where  $\beta$ -catenin functions as a transcriptional co-activator with members of the TCF/LEF family of transcription factors (Fig. 1).

It is well-known that UBE3A reversibly associates with the proteasome and impacts its processivity (23-27). Moreover, proteasome subunits and proteasome-associated proteins physically interact with and are ubiquitinated by UBE3A (28-34). The relevance of these UBE3A substrates to nervous system development is largely unclear, with the notable exception of UCHL5 whose deletion leads to disorganized brain development (35), and PSMD4 (S5A/RPN10) which promotes dendrite development (36). It is also well known that proteasome inhibitors, such as MG-132, block proteasomal processing of  $\beta$ -catenin and potentially activate Wnt signaling (37,38). As part of a genome-wide small interfering RNA screen, we found that Wnt pathway activation is highly sensitive to knockdown of specific proteasomal subunits (39) (and see new analyses of these data below). Moreover, others found that wild-type (WT) UBE3A can stimulate Wnt pathway activation and protect  $\beta$ -catenin from proteasomal degradation, with these activities dependent on the ubiquitin ligase activity of UBE3A (40-42).

Recently, we characterized a *de novo* autism-linked UBE3A<sup>T485A</sup> mutation that disrupts phosphorylation control of UBE3A and enhances UBE3A ubiquitin ligase activity (6). This UBE3A T485A mutation, along with an engineered UBE3A T485E mutation that inhibits UBE3A by mimicking phosphorylation, gave us new molecular tools to probe the link between UBE3A activity and Wnt signaling. Through extensive proteomic and functional experiments, we show that UBE3A and Wnt signaling converge at the proteasome—with UBE3A impacting overall protein homeostasis, including  $\beta$ -catenin turnover, by ubiquitinating multiple protea-

some subunits. Intriguingly, subunits that interact with UBE3A and affect Wnt signaling are located along one side of the 19S regulatory particle, suggesting functional organization of the proteasome. The UBE3A T485A mutant activated Wnt signaling more effectively than WT UBE3A, and ligase-dead UBE3A failed to activate Wnt signaling, raising the novel possibility that abnormal Wnt signaling contributes to neurodevelopmental disorders involving UBE3A loss- or gain-of-function.

## RESULTS

*UBE3A<sup>T485A</sup> enhances Wnt signaling in a cell-context dependent manner.* WT UBE3A, but not ligase-dead (LD) UBE3A, was previously found to stimulate Wnt reporter gene expression in HEK293T cells and to do so independent of Wnt ligand (40,42). Given that a significant number of autism-linked *de novo* mutations are found in genes associated with the Wnt pathway (14,17), we sought to determine if the autism-linked UBE3A<sup>T485A</sup> mutation, which disables phosphorylation control and hyperactivates ubiquitin ligase activity (6), had an equal or greater effect on Wnt pathway activation. To test this possibility, we transfected HEK293T cells with the  $\beta$ -catenin-activated luciferase reporter (BAR)(43), along with UBE3A expression constructs: WT UBE3A, UBE3A-LD, UBE3A<sup>T485A</sup>, or UBE3A<sup>T485E</sup> (phosphomimetic mutant; reduces UBE3A activity to near UBE3A-LD levels). We previously characterized the protein level and ubiquitin ligase activity of each construct in HEK293T cells (6). Cells were then acutely (12-16 h) treated with control (L-cell) or Wnt3a-conditioned medium (CM) prior to quantifying luciferase activity. We found that WT UBE3A and the UBE3A<sup>T485A</sup> mutant strongly stimulated Wnt pathway activation in the absence (Fig. 2A) or in the presence of Wnt ligand (Fig. 2B). The autism-linked UBE3A<sup>T485A</sup> construct activated Wnt signaling to a greater extent than WT UBE3A, particularly as the amount of plasmid transfected was increased (Fig. 2C). The phosphomimetic UBE3A<sup>T485E</sup> mutant, which impairs UBE3A ubiquitin ligase activity, was much less effective at stimulating Wnt pathway activation. Consistent with a previous study (40), UBE3A-LD did not activate the pathway (Fig. 2A and 2B), indicating that UBE3A ubiquitin ligase activity is re-

quired to stimulate Wnt pathway activation. Lastly, we found that UBE3A<sup>T485A</sup> stimulated Wnt pathway activation more than 11-fold (relative to T485E) in cells with low baseline Wnt pathway activation (L-cell control media), and was relatively less effective in cells treated acutely (12-16 h; 2.6-fold relative to T485E) or chronically (one passage prior to transfection through to lysis; 1.8-fold relative to T485E) with Wnt ligand (Fig. 2D). These data suggest UBE3A stimulates Wnt signaling in a cell-context dependent manner, and is relatively more effective in cells with low levels of ongoing Wnt signaling, consistent with findings by Sherman's group (42).

*UBE3A and the Wnt pathway converge at the proteasome.* Epistasis experiments suggested that UBE3A stimulated the Wnt pathway independent of GSK3 $\beta$  or APC (41). We performed additional epistasis experiments, probing different parts of pathway (Fig. 1) with the O-acyltransferase inhibitor C59 (inhibits Wnt palmitoylation and secretion), tankyrase inhibitor XAV939 (stabilizes the destruction complex), GSK3 $\beta$  inhibitors CT99021 and LiCl, the  $\beta$ -catenin transcriptional activator valproic acid (VPA), and the  $\beta$ -catenin transcriptional inhibitor iCRT14 (Fig. S1). These studies suggested that UBE3A acts at or downstream of the destruction complex and influences the transcriptional activity of  $\beta$ -catenin, consistent with work by Kuslansky and colleagues (42).

We next sought to identify a molecular link between UBE3A and the Wnt/ $\beta$ -catenin signaling pathway. Given that UBE3A is an E3 ubiquitin ligase that targets proteins for destruction, we hypothesized that any protein that 1) interacts with UBE3A, 2) is ubiquitinated by UBE3A, and 3) activates the Wnt pathway when knocked down could represent bona fide UBE3A substrates and serve as core components of this signaling pathway. Published data sets addressed two of these criteria. Martinez-Noel et al. identified a comprehensive list of proteins that selectively interact with UBE3A in HEK293T cells (relative to NEDD4, another HECT domain E3 ubiquitin ligase) (24). Of these proteins, 63 interact with all three UBE3A isoforms (WT and LD; Figure 3A, Table S1). We recently identified a comprehensive list of positive and negative regulators of WNT signaling in HEK293T cells from a near-saturation

genome-wide siRNA screen (39). Of these regulators, 1,958 proteins significantly increased Wnt pathway activation when knocked down (Figure 3A and Table S2). DAVID analysis predicted that "Proteasome" was the top gene ontology term for both protein lists ( $P = 3 \times 10^{-54}$  and  $8 \times 10^{-8}$ , respectively). Remarkably, only 24 proteins were shared between these two lists, and each of these proteins was a component of the 19S regulatory particle, the 20S core complex, or a proteasome accessory protein (Fig. 3B). Intriguingly, when mapped to the structure of the proteasome (44), the shared 19S regulatory subunits were located on one side of the complex (Fig. 3C red). Subunits on the opposite side largely did not alter Wnt pathway signaling when knocked down (Fig. 3C, blue). These data suggest proteasome subunits are functionally organized, with one side of the 19S regulatory particle associated with Wnt/ $\beta$ -catenin signaling.

UBE3A physically associates with the proteasome (24-26), and this association can be reversibly altered by certain stimuli (23). To evaluate whether T485 phosphorylation affects the association of UBE3A with the proteasome, we isolated UBE3A protein complexes from HEK293T cells overexpressing LD versions of UBE3A<sup>T485A</sup>, which cannot be phosphorylated, and UBE3A<sup>T485E</sup>, a mutant that mimics phosphorylation. Using mass spectrometry and spectral counts to quantify interactions, we found that the UBE3A<sup>T485A</sup> and UBE3A<sup>T485E</sup> mutants interact with the same proteasome subunits, with a slight bias for UBE3A<sup>T485A</sup>:proteasome subunit interactions (Table S3 and Figure S2A). To further evaluate whether phosphorylation at T485 affects UBE3A binding to the proteasome, we biochemically purified proteasomes from HEK293T cells expressing LD versions of UBE3A<sup>T485A</sup> and UBE3A<sup>T485E</sup> mutants. UBE3A abundance was measured in proteasome-bound and unbound fractions by western blot analysis. We found no difference between UBE3A<sup>T485A</sup> and UBE3A<sup>T485E</sup> mutants, suggesting that phosphorylation does not strongly influence UBE3A-proteasome interaction (Fig. S2B and S2C).

*Multiple proteasome subunits are ubiquitinated by UBE3A.* We next sought to comprehensively identify proteins that are ubiquitinated by UBE3A in

HEK293T cells. We combined quantitative SILAC (stable isotope labeling of amino acids in cell culture) with ubiquitin remnant immunoaffinity profiling (45,46). This approach allowed us to isolate ubiquitinated peptides containing the characteristic di-glycine motif exposed after tryptic digestion, and to perform ratiometric analysis in cells expressing active UBE3A<sup>T485A</sup> relative to inactive UBE3A<sup>T485E</sup> (Table S4). We found that UBE3A ubiquitinated multiple components of the proteasome, including eight core proteasome subunits that physically interact with UBE3A and that negatively regulate WNT signaling (Fig. 3B, shown in bold).

Of these eight subunits, we examined endogenous protein abundance of five (PSMA2, PSMB1, PSMD2, PSMD4, PSMD11) by western blot analysis in cells transfected with UBE3A<sup>T485A</sup> or UBE3A<sup>T485E</sup> expression constructs. Protein levels of most of these proteasome subunits were reduced in UBE3A<sup>T485A</sup>-expressing cells relative to UBE3A<sup>T485E</sup> controls, in the absence or presence of Wnt ligand (Fig. 4A – 4C). We also examined endogenous levels of these five subunits in immortalized human lymphocytes derived from the UBE3A<sup>T485A</sup> autism proband and the unaffected (WT UBE3A) parents (47). We previously found that UBE3A ubiquitin ligase activity is elevated in cells from this proband using PSMD4 (also known as S5A or RPN10), RAD23A (HHR23A) and UBE3A as endogenous substrates (6). Several proteasome subunits were reduced in cells from the proband relative to the unaffected parents (Fig. 4D and 4E). Note that these lymphocytes showed no evidence of Wnt pathway activation, based on quantitative PCR experiments with Wnt target genes AXIN2, BMP4, NKD1, and SOX17 (Fig. S3A – D), likely because these non-adherent cells have undetectable levels of  $\beta$ -catenin protein (Fig. S3E). Thus, we could not evaluate the extent to which endogenous Wnt signaling was affected in this patient-derived cell line.

*PSMD2 is a substrate of UBE3A.* Our experiments suggested that PSMD2 (also known as RPN1) was a novel UBE3A substrate. Unlike other 19S components, PSMD2 is located at the edge of the regulatory complex (Fig. 5A, green) and anchors several accessory proteins to the proteasome (48). These acces-

sory proteins include multiple UBE3A substrates: RAD23A and RAD23B (28,49), ADRM1, PSMC3, UCHL5 (25), PSMD4 (6), MCM7 (50), as well as proteins that associate with UBE3A, including the E2 enzyme UBE2L3 (51). In titration experiments with increasing amounts of PSMD2 plasmid, we found that PSMD2 reduced UBE3A<sup>T485A</sup>-mediated Wnt pathway activation, in the absence or presence of Wnt ligand (Fig. 5B and 5C). These findings suggest that overexpression of PSMD2 can functionally rescue or compensate for the loss of endogenous PSMD2 in UBE3A<sup>T485A</sup>-expressing cells (Fig. 4A-C). Moreover, high molecular weight ubiquitin-conjugated forms of PSMD2 were detected in HEK293T cells expressing UBE3A<sup>T485A</sup>, PSMD2, and HA-tagged ubiquitin, after treatment with the proteasome inhibitor MG-132 (30  $\mu$ M, 4 h) (Fig. 5D). In contrast, polyubiquitinated PSMD2 was not detected in cells expressing the inactive UBE3A<sup>T485E</sup> mutant (Fig. 5D). We also performed in vitro ubiquitination reactions with recombinant ubiquitin, E1, E2, and UBE3A enzymes along with PSMD2 purified from HEK293T cells. A single distinct ubiquitin-conjugated PSMD2 species was detected in reactions containing UBE3A (Fig. 5E), which differed from what was observed in cells (Fig. 5D), suggesting additional cellular factors promote polyubiquitin chain formation on PSMD2. Collectively, these data indicate that PSMD2 is a substrate of UBE3A and that overexpression of PSMD2 dampens Wnt pathway activation in UBE3A<sup>T485A</sup>-expressing cells.

*UBE3A regulates Wnt signaling through multiple proteasome subunits.* We next evaluated the extent to which additional proteasomal subunits rescue Wnt pathway activation in UBE3A<sup>T485A</sup>-expressing cells. Plasmids encoding subunits that interact with UBE3A were transfected into HEK293T cells together with UBE3A<sup>T485A</sup> at 1:1 and 1:2 UBE3A:subunit ratios, or alone to confirm overexpression (Fig. S4). We were particularly interested in PSMD4, as PSMD4 is a well-characterized UBE3A substrate (6,25,26,29). However, PSMD4 failed to reduce Wnt pathway activation in UBE3A<sup>T485A</sup>-expressing cells at the 1:1 or 1:2 transfection ratio (Fig. 6; ranked results shown in Table S5). Instead, PSMD4 augmented

Wnt signaling by nearly 200% at the 1:2 transfection ratio. Of the remaining subunits tested, PSMB1, PSMC2, PSMD2, and PSMD7 consistently reduced Wnt pathway activation in UBE3A<sup>T485A</sup>-expressing cells, whereas PSMC6 augmented the Wnt response (at 1:1 and 1:2 ratios; Fig. 6, Table S5). PSMD2 was one of the most effective subunits to rescue UBE3A-dependent Wnt activation in HEK293T cells (Table S6). PSMD1, PSMD3, PSMD6, and PSMD11 had no effect or inconsistent effects across the four conditions. Our experiments collectively suggest that UBE3A stimulates Wnt pathway activation by interacting with, ubiquitinating, and reducing the levels of multiple (PSMB1, PSMC2, PSMD2, and PSMD7) proteasome subunits.

*UBE3A inhibits proteasome function and stabilizes  $\beta$ -catenin* Several groups found that WT UBE3A inhibits the proteasome in cells (25,34). To determine if UBE3A<sup>T485A</sup> similarly affected proteasome activity, we transfected HEK293T cells with UBE3A<sup>T485A</sup> or UBE3A<sup>T485E</sup> mutants along with a destabilized G76V-Ubiquitin(Ub)-GFP fusion reporter protein and mCherry as the normalization control. This G76V-Ub-GFP construct was previously used to quantify proteasome-dependent activity in cells (52). We found that the levels of this G76V-Ub-GFP reporter were higher in cells transfected with UBE3A<sup>T485A</sup> relative to the inactive UBE3A<sup>T485E</sup> mutant, suggesting that elevated UBE3A<sup>T485A</sup> activity inhibits proteasome function in cells (Fig. 7A and 7B). This difference in G76V-Ub-GFP reporter levels was abolished in cells treated with MG-132 (10  $\mu$ M 12 h; Fig. 7A and 7B), further suggesting a proteasome-dependent mechanism.

$\beta$ -catenin is ubiquitinated and then degraded by the proteasome, which limits entry to the nucleus and transcriptional activity (Fig. 1). Given that UBE3A<sup>T485A</sup> reduces proteasome activity, we hypothesized that nuclear  $\beta$ -catenin levels might be elevated in UBE3A<sup>T485A</sup>-expressing cells. Indeed, we found that nuclear  $\beta$ -catenin levels were slightly elevated in cells expressing the UBE3A<sup>T485A</sup> mutant relative to cells expressing UBE3A<sup>T485E</sup>, in the absence or presence of Wnt ligand (Fig. 7C and 7D). Additionally, UBE3A<sup>T485A</sup> less effectively stimulated Wnt signaling (relative to UBE3A<sup>T485E</sup>) in cells overexpressing WT  $\beta$ -catenin and did not

augment Wnt signaling (relative to UBE3A<sup>T485E</sup>) in cells overexpressing a constitutively active  $\beta$ -catenin construct (Fig. 7E). These data further demonstrate that UBE3A<sup>T485A</sup> most effectively stimulates Wnt signaling in cells with sub-saturating levels of active  $\beta$ -catenin, that is, when baseline Wnt pathway activation is low. Altogether, our results suggest a mechanism whereby UBE3A ubiquitinates multiple subunits of the proteasome, leading to impairment of proteasome-mediated degradation of  $\beta$ -catenin and increased  $\beta$ -catenin/Wnt signaling (Fig. 7F).

## DISCUSSION

Despite the well-known association between excess UBE3A and autism risk (4,5,53), including autism-linked mutations that elevate UBE3A alone (6,7), and extensive research on UBE3A (10), it is currently unclear how excess UBE3A affects mechanisms linked to autism and neurodevelopment. UBE3A is thought to be a promiscuous enzyme (54), and this seeming promiscuity has confounded attempts to identify the physiological substrates of UBE3A. Recently, we found that an autism-linked point mutation in UBE3A (UBE3A<sup>T485A</sup>) disables a phosphorylation control switch and elevates UBE3A ubiquitin ligase activity (6). Here, we found that UBE3A<sup>T485A</sup> exacerbates Wnt signaling by inhibiting the proteasome and stabilizing nuclear  $\beta$ -catenin. Numerous autism-linked genes are implicated in Wnt signaling (14,15,17), valproic acid—an environmental risk for autism—activates Wnt signaling (Fig. S1E)(55,56), and Wnt signaling regulates proliferation and differentiation of neuronal progenitors (57,58). Our study, and the work of others (40–42), firmly places UBE3A within the Wnt signaling pathway, and provides fundamental new insights into how excess UBE3A could impair brain development and increase risk for autism.

Our biochemical and proteomics findings suggest that UBE3A stimulates Wnt signaling by ubiquitinating multiple proteasome subunits and reducing proteasome activity, which ultimately stabilizes  $\beta$ -catenin. UBE3A<sup>T485A</sup>-mediated stimulation of WNT signaling was reduced in cells overexpressing different proteasome subunits, suggesting that proteasome subunits are functionally relevant UBE3A substrates. Multiple labs found that

UBE3A can ubiquitinate proteasome subunits and inhibit proteasome function (25,34), although one group found that UBE3A stimulated proteasome proteolytic activity (27). Discrepancies could relate to differences in cell context or experimental approach. Jacobson and colleagues found that UBE3A inhibited the proteasome by in situ ubiquitination provided the levels of polyubiquitinated proteins in the cell were low (25). Cellular stresses that increase polyubiquitinated protein levels (oxidative stress, serum starvation) blocked UBE3A from ubiquitinating the proteasome, leading to increased proteasome activity (25). In a simplistic sense, the proteasome can “sense” whether global polyubiquitinated protein levels are low or high and, via UBE3A, adjust proteasomal activity accordingly. Thus, differences in cell culture conditions (such as differences in serum composition, oxygen tension, plating density) have the potential to influence polyubiquitinated protein levels and alter proteasome activity via UBE3A.

We found that knockdown of different proteasome subunits, or overexpression of UBE3A<sup>T485A</sup>, led to activation of Wnt signaling in HEK293T cells, a cell line with low baseline Wnt pathway activation. In contrast, UBE3A<sup>T485A</sup> was relatively less effective at stimulating Wnt signaling in HEK293T cells exposed (acutely or chronically) to Wnt ligand, or in HEK293T cells expressing elevated levels of  $\beta$ -catenin. It is well-known that cell context can affect Wnt signaling (59,60). Our study suggests that the proteasome, which lies at the heart of the Wnt signaling pathway (Fig. 1), and UBE3A, which regulates proteasome function and subunit composition, can influence active  $\beta$ -catenin levels in a cell-context dependent manner.

This cell-context dependent effect of UBE3A has implications for brain development. Wnt signaling weakens over the course of development in cortical neuron progenitors, which express UBE3A biallelically (61), and varies by cell and tissue type (62,63). It is thus conceivable that cells with latent but low levels of Wnt pathway activation may be more vulnerable to UBE3A excess. Future experiments will be needed to test this possibility.

To our knowledge, we are the first to identify a proteasomal subdomain that associates with the Wnt signaling pathway. Proteasome subunits

that enhance Wnt signaling upon siRNA-mediated knockdown localize to one side of the 19S regulatory complex. While this observation is based on data from a primary siRNA screen and will need to be independently verified, we speculate that such an arrangement could spatially restrict docking or shuttling of WNT pathway components to the proteasome. The destruction complex scaffolding protein AXIN1 can directly bind to  $\beta$ -catenin and PSMD1, while  $\beta$ -catenin stabilization is dependent on its sequestration away from the proteasome (64,65). Post-translational modifications of proteasome subunits, such as ubiquitination, could block the association of  $\beta$ -catenin with the proteasome, which would stabilize  $\beta$ -catenin and promote Wnt signaling. Thus, cells might physically segregate Wnt signaling from other ubiquitin-dependent signaling pathways, to permit independent control of cellular process that depend on proteasomal proteolysis.

Numerous pathways require protein degradation, but it has long remained unknown how the proteasome simultaneously processes signaling components to maintain homeostasis. Our study raises the possibility that distinct pathways are compartmentalized at the proteasome, and this provides the necessary molecular platforms to execute multiple parallel functions.

Lastly, our study has broader implications and suggests that UBE3A could affect additional proteins, signaling pathways, and cellular functions that are particularly sensitive to ubiquitination and proteasomal degradation. Our findings hint that the apparent promiscuity of UBE3A may relate to its inhibitory effect on the proteasome, as proteasome inhibition would elevate the levels of many ubiquitinated proteins, including those that are not direct UBE3A substrates.

## EXPERIMENTAL PROCEDURES

**Molecular Biology.** All UBE3A constructs used in this study were derived from human UBE3A isoform II (accession: NP000453.2). Myc epitope tags were placed on the N-terminus of UBE3A by polymerase chain reaction and cloned into pCIG2 using SacI and XmaI or into pEGFP-N1 using BamHI and NotI. All constructs were verified by sequencing. HA-tagged

ubiquitin, pGL3-BAR, TK-*Renilla*, pHAGE  $\beta$ -catenin WT, and pHAGE  $\beta$ -catenin mutant constructs were previously described (39,66,67). G76V-Ubiquitin-GFP was obtained from Addgene (23969). pCMV6 Myc-DDK-tagged constructs for PSMB1 (RC201798), PSMC2 (RC200945), PSMC5 (RC201251), PSMC6 (RC202809), PSMD1 (RC210486), PSMD2 (RC203204), PSMD3 (RC202307), PSMD6 (RC202292), PSMD7 (RC203133), PSMD11 (RC201201), and untagged PSMD4 (SC111678) were all purchased from Ori-gene.

**Antibodies and reagents.** Primary antibodies used were mouse anti-Myc (1:1000, EMD Millipore, 05-724), mouse anti-HA (1:1000, EMD Millipore, 05-904), rabbit anti-GAPDH (1:1000, Genetex, GTX100118), mouse anti-FLAG M2 (1:1000, Sigma, F3165), rabbit anti-PSMA2 (1:1000, Thermo Fisher, PA5-17294), rabbit anti-PSMB1 (1:1000, Thermo Fisher, PA5-49648), rabbit anti-PSMD2 (1:1000, Thermo Fisher, PA5-27663), goat anti-PSMD4 (1:1000, Boston Biochem, AF5540), rabbit anti-PSMD11 (1:1000, Thermo Fisher, PA5-27447); mouse anti-GFP (1:5000, Clontech, 632281), rabbit anti-mCherry (1:2500, Abcam, ab167453), rabbit anti- $\beta$ -catenin (1:1000, Cell Signaling, 9562), Mouse anti-HDAC1 (1:1000, Cell Signaling, 5356). LiCl, XAV939, iCRT14, and valproic acid were purchased from Sigma, CT99021 was purchased from Axon Med Chem, and C-59 was purchased from Cellagen Technology. MG-132 was purchased from Calbiochem. Wnt3a media was made according to the ATCC protocol (68).

**Tissue Culture.** HEK293T cells (ATCC) were maintained in a 5% CO<sub>2</sub> humidified incubator in DMEM (Thermo Fisher) containing 4.5g/L glucose, 584 mg/L glutamine, 110 mg/L sodium pyruvate, and supplemented with 10% (v/v) fetal bovine serum (Hyclone), and 1x Antibiotic-Antimycotic (penicillin, streptomycin, amphotericin b; Life Technologies). For biochemical analyses, transfections were performed in 6-well dishes using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. HEK293T cells were lysed in NuPAGE Lithium dodecyl sulfate (LDS) sample buffer (Life Technologies) supplemented with 1%  $\beta$ -mercaptoethanol and 1x

Complete Mini protease inhibitor cocktail (Roche). Lysates were boiled and proteins resolved by 4% to 20% SDS/PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in Odyssey blocking buffer (Li-Cor), and probed with the appropriate primary antibodies overnight. Protein bands were visualized using the Odyssey CLx Infrared Imaging System (LI-COR) with the following secondary antibodies (donkey anti-rabbit 800CW, 926-32213; donkey anti-rabbit 680RD, 925-68073; donkey anti-mouse 680RD, 926-68072, all from LI-COR, used at 1:10,000). Nuclear fractionation was performed using the NE-PER kit (Thermo) according to the manufacturer's instructions. Immortalized human lymphocytes (Simons Simplex Collection) were maintained in a 5% CO<sub>2</sub> humidified incubator in RPMI media supplemented with 15% (v/v) fetal bovine serum (Hyclone), 1x Glutamax (Gibco), and 1x Antibiotic-Antimycotic. The following cell lines were used for our study: 13873.Fa, 13873.Mo, and 13873.P1. For protein analysis, 10 million lymphocytes were collected by centrifugation and lysed for 20 minutes on ice with periodic rocking in a lysis buffer consisting of 20 mM HEPES, pH 7.4, 1% NP-40, 50 mM KCl supplemented with protease and phosphatase inhibitors. Samples were cleared at 1000 x g for 5 minutes and the supernatant collected and subjected to a protein assay (Bio-Rad) to determine concentration. Samples were prepared by diluting in sample buffer supplemented with 1%  $\beta$ -mercaptoethanol, boiled, and resolved by SDS-PAGE.

**Luciferase Assays.** BAR assays were performed in 96 well plates for all experiments except those shown in Fig. S1B-F (see below). HEK293T cells were plated at a density of 10,000/well. Cells were transiently transfected with 10 ng pRL-TK-*Renilla*, 30 ng BAR-pGL3, and 60 ng or 80 ng of indicated constructs using the TransIT®-2020 Transfection Reagent (Mirus) or TransIT®-LT1 Transfection Reagent (Mirus). After 2 h, media containing transfection reagents was replaced with fresh media. The following day, cells were serum deprived (0.2% FBS) for 6 h and stimulated with Wnt3a conditioned media or control L-cell media for 12-16 h. Reporter gene expression was assessed using the Dual-Luciferase Reporter Assay System (Promega) and measured on the Enspire plate reader from Perkin

Elmer (Waltham, MA). For Figs. S1B-F, HEK293T cells were plated at a density of 5,000/well in 384 well plates, and drugs were added four hours post-plating. 24 h after plating, cells were transfected with 5 ng pRL-TK-*Renilla*, 15 ng BAR-pGL3, and 30 ng of the indicated UBE3A expression constructs using FuGene HD (Promega). Reporter gene expression was assessed 24 h post transfection using the Dual-Glo Luciferase Assay System (Promega). For all experiments, the luciferase activity was normalized against *Renilla* activity.

**Ubiquitin remnant immunoaffinity profiling.** HEK293T cells were grown in high glucose DMEM media (Caisson Labs) supplemented with 10% dialyzed FBS (Gibco), 3.7 g/L sodium bicarbonate, 1x Glutamax, 1x Antibiotic-Antimycotic. For heavy media, 50  $\mu$ g/ml of L-lysine (Lys8) and 40  $\mu$ g/ml of L-arginine (Arg10) were added (Cambridge Isotope Lab). 50  $\mu$ g/ml of standard L-lysine and 40  $\mu$ g/ml of standard L-arginine (Thermo) were added for light media. For complete labeling, cells were passaged for at least seven generations in the above media. Cells were lysed in Lysis buffer (8 M urea in 50 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM EDTA, 2  $\mu$ g/ml apoprotinin, 10  $\mu$ g/ml leupeptin, 1 mM PMSF, 50  $\mu$ M PR-619 and 1 mM iodoacetamide) and sonicated to clarify lysate. The extract was centrifuged at 20,000 rpm at 4°C for 15 min and protein was estimated using Bradford method. Both heavy and light protein samples were mixed in 1:1 ratio (mg:mg) and diluted in 50 mM Tris-HCl, pH 7.5 to reduce the urea concentration to 2 M. The total protein was digested overnight with sequencing grade trypsin (Promega). The trypsin to protein ratio was maintained at 1:100. Total peptides were purified on Sep-Pack column (Waters) and eluted with 40 mL step gradient of 10-50% acetonitrile (MeCN) and 0.1% trifluoroacetic acid gradient (10%, 15%, 20%, 25%, 30%, 35%, 40% and 50%) 5 mL each. The eluents fractions were lyophilized overnight till dry powdery flakes were obtained.

Di-Glycine antibody kit was purchased from Cell Signaling Technologies (PTMScan Ubiquitin Remnant Motif (K- $\epsilon$ -GG), Kit 5562). Dried total cell peptide samples were solubilized in 1X IAP buffer and peptide IPs performed at 4°C for 4-6 h. The bead slurry was washed three times with PBS and two times with water. K- $\epsilon$ -GG peptides were

eluted with two washes of 50  $\mu$ L of 0.15% trifluoroacetic acid (TFA). Eluted peptides were further purified on Pierce C-18 spin columns (Cat. 89870) using the manufacturer's protocol. Peptides were eluted using 70% MeCN 0.1% TFA solution in 50  $\mu$ L volumes twice and dried in a speed vac at room temperature.

**Mass Spectrometry.** Mass spectrometry analysis was performed at the Michael Hooker Proteomics Center at the University of North Carolina. Dried peptide samples were reconstituted in 1% ACN, 0.1% formic acid. Each sample was analyzed by LC/MS/MS using an Easy nLC 1000 coupled to a QExactive HF mass spectrometer (Thermo Scientific). Samples were injected onto an Easy Spray PepMap C18 column (75  $\mu$ m id, x 25 cm, 2  $\mu$ m particle size) (Thermo Scientific) and separated over a 2 hr method. The gradient for separation consisted of 5–35% mobile phase B at a 250 nl/min flow rate, where mobile phase A was 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The QExactive HF was operated in data-dependent mode where the 15 most intense precursors were selected for subsequent fragmentation. Resolution for the precursor scan ( $m/z$  400–1600) was set to 120,000 with a target value of  $3 \times 10^6$  ions. MS/MS scans resolution was set to 30,000 with a target value of  $1 \times 10^5$  ions. The normalized collision energy was set to 27% for higher-energy collisional dissociation (HCD), and the isolation window was set to 1.6  $m/z$ . Peptide match was set to preferred, and precursors with unknown charge or a charge state of 1 and  $> 7$  were excluded.

Raw data files were processed using Proteome Discoverer version 2.1 (Thermo Scientific). Peak lists were searched against a reviewed human UniProt database (containing 20,203 entries), appended with a contaminants database, using Sequest. The following parameters were used to identify tryptic peptides for protein identification: 10 ppm precursor ion mass tolerance; 0.02 Da product ion mass tolerance; up to two missed trypsin cleavage sites; carbamidomethylation of C was set as a fixed modification; oxidation of M, glygly of K, phospho of S,T,Y, 13C6-R, -K and acetyl of N-terminus were set as variable modifications. Percolator was used to estimate the number of false positive identifications, and a q-value (false discovery rate, FDR) was

assigned; a q-value of <0.05 (5% FDR) which was used to filter all results. The ptmRS node was used to assign the PTM site localization probability. Peptide quantitation was enabled to determine SILAC ratios for ubiquitin-modified peptides.

**Ubiquitination assays.** In vitro ubiquitination assays were performed using immunopurified PSMD2 in combination with a commercially available recombinant UBE3A ubiquitin ligase kit (Boston Biochem K-230) according to the manufacturer's instructions. In brief, HEK293T cells grown in 25 cm dishes were transfected with a plasmid encoding Myc-DDK-PSMD2 and allowed to grow for 48 h. Cells were lysed on ice for 20 minutes with gentle agitation using an immunoprecipitation buffer containing 20 mM HEPES, pH 7.4, 1% NP-40, 50 mM KCl, and 100 mM NaCl. Lysates were cleared by centrifugation, and PSMD2 was immunoprecipitated using an anti-myc affinity gel (Sigma A7470) at 4°C for 2 h. The resulting beads were washed two times each (eight washes total) in IP buffer containing 100 mM, 250 mM, 500 mM NaCl, as well as two final washes in PBS. PSMD2 was eluted from

the beads in PBS containing 10 µg/ml myc peptide (Sigma). For cell-based assays, HEK293T cells were transfected with constructs encoding UBE3A, Myc-DDK-PSMD2, and HA-ubiquitin. Cells were allowed to grow for 48 h and treated with 30 µM MG-132 for 4 h. The cells were then lysed in RIPA buffer containing 1% SDS and 30 µM MG-132. Cell lysates were boiled for 20 minutes and clarified by centrifugation at 15,000 x g for 10 minutes. The resulting supernatant was diluted 1:10 (v/v) in an immunoprecipitation buffer (20 mM HEPES pH 7.4, 50 mM KCl, 1% Triton X-100) and PSMD2 immunoprecipitated using the EZ View anti-FLAG affinity gel (Sigma, F2426) at 4°C for 1 h. The final complex was washed three times with immunoprecipitation buffer containing 125 mM NaCl, resuspended in sample buffer, and processed for SDS-PAGE and immunoblot analysis.

**Statistical Analysis.** Statistical analyses were performed using GraphPad Prism software. Statistical treatments for each experiment are indicated in the figure legends. P<0.05 was considered significant.

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**Author contributions:** JJY conceived, designed and performed experiments and wrote the manuscript; SRP performed luciferase experiments; MPW and JMW performed epistasis experiments; RC performed ubiquitin remnant immunoaffinity profiling; GF performed quantitative PCR; MJE, MBM, MJZ conceived and designed experiments and wrote the manuscript.

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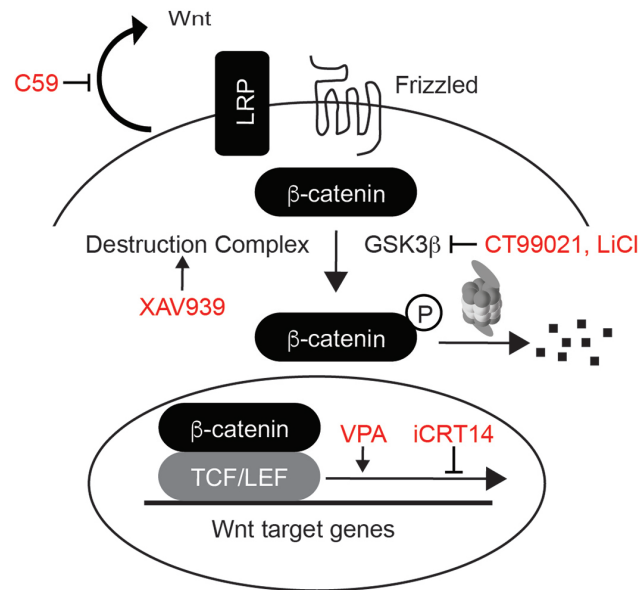
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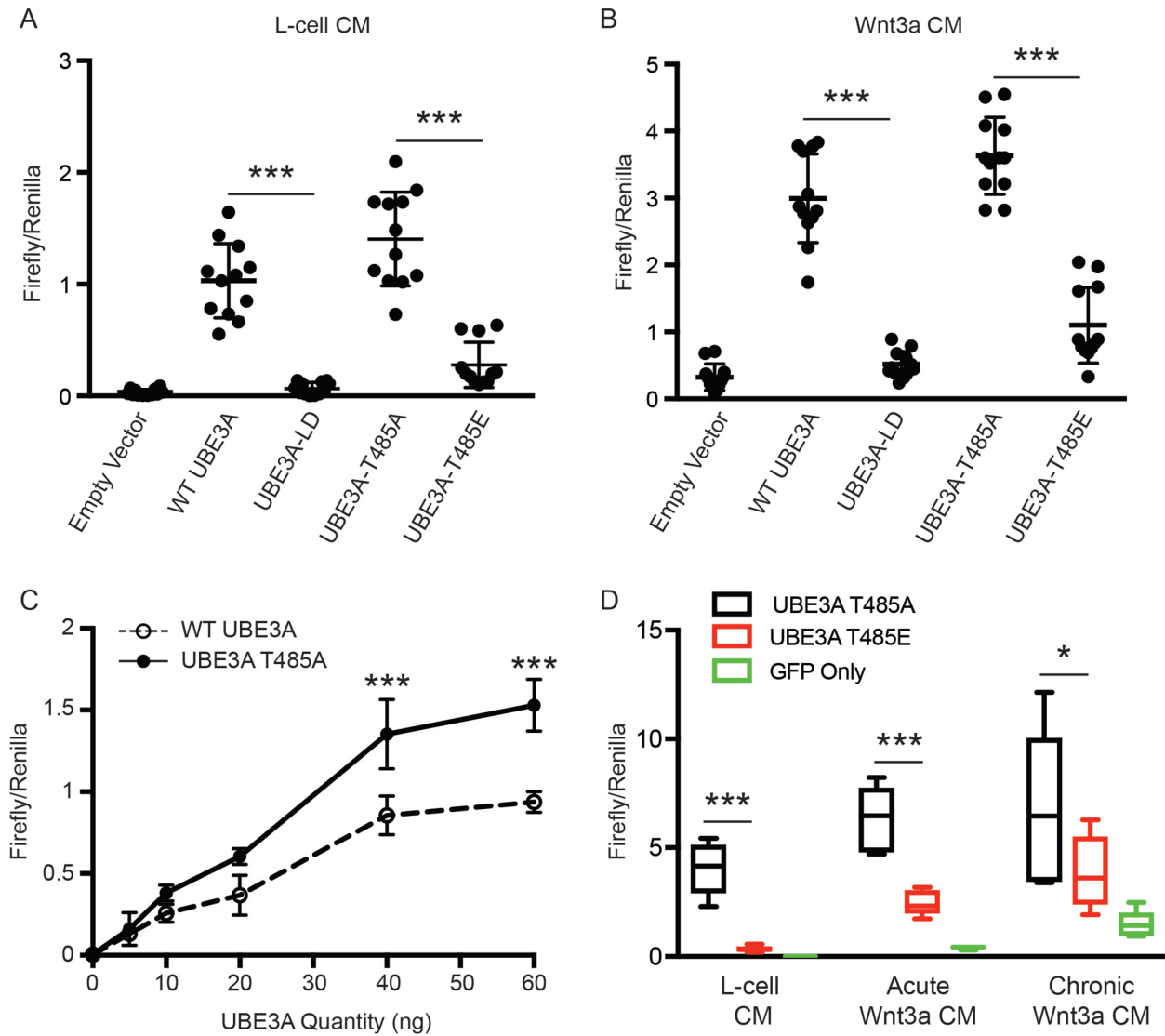
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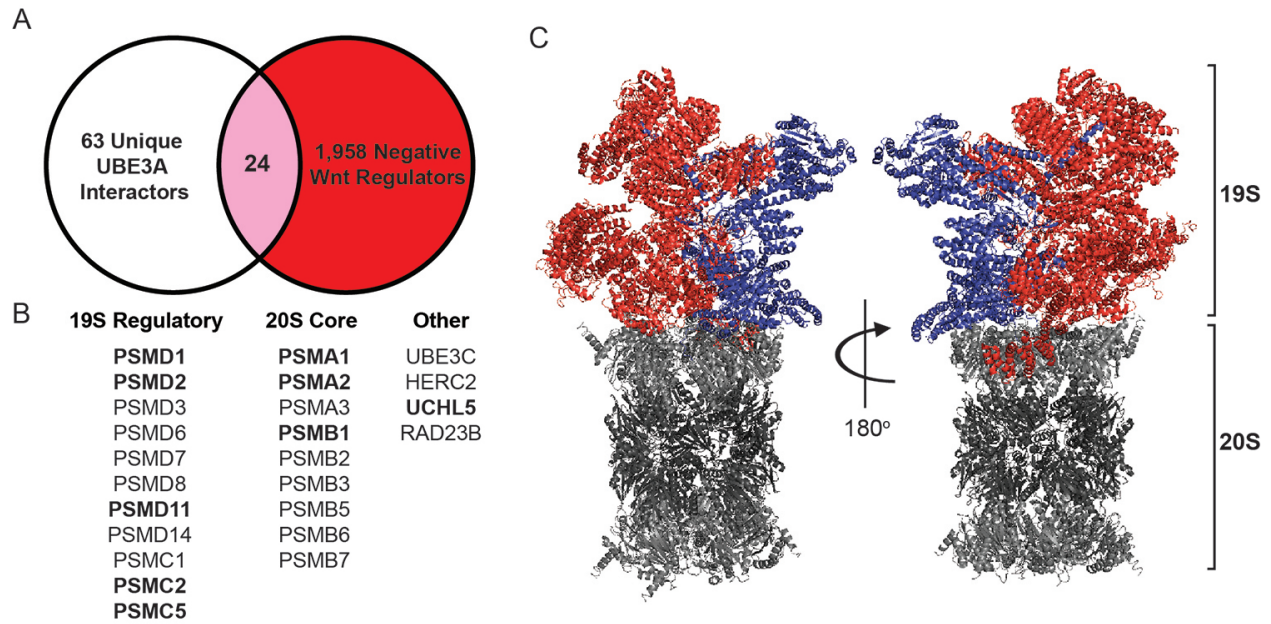
## FIGURES



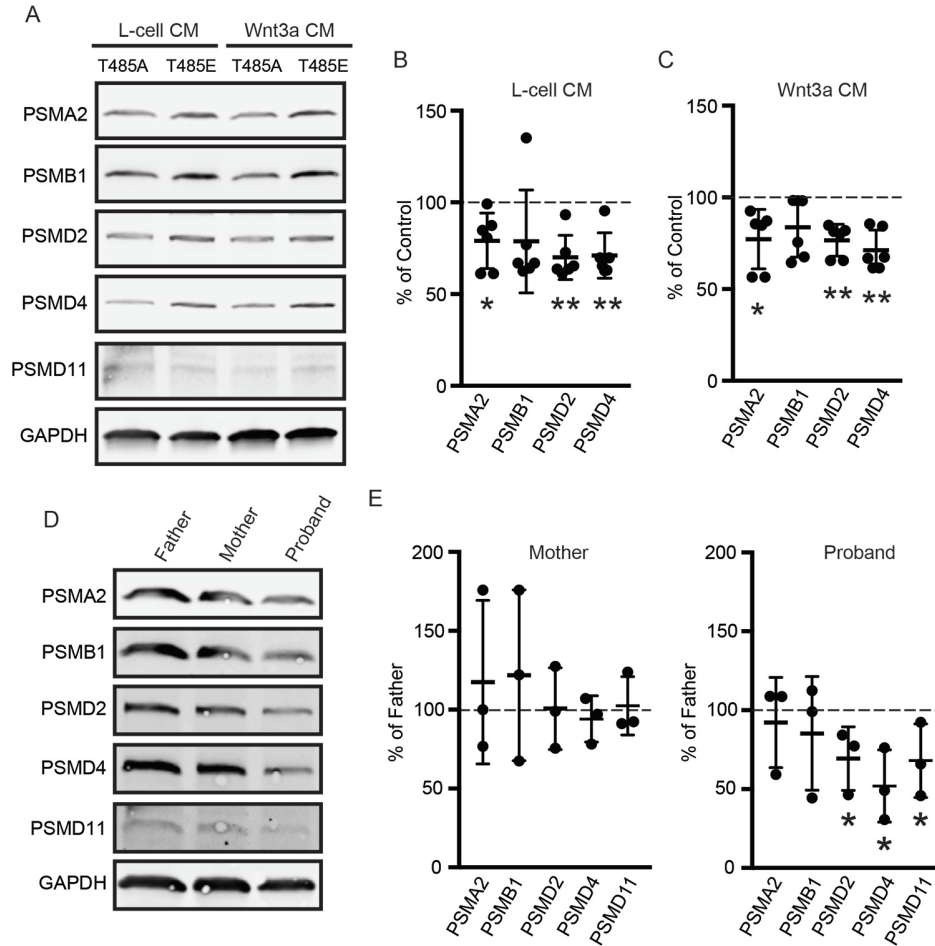
**Figure 1:** The canonical Wnt signaling pathway. Drugs used in epistasis experiments are indicated (red). C59 is a Porcupine inhibitor that suppresses Wnt secretion, XAV939 is a Tankyrase inhibitor that stabilizes the destruction complex, LiCl and CT99021 are inhibitors of GSK3, VPA is an activator of  $\beta$ -catenin-dependent transcription, and iCRT14 inhibits  $\beta$ -catenin-dependent transcription.



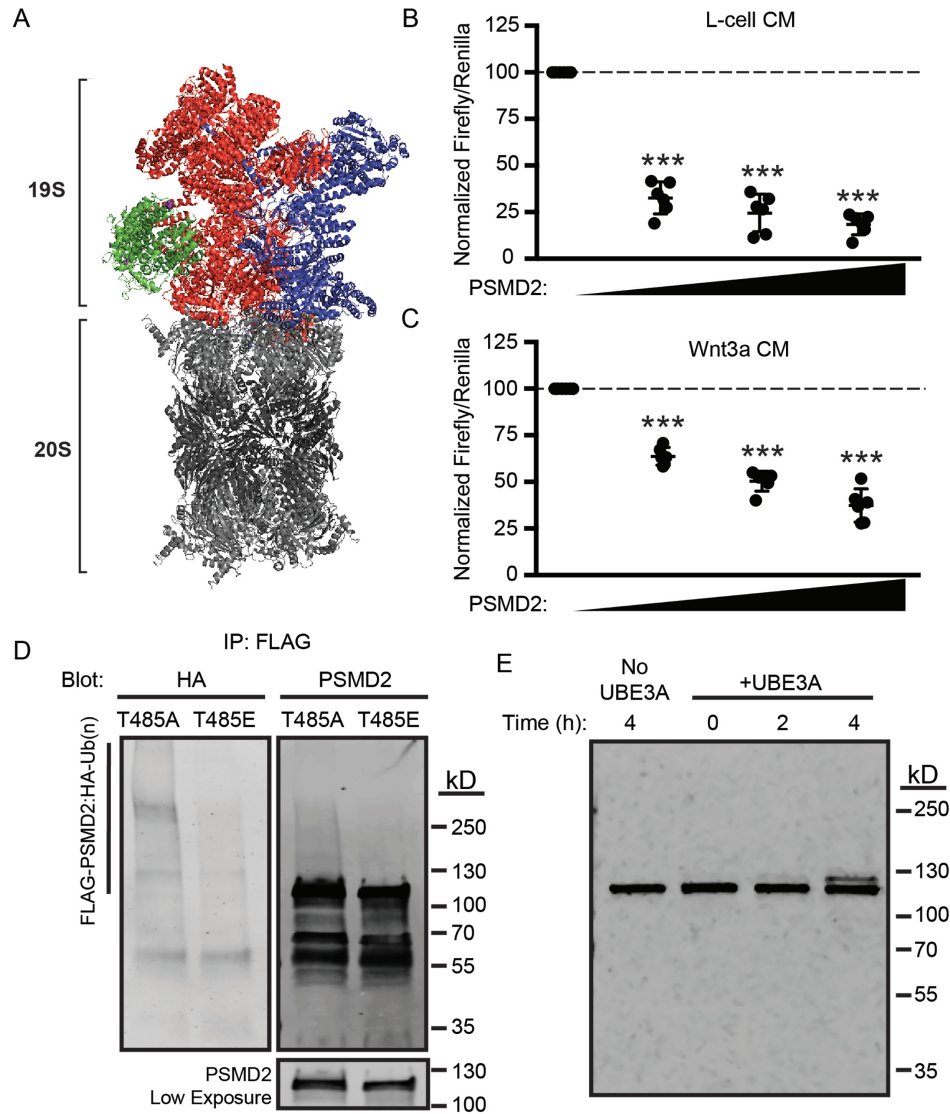
**Figure 2:** The autism-linked UBE3A<sup>T485A</sup> mutant exacerbates Wnt signaling. (A) HEK293T cells were transfected with the BAR reporter, TK-Renilla, and empty vector or the indicated UBE3A plasmid. Cells were serum deprived and treated with control (L-cell) CM or (B) Wnt3a CM. Values are shown as the mean  $\pm$  standard deviation of firefly/Renilla ratios (n = 9). Statistical analysis was performed using one-way ANOVA with Bonferroni post-hoc correction; \*\*\*p<0.0005. (C) HEK293T cells were transfected with increasing amounts of plasmid encoding WT UBE3A or UBE3A<sup>T485A</sup>. Values are shown as the mean  $\pm$  standard deviation of firefly/Renilla ratios (n = 9). Statistical analysis was performed using two-way ANOVA with Bonferroni post-hoc correction \*\*\*p<0.0005. (D) Transfected HEK293T cells were grown for 12-16 h in L-cell CM or Wnt3a CM (acute), or in Wnt3a CM for one passage prior to transfection through lysis (chronic). Values are shown as box and whisker plots for firefly/Renilla ratios (n = 6). Statistical analysis was performed using a two-sample t-test (two-tailed); \*p<0.05, \*\*\*p<0.0005.



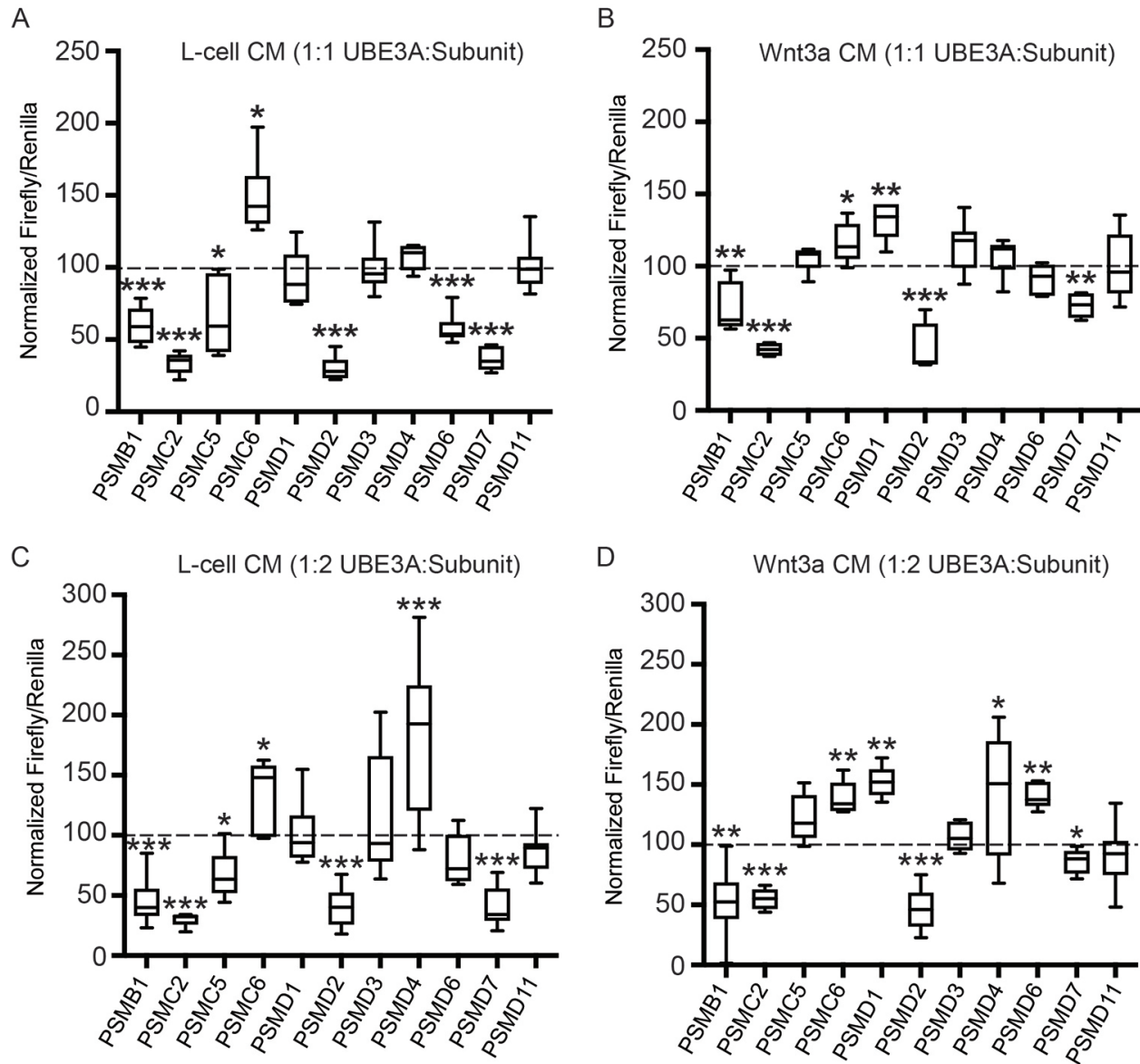
**Figure 3:** UBE3A and Wnt signaling converge at the proteasome. **(A)** High-confidence unique UBE3A interacting proteins identified by proteomics (63, white) were cross-referenced with negative regulators of Wnt signaling identified from a genome-wide siRNA screen (1,958, red). Venn diagram shows 24 common proteins (pink) that complex with UBE3A and negatively regulate Wnt signaling. **(B)** List of the 24 common proteins. Each protein was a component of the 19S regulatory particle of the proteasome, the 20S core complex, or an accessory protein associated with the proteasome (other). Proteins in bold were identified as putative UBE3A substrates from ubiquitin remnant immunoaffinity profiling (See Table S4). **(C)** Spatial organization of Wnt regulators in the 19S proteasome complex. Cryo-EM structure showing the 19S and 20S proteasome complex (PDB: 5GJQ). 20S core complex subunits are shown in gray. 19S subunits that negatively regulated Wnt signaling when knocked down in HEK293T cells are shown in red, while subunits that did not affect WNT signaling are shown in blue.



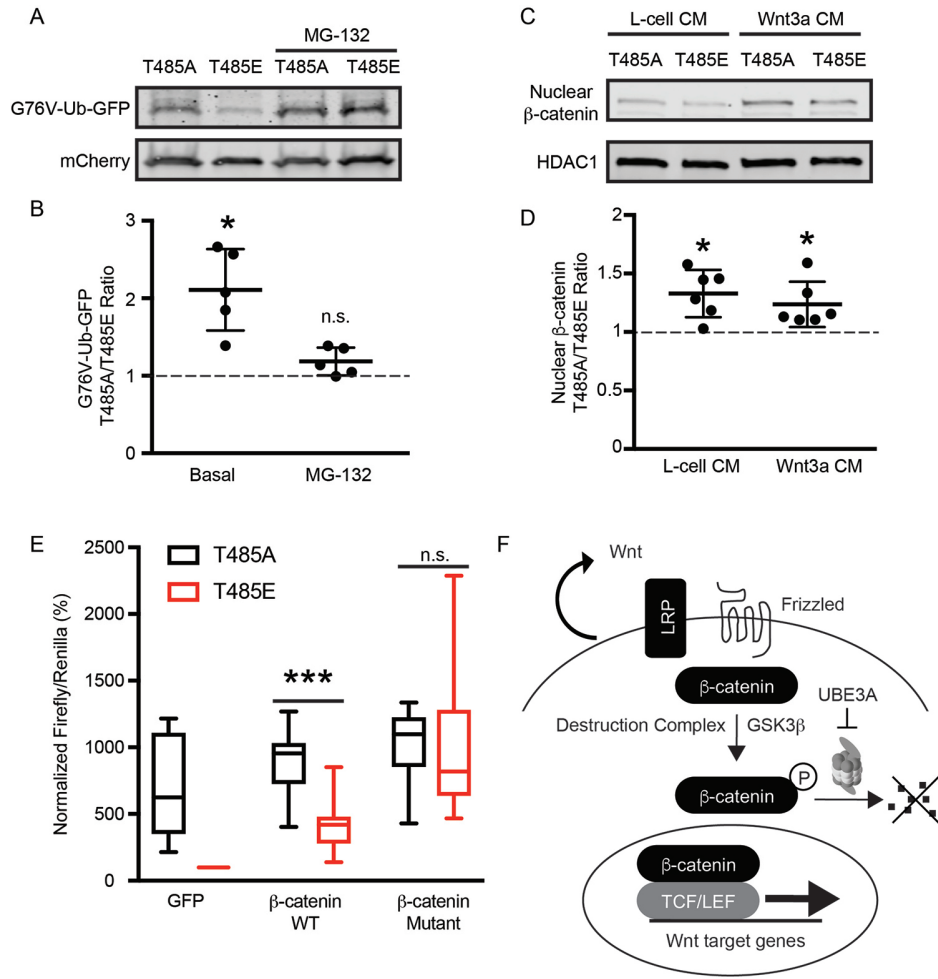
**Figure 4:** UBE3A<sup>T485A</sup> depletes multiple endogenous proteasome subunits. **(A)** Protein lysates from HEK293T cells expressing UBE3A<sup>T485A</sup> or UBE3A<sup>T485E</sup> mutants were analyzed by western blot for proteasome subunit protein levels. Analysis was performed on cells grown in L-cell CM or Wnt3a CM. **(B and C)** Quantification of western blots for cells grown in L-cell CM **(B)** or Wnt3a CM **(C)**. Values are shown as the mean percent abundance in UBE3A<sup>T485A</sup> samples relative to UBE3A<sup>T485E</sup> samples  $\pm$  standard deviation ( $n = 6$ ). Statistical analysis was performed using a one-sample t-test (two-tailed); \* $p < 0.05$ , \*\* $p < 0.005$ . PSMD11 was not analyzed due to its low abundance in HEK293T cells. **(D and E)** Representative western blot of proteasome subunits in immortalized lymphocyte cell lines from the father, mother, and autism proband (Simon Simplex Collection; Family ID: 13873), and quantification **(E)**. Values are shown as the mean percent relative to the level of the father  $\pm$  standard deviation ( $n = 3$ ), \* $p < 0.05$ , one-sample t-test.



**Figure 5:** PSMD2 is a substrate of UBE3A. **(A)** Cryo-EM structure of the proteasome showing the location of PSMD2 (green) in the 19S regulatory particle (PDB: 5GJQ). **(B and C)** PSMD2 dose-dependently rescues UBE3A-stimulated Wnt signaling. HEK293T cells were co-transfected with plasmids encoding UBE3A<sup>T485A</sup> and increasing quantities of PSMD2 (0, 20, 40, 60 ng). Cells were then grown in L-cell CM **(B)** or Wnt3a CM **(C)**. Values are shown as percent firefly/*Renilla* ratios  $\pm$  standard deviation relative to cells transfected with UBE3A + empty vector (n = 6). \*\*\*p<0.0005, one-sample t-test (two-tailed). **(D)** HEK293T cells transfected with the indicated UBE3A, Myc-DDK-PSMD2, and HA-ubiquitin constructs were treated with the proteasome inhibitor MG-132 (30  $\mu$ M, 4 h). PSMD2 was immunoprecipitated using an anti-FLAG antibody, and western blot probed with an anti-HA antibody (left panel) or PSMD2 antibody (right panels) to detect ubiquitinated PSMD2. **(E)** In vitro ubiquitin assay was performed using recombinant E1, E2 (UBE2D3), UBE3A, and PSMD2 expressed and purified from HEK293T cells. Reactions were stopped at the indicated time and the formation of ubiquitinated PSMD2 was monitored using a PSMD2 antibody. WT UBE3A was omitted from the reaction as a negative control (first lane).



**Figure 6:** Overexpression of proteasome subunits reversed the effect of UBE3A<sup>T485A</sup> on Wnt signaling. (A and B) BAR assays were performed in HEK293T cells expressing the UBE3A<sup>T485A</sup> mutant and the indicated proteasome subunits. Cells were transfected using a 1:1 UBE3A:subunit ratio and grown in L-cell CM (A) or Wnt3a CM (B). Values are shown as box and whisker plots for percent firefly/*Renilla* ratios relative to cells transfected with UBE3A<sup>T485A</sup> + empty vector. (C and D) Cells were transfected using a 1:2 UBE3A:subunit ratio and grown in L-cell CM (C) or Wnt3a CM (D). Values are shown as box and whisker plots for percent firefly/*Renilla* ratios relative to cells transfected with UBE3A<sup>T485A</sup> + empty vector. (A-D) N=9 (3 independent experiments, 3 biological replicates/experiment), \*p<0.05, \*\*p<0.005, \*\*\*p<0.0005, one-sample t-test (two-tailed).



**Figure 7:** UBE3A inhibits the proteasome and increases nuclear  $\beta$ -catenin accumulation. **(A and B)** Representative western blot and quantification **(B)** of protein lysates from HEK293T cells expressing UBE3A T485A or UBE3A T485E, the destabilized G76V-Ub-GFP reporter protein, and mCherry as transfection and loading control. The proteasome was inhibited with MG-132 (10  $\mu$ M, 12 h). Values are shown as the mean percent relative to UBE3A T485E expressing cells  $\pm$  standard deviation. N=5, \* $p$ <0.05, two-sample t-test (two-tailed). **(C and D)** Representative western blot and quantification **(D)** of nuclear lysates from HEK293T cells expressing UBE3A T485A or UBE3A T485E mutants. Cells were grown in L-cell CM or Wnt3a CM and analyzed for  $\beta$ -catenin abundance. Values are shown as the mean percent relative to UBE3A T485E expressing cells  $\pm$  standard deviation. N=6, \* $p$ <0.05, two-sample t-test (two-tailed) **(E)**  $\beta$ -catenin degradation is required for UBE3A-dependent Wnt pathway activation. BAR assays were performed with HEK293T cells expression UBE3A<sup>T485A</sup> or UBE3A<sup>T485E</sup> and co-transfected with plasmids encoding GFP, WT  $\beta$ -catenin, or a stabilized  $\beta$ -catenin mutant. Values are shown as box and whisker plots for percent firefly/*Renilla* ratios relative to cells transfected with GFP alone (n=9), \*\*\* $p$ <0.0005; n.s., not significant; two-sample t-test (two-tailed). **(F)** Mechanism. Enhanced UBE3A activity at the proteasome reduces protein turnover, leading to  $\beta$ -catenin stabilization and enhanced transcription of Wnt target genes

**The autism-linked UBE3A T485A mutant E3 ubiquitin ligase activates the Wnt/ $\beta$ -catenin pathway by inhibiting the proteasome**

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