A MUB E2 structure reveals E1 selectivity between cognate ubiquitin E2s in eukaryotes

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Ubiquitin (Ub) is a protein modifier that controls processes ranging from protein degradation to endocytosis, but early-acting regulators of the three-enzyme ubiquitylation cascade are unknown. Here we report that the prenylated membrane-anchored ubiquitin-fold protein (MUB) is an early-acting regulator of subfamily-specific E2 activation. An AtMUB3:AtUBC8 co-crystal structure defines how MUBs inhibit E2 ~ Ub formation using a combination of E2 backside binding and a MUB-unique lap-bar loop to block E1 access. Since MUBs tether Arabidopsis group VI E2 enzymes (related to HsUbe2D and ScUbc4/5) to the plasma membrane, and inhibit E2 activation at physiological concentrations, they should function as potent plasma membrane localized regulators of Ub chain synthesis in eukaryotes. Our findings define a biochemical function for MUB, a family of highly conserved Ub-fold proteins, and provide an example of selective activation between cognate Ub E2s, previously thought to be constitutively activated by E1s.

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The covalent attachment of ubiquitin (Ub) to a protein substrate occurs through the sequential activity of Ub E1-activating, E2-conjugating and E3-ligating enzymes \(^1\)\(^-\)\(^4\), and has diverse regulatory functions in protein degradation, DNA repair, endocytosis and many other aspects of cell biology \(^5\)\(^-\)\(^7\). Each Ub must be activated by an E1, an 80–100 kDa protein composed of two E2 coordinating arms atop an activation body. The arms consist of the ubiquitin-fold domain (UFD) on one side, and the Cys domain, plus, first catalytic cysteine half domain on the other, bridged by the active adenylation domain (AAD) and inactive adenylation domain \(^8\). The AAD forms Ub-AMP using Mg\(^2\+)\+-ATP and ubiquitin, which the Cys domain then acquires via a thioester linkage before rotating away to allow a second activation in the vacated AAD, resulting in E1(Ub)\(_2\) (ref. 6).

E1(Ub)\(_2\) is thought to recruit an E2 (refs 7,8) and catalyse an E2 thioester linkage to Ub (E2 ~ Ub). Current models suggest the E1 UFD first interacts with the E2 in a distal conformation before rotating towards the opposed Cys domain to form a proximal configuration, placing the E1 and E2 active sites in range for Ub transfer \(^9\) (Fig. 1). This elegant mechanism affords regulation potential, which is indeed used between Ub-like protein tags (Ubls), but not traditionally within a Ub family. For instance, Ub E1-like enzymes for SUMO, Nedd8, and other Ubls do not activate Ub E2s, or vice versa \(^10\). In contrast, current models hold that the 37 diverse E2 enzymes of Arabidopsis are constitutively activated by either of two closely related E1s, UBA1 or UBA2 (refs 11,12). The extent to which Ub E1s select Ub E2s is largely unexplored; although it would impact basal choices in the assembly of highly combinatorial ubiquitylation complexes.

In large part, Ub signalling outcomes are determined by the Ub-topology assembled on a given substrate \(^12\); for example, the addition of one Ub, or a Ub chain shaped by the use of seven internal Ub lysines \(^13\)\(^-\)\(^14\). Functionally, mono-ubiquitylation is associated with the endocytosis apparatus and DNA maintenance \(^15\)\(^-\)\(^16\). Lys48-linked Ub chains drive substrate degradation by the 26S proteasome \(^17\), and Lys63-linked Ub chains scaffold non-degradative protein complexes \(^18\). In \textit{in vitro}, E2s exhibit characteristic chain-building preferences that are strong for some E2 subfamilies such as Ube2S for Lys11-linked chains, Ube2K for Lys48-linked chains, Ube2N-Ube2V1 for Lys63 chains \(^19\), while the Ube2D subfamily requires additional guidance from an E3 (refs 20,21).

In emerging studies, some E2s appear to control chain-building by scaffolding higher-order complexes through a non-covalent backside binding site (BBS) distant from the active-site Cys. For example, the BBS of Ubc13 positions the UEV (ubiquitin E2 variant) Mms2 to orchestrate K63 chain assembly \(^22\). Ube2D3 and variant) Mms2 to orchestrate K63 chain assembly \(^22\). Ube2D3 and, for some E2 subfamilies such as Ubc13, the UEV interacts with the E2 group, promoting chain-activation \(^23\),24, the BBS of Rad6 promotes Ub chain assembly unless blocked by Rad18 (ref. 25) and Ube2E3, which requires an intact BBS to add monoUbs \(^26\). Recent advances in understanding the HECT, RBR and APC E3s continue to reveal new E2 activities during the Ub-ligation cycle including unexplored avenues for Ub delivery into E3/substrate reactions and subsequent Ub chain-assembly processes \(^17\)\(^-\)\(^17\). Since E2s are dynamic members of the substrate modification complex, the nature of chain formation remains elusive \(^26\). How Ub chain topologies arise mechanistically, and whether the timing and positioning of E2 activation plays a role are key outstanding questions.

An interest in early regulation of the ubiquitylation axis drives our characterization of one representative family called membrane-anchored ubiquitin-fold (MUB) proteins. Although small at ~ 120 amino acids, MUBs are unlike other ubiquitin-fold proteins including SUMO and NEDD8, because they do not have a C-terminal GlyGly motif for substrate attachment, and are probably not ubiquitin-like substrate modifiers. Instead, the MUB C-terminal CaaX box motif is prenylated and drives plasma membrane (PM) association in \textit{in vivo} \(^27\). Arabidopsis MUBs \(_{1-6}\) interact specifically with the Arabidopsis group VI E2 family, and the single \textit{Homo sapiens} HsMUB interacts only with the homologous Ubc2D subfamily \(^30\). This E2 subfamily is a significant source of conjugating activity containing ~ 20% of \textit{Arabidopsis} E2 genes (7/37) and ~ 10% of human E2 genes (3/24) and includes many examples with abundant expression \textit{in vivo} \(^19\)\(^,\)\(^31\). The E2 subfamily is versatile, lacking lysine preference for chain-building, lacking N- or C-terminal extensions to the E2 core \(^21\) and promiscuous; functioning with all E3 families tested \textit{in vitro} including HECT \(^22\), RING \(^24\)\(^,\)\(^33\), BBR \(^34\), SCF \(^35\) and APC \(^36\).

It was previously reported that MUBs function as PM adaptors for an E2 subfamily, but here, the analysis reveals that MUBs specifically inhibit activation of these critical Ub E2s. This unique finding led us to obtain a 2.8-Å co-crystal structure of AtMUB3 bound to AtUBC8. The structure reveals a mechanism for inhibition of E2 ~ Ub formation that discriminates between cognate Ub E2s. This establishes a previously unappreciated point of regulation in the combinatorial complexity of ubiquitylation. The E2 active site remains accessible in the AtMUB3:AtUBC8 structure and should play an integral role in ubiquitylation events near the PM.

Results

**MUBs inhibit \textit{Arabidopsis} Group VI E2 activation.** To assess whether MUB binding regulates E2 function, we performed E2 ~ Ub thioester-formation assays on AtUBC8. For all time points tested, E2 ~ Ub formation was strongly reduced by AtMUB3 (Fig. 2a, top panel, –βme). AtUBC8-βme peptide linkages, which could complicate the analysis, were observed after 10 min (Fig. 2a, bottom panel, + βme), so three minute reactions were used in subsequent assays. Ser22 on the BBS of AtUBC8 is distant from the active-site Cys85, and was previously determined to interfere with MUB binding \(^36\). E2 ~ Ub formation on AtUBC8\(_{ser22Ar8}\) was unaffected by AtMUB3, supporting the requirement of a direct interaction for inhibition (Fig. 2b). Since MUBs interact specifically with \textit{Arabidopsis} group VI E2s, two additional subfamily members, AtUBC10 and AtUBC28 were tested. Significantly, AtMUB3 strongly inhibits their activation, however, AtUBC4 from group IV and AtUBC36 from group XV are unaffected (Fig. 2c). Collectively, MUB discriminates between cognate Ub E2s to inhibit the activation of a specific subfamily.

**Structure of the AtMUB3:AtUBC8 complex.** We framed two models for MUB competition against E1 with different

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**Figure 1 | Ubiquitin (Ub) conjugating enzyme (E2) activation by Ub-activating enzyme (E1).** Conformational changes are crucial to E1 activity including rotation (closed arrow) of the UFD, which is required to efficiently position E2 for ubiquitin (Ub) thioester (E2 ~ Ub) formation. Active-site cysteine residues (C, circled) move into proximity for trans-thiolation of Ub from E1 to E2.
downstream implications. First, that MUBs block E1 access to the E2 active site, which would imply that a MUB-bound E2 would neither be activated nor participate in downstream substrate ubiquitylation; and second, that MUBs block E1 association using a surface other than the E2 active site, supporting the possibility that MUB-bound E2s activated before joining the complex could participate in substrate ubiquitylation. To distinguish between these two models, we crystallized the AtMUB:AtUBC8 complex and obtained a 2.8-Å co-crystal structure (Table 1 and Fig. 3a). AtMUB3 occupies 800 Å² of the AtUBC8 surface including the

**Figure 2 | AtMUB3 specifically inhibits activation of AtUBC8-like E2s.** (a) Thioester formation time course demonstrates the delayed charging of AtUBC8 in the presence of AtMUB3. Reactions were started with Mg-ATP addition, and stopped with Laemmli sample buffer at indicated time points and analysed by non-reducing (-βme, β-mercaptoethanol) and reducing (+βme) immunoblot (IB) with the indicated antibodies. (b) AtMUB3 inhibition of thioester formation was examined for AtUBC8 and AtUBC8Ser22Arg using immunoblot (IB) analysis with AtUBC8, AtMUB3 or Ub antibodies. All samples contained Mg-ATP, Ub, reaction buffer and other reaction components as indicated. (c) Thioester-formation assays of representative Group VI E2s (top panel IB), and non-Group VI E2s (bottom panel IB). See also Supplementary Figs 9, 10 and 11).
MUB uses the Ub E2 BBS for interaction. To define a mechanism for AtMUB3 inhibition of E2~Ub formation, we examined known functional E2 surfaces, most obviously, the non-covalent BBS. In detail, the MUB BBS interaction uses the AtUBC8 z1/β1-loop (Asp16, Pro17, Pro18, Thr19), β1-strand (Ser22, Ala23), β2-strand (Gln34, Ala35, Thr36 and Met38), β3-strand (Val49) and the C-terminal residue Met147. Meanwhile, AtUBC8 interacts with AtMUB3 β1-, β2-, β4-, β5-strands (Arg12, Gly16, Ile58, Val86, His88 and Val90), β4z2-loop (Ser60, Gly61 and Ile63) and one residue on the C-terminal tail (Gln92; Fig. 3f and Supplementary Fig. 3). The surface-forming residues are highly conserved in this family of E2s from plants, fungi and humans. These residues are not conserved in other major Arabidopsis E2 groups, revealing why MUBs are specific to the Group VI subfamily (Supplementary Figs 4 and 5).

Comparing AtMUB3:AtUBC8 with the most related structure, Ub:Ube2D3 (2FUH)24, reveals that MUB interacts with E2s using a similar configuration (Supplementary Fig. 3), albeit with several critical differences. MUB and Ub interact with the BBS using nine analogous residues. Each protein also has three unique interaction residues. For Ub, AtUBC8-analogous residues Ser20, Val26 and Leu51 (Fig. 3d, dark grey) would pull Ub towards the E2 N-terminus, whereas AtMUB3 unique interactions using Pro18, Ala23 and Ala35 tip AtMUB3 towards the AtUBC8 N-terminus (Fig. 3d, green). Collectively, the MUB to E2-BBS surface specifies and anchors the interaction, but has no clear role in inhibition of E2~Ub formation, and so we turned our attention to the adjacent interaction surface.

A MUB lap-bar loop extends beyond the E2–BBS. Detailed examination of MUB binding outside of the Ub-analogous BBS reveals a predominant role for the AtMUB3 z2/β5-loop, referred to, from here on, as the lap-bar loop (LBL). The LBL reaches from the BBS towards the E2 N-terminus to occupy an additional ~290 Å² surface, highlighted in cyan in Fig. 3d,e. It is likely that the increased area accounts for the significantly stronger interaction that we detect experimentally, relative to E2 and Ub. For instance, the AtUBC8 BBS is an efficient bait for MUB pull-down, but not for Ub pull-down (Fig. 3e). Similarly, we have routinely detected the interaction of MUB and E2 by yeast two-hybrid, but have never detected a Ub E2 interaction30. In addition, previous reports of both Ub and SUMO BBS interactions have used elegant structural methodologies, but to our knowledge have not successfully used pull-downs or yeast two-hybrid techniques to demonstrate interaction21,23,40–42. Further analysis revealed a more negative AG for MUB:E2 compared with other known BBS interactions (Supplementary Table 1). These results suggest that the LBL enhances the stability of the MUB:E2 interaction.

Compared with Ub, the MUB-unique LBL has expanded by 3 and 7 residues in animals and plants, respectively (Fig. 4a). In cases examined, the LBL contains the consensus (Lys/Arg) xProPheGly((+)/(−)) motif. The larger plant LBL follows with (Ile/Leu/Phe/Val) that contributes hydrophobic contacts and a main chain salt bridge to AtUBC8 (Figs 3f and 4a). In all cases, the invariant Pro and highly conserved Phe are the core of the LBL:E2 interaction. Adjacent to the MUB fold of BBS interaction, the LBL loop flattens and conforms to the AtUBC8 surface (Fig. 4b). A detailed view of the interaction reveals how the LBL nestles into a hydrophobic AtUBC8 pocket contacting the β1β2-loop residues Pro25, Ala27, Asp28, Met30 on one side and the N-terminal α-helix residue Leu7 on the other (Fig. 4c). Gln14, also on z1, stabilizes the interaction forming salt bridges to the Phe77 and Ile80 main chain positions, possibly contributing to the distinctive sigmoidal shape of the distal ‘bar’ of the LBL.

### Table 1 | Crystallographic data and refinement statistics.

| Data collection |  |
|---|---|
| Wavelength (Å) | 0.97 |
| Space group | P 63 2 2 |
| Unit cell dimensions (Å) |  
| a = b = 135.72 |  |
| c = 202.13 |  |
| Molecules/asymmetric unit | 4 |
| Resolution range (Å) | 50.00–2.8 |
| Unique observations | 27,721 |
| Completeness (%) | 99.8 (100) |
| Rsym (%) | 0.22 (0.39), 0.26 (0.36) |
| Reflections (working/test) | 25,543/1,346 |
| Protein atoms | 3,720 |
| Sulfate atoms | 30 |
| R.m.s.d. bond lengths (Å) | 0.01 |
| R.m.s.d. angles (Å²) | 1.5 |
| <β> protein (Å²) | 75 |
| Ramachandran plot | |
| Most favoured (%) | 90.7 |
| Allowed (%) | 7.8 |
| Generously allowed (%) | 1.5 |

* R.m.s.d., root mean-squared deviation.
* High-resolution shell for data collection is 2.8–2.85 and for refinement 2.8–2.87.
* Calculated by PROCHECK.

BBS and adjoining N-terminal residues, but does not encroach on the active-site Cys85 (Fig. 3b). Indeed, AtMUB3 pulls-down AtUBC8 Cys85Ser with Ub linked to the active site through an oxyester bond (Fig. 3c). Importantly, AtUBC8 Ser22 forms a salt bridge with AtMUB3 Gly61, central to additional salt bridges and extensive hydrophobic interactions (Fig. 3b,f) consistent with the submicromolar dissociation constant Kd as measured by iso-thermal titration calorimetry (ITC, Supplementary Fig. 1) and enzymatic assays30 (Fig. 2b). Comparing AtUBC8 with Human Ub:2D3 (3UGB)37, which is 80% identical and 89% similar at the positions and flexibility (see detailed description below) between proximal proteins, yet with E2B a MUB-anchored E2 with its active site exposed to cytosolic PM in planta converted to a C-terminal geranylgeranyl carboxymethyl Cys suggesting a flexible tether to the C-terminal Cys115, which is C-terminal nineteen residues of AtMUB3 are unstructured, C-terminus, AtMUB3 aligns closely with Ub, r.m.s.d. 1.8 Å for 71 α-atoms suggesting that AtUBC8 does not undergo a large MUB-induced conformational change (Supplementary Fig. 2).

In the complex, AtMUB3 bears strong resemblance to known Ub-fold structures, featuring a central β-sheet cradling a major α-helix, and a second minor α-helix residing near the N-terminus. Excluding the connecting loops and the unstructured C-terminus, AtMUB3 aligns closely with Ub, r.m.s.d. 1.8 Å for 71 Cx atoms (2FUH)24, and SUMO, r.m.s.d. 1.6 Å for 74 Cx atoms (2UYZ)38. There are no MUB crystal structures for comparison, but the Ub-fold core of AtMUB3 aligns well with an NMR structure of AtMUB1 in solution, r.m.s.d. equivalent 0.8 Å (1SE9) for 66 Cx atoms39. Yet there are significant differences in loop positions and flexibility (see detailed description below) between the AtMUB1 and AtMUB3 structures despite strong sequence conservation of 46% identity and 68% similarity (Supplementary Fig. 2). Consistent with efficient MUB prenylation39, the C-terminal nineteen residues of AtMUB3 are unstructured, suggesting a flexible tether to the C-terminal Cys115, which is converted to a C-terminal geranylgeranyl carboxymethyl Cys in planta and used for PM-anchoring. The model that emerges is a MUB-anchored E2 with its active site exposed to cytosolic PM proximal proteins, yet with E2~Ub turnover biochemically suppressed possibly to guide E2 chain-building preferences of the E2 subfamily.

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When bound to AtUBC8, the bar is rigid, but flanked by flexible segments indicated by the b-factor values of the structure (Fig. 4d). Superposition with Ub reveals how extension of the MUB LBL contacts the hydrophobic E2 patch (Fig. 4e). Small differences occur in the conformation of the short a2-helix that leads into the LBL and the conformation of β2-strand at the other end of the LBL (Supplementary Fig. 2). These differences occur in the conformation of the short a2-helix that leads into the LBL and the conformation of β2-strand at the other end of the LBL (Supplementary Fig. 2). Thus, MUB undergoes a conformational change to snap the LBL onto the E2 surface.

MUB inhibition is conserved between plants and humans. To test whether the differences between plant and animal LBLs influence their activity, we compared them in reciprocal inhibition experiments. The human homologue of MUB, HsMUB, reveals potent inhibition of not just Ube2D3~Ub formation but also AtUBC8~Ub formation, suggesting that the smaller HsMUB LBL is sufficient. AtMUB3 does not inhibit Ube2D3~Ub, suggesting that the four residue expansion of plant-specific LBLs prohibits access to the Ube2D3 surface (Fig. 5a).

MUB inhibition occurs at physiological concentrations. To determine the magnitude of MUB inhibition, we investigated HsMUB inhibition of Ube2D3 using an independent method based on Homogeneous Time Resolved Fluorescence–Förster Resonance Energy Transfer (HTRF–FRET). In this assay, fluorescein-labelled Ub behaves as a FRET acceptor once the thioester is formed with biotinylated E2 labelled with a streptavidin-terbium FRET donor. This assay establishes a Ki of 16 nM, and demonstrates that up to 70 per cent of Ube2D3 activation can be inhibited (Fig. 5b). These results are highly consistent with the PAGE-based assays where we typically see low levels of E2 activation even in the presence of saturating MUB. Taken together, they indicate MUB inhibition of E2~Ub formation is conserved and broadly relevant at physiological MUB concentrations in organisms that use the ubiquitylation system.

The LBL interferes with E2 charging. To further test the prediction that LBL is sufficient for inhibition, we applied a minimal human LBL (LeuLysLeuProPheGlyLysThr) peptide to E2~Ub-formation assays. This peptide has potent inhibitory activity when added to assays at 5 mM, similar to 30 μM complete HsMUB. Three scrambled peptides (5 mM, each) or equimolar free amino acids have no effect. In a complementary experiment,
HsMUB with the LBL replaced with the Ub \(\alpha2\beta5\)-loop, named HsMUBX, completely loses the ability to inhibit E2~Ub formation (Fig. 5c). Further, AtMUB3 with the core LBL residues ProPheGlyAspIle all converted to alanines fails to inhibit, while retaining binding in pull-down assays. Individual mutations of this sequence reveal that the highly conserved ProPhe motif is most responsible for inhibition (Fig. 5d,e and Supplementary Figs 6 and 7).

**E1 and MUB require access to the same E2 residues.** To better define the mechanism used by the LBL to inhibit E2~Ub thioester formation, we compared the LBL:E2 surface and a previously characterized E1:E2 surface (PDB code 4I12), which reveals that LBL interferes with E1:E2 interaction. The *S. pombe* E1 (SpUba1) uses the conserved UFD domain to coordinate E2(SpUbc4)~Ub formation. The interface is formed by SpUba1 \(\beta28, \beta29\) and \(\alpha32\), and includes two key E2-binding residues, Leu951 and Phe956 in *S. pombe*, which are conserved in *Arabidopsis* UBA1/2 as Leu1020 and Met1025 (Fig. 6a, top). Mutation of these SpUba1 residues decrease E2~Ub formation by at least 70 percent\(^9\). This is strikingly similar to the decrease observed with saturating MUB (Fig. 5b).

Notably, plotted in Fig. 6a, middle, the N-terminal twenty-nine E2 residues of the \(\alpha1\) helix through the \(\beta1\beta2\)-loop contain residues for both (i) E2~Ub formation\(^9\) and (ii) LBL binding (Fig. 4c). Positions Leu7, Glu28 and Met30 are key contacts for Phe77 and Ile80 of AtMUB3 LBL, and Leu951 and Phe956 of the SpUb1 UFD, respectively (magenta versus green circles, Fig. 6a). Positions Leu7, Glu28 and Met30 are key contacts for Phe77 and Ile80 of AtMUB3 LBL, and Leu951 and Phe956 of the SpUb1 UFD, respectively (magenta versus green circles, Fig. 6a). Most of the conflict occurs in two focal regions, the C-terminal half of the \(\alpha1\)-helix and the \(\beta1\beta2\)-loop, which form a conserved contiguous surface in both AtUBC8 and SpUb4c structures.

To examine the conflict-implicated residues in three dimensions, a model was generated superimposing the AtMUB3:AtUBC8 complex onto the SpUb1:SpUb4c structure (Fig. 6b), which reveals that the conflict is exclusive to the UFD of the E1. Here, the Phe77 and Ile80 of the LBL are imposed directly into
The main finding of this work is that MUB exerts high-level regulation on the ubiquitin system in plants and mammals by inhibiting E2 activation of an important E2 subfamily (Fig. 2).

The hydrophobic groove used by SpUbA1 residues Leu951 and Phe956 (Fig. 6c). The central position of Phe77 in the conflict explains the strong loss of inhibition seen with Phe77Ala (Fig. 6d). Aside from Phe77, Pro76 appears to play a key role positioning these residues in the conflict zone. Taken together, E1:MUB:E2 model reveals direct conflicts between the E1 residues required for efficient E2~Ub formation and the MUB LBL residues found to inhibit E2~Ub formation, and in conjunction with the BBS interaction explains why MUBs can specifically inhibit the E2 subfamily.

**Figure 5 | Functionally conserved MUB LBL is sufficient for inhibition of E2 ~ Ub formation.** (a) Thioester-formation assays, as described in Fig. 2, of AtUBC8 (left) and Ube2D3 (right), each exposed to both Arabidopsis and human MUBs. (b) Using HTRF-FRET, Ube2D3 activation was monitored with indicated concentrations of HsMUB to establish an inhibition curve. The initial velocity was linear over the first 10 min and is represented as percent E2 activated, with 100% velocity taken in the absence of MUB and 0% in the absence of ATP. Plot represents three independent assays ± s.e.m., fit to the one-site binding equation. An additional three assays fit to the Hill equation with coefficient n produced nearly identical results with n = 1. See also Supplementary Fig. 1. (c) Top panel, thioester-formation assays of AtUBC8 exposed to N-LKLPFGKT-C (HsMUB LBL), three scrambled peptides or an amino acid mixture equimolar to the human LBL (AA control). Bottom panel, thioester-formation assays of UBC8 exposed to HsMUB chimeric for the Ub α2β5-loop (HsMUBX). (d) Quantification of AtMUB3 LBL mutant protein inhibition was performed by normalization to WT as 100% (dashed line), ± s.e.m. and n = 3 for all data points (Supplementary Figs 6 and 7). AtMUB3 with the LBL core converted to alanines is P76A F77A G78A D79A I80A (All A). (e) An interaction study between AtUBC8 and AtMUB3 All A LBL mutant proteins was carried out using GST pull-down assays. The input and output proteins were visualized by immunoblotting (IB) as indicated. See also Supplementary Figs 14, 15 and 16).

**Discussion**

The main finding of this work is that MUB exerts high-level regulation on the ubiquitin system in plants and mammals by inhibiting E1 activation of an important E2 subfamily (Fig. 2). E2 activation is required for all subsequent ubiquitylation events; however, it has not been previously documented as a point of regulation. These studies provide a functionally distinguishing characteristic for the E2 family as a whole; whether a subfamily is susceptible to MUB inhibition when near the PM or not. Moreover, the identification of this regulatory mechanism has broad implications for the ubiquitylation system because of the critical E2 clade affected. These E2s include Arabidopsis Group VI and human Ube2D, which are abundant, highly conserved in eukaryotes and implicated in chain initiation. Considering the E1 interaction, the inhibition caused by MUB gives new perspective on UFD surface requirements for E2 activation.

A mechanistic definition of the inhibition we have observed was made possible by a 2.8 Å structure of AtMUB3:AtUBC8, which revealed that MUB differentially recruits Ub E2s using the BBS (Fig. 3). The MUB E2-BBS interaction provides a fourth different Ub or Ubl BBS combination implicated in E2 regulation (Supplementary Fig. 3), which currently includes Ub, SUMO and Nedd8 (refs 23,24,40,41,44). However, unlike these proteins, MUB is not a protein modifier and thus its position on the BBS likely exerts native regulation of the Ub E2 by occluding Ub access, which would have ramifications for chain building and reaction dynamics. This notion is supported by Rad18, and Ube2D3Ser22Arg suppression of polyubiquitylation. However, studies of chain assembly are beyond the scope of this study, since it is critical to first understand inhibition of E2~Ub formation by a MUB E2 complex.
Figure 6 | The MUB LBL conflicts with E1 E2 association. (a) Structure-based sequence alignment for E1s (ref. 58) in Arabidopsis thaliana (At), Homo sapiens (Hs) and Schizosaccharomyces pombe (Sp) focused on the UFD E2 interacting region. Cyan circles indicate residues that are most critical for E1 UFD:E2 docking and E2 ~ Ub formation9. A structure-based sequence alignment for E2s (UBCs) in At, Hs, Sp and Saccharomyces cerevisiae (Sc) focused on the region that interfaces with the MUB LBL. Residues in contact with E1 UFD and MUB are indicated by circles above and below the alignment, respectively. Magenta circles indicate residues that interact with MUB LBL. Green circles indicate residues that are most critical for E1 UFD docking9. Residues that contact both MUB and E1 are indicated with red. The alignments are coloured as described in Fig. 4. Lines between E1, E2 and MUB indicate structurally determined interactions. Red triangles indicate spatially conflicting residues. (b) Structural superposition model of E1, E2 and MUB. AtMUB3:AtUBC8 complex (in magenta and cyan) was superimposed to the SpUba1:SpUbc4:Ub:Mg-ATP complex using E2s for alignment. SpUba1 is featured in dark green with UFD highlighted in light green, while the Ub, SpUbc4 and Mg-ATP are not shown (PDB code 4II2). Secondary structures for Uba1 are labelled. Arrow indicates perspective for c detail. Yellow star indicates the UFD hinge. (c) Detailed cartoon structure of MUB LBL region (magenta) and E1 UFD (green) conflict region near the E2 N-terminal α-helix (AtUBC8 in cyan, SpUbc4 in dark blue, arrow indicates loop displacement in the current structure). The critical residues involved in contact and conflict are labelled and shown as stick representations. (d) No additional conflicts were obvious in structure models of the AtMUB3:AtUBC8 complex (magenta, cyan, respectively) docked to various Ub system Ube2D3 containing complexes constructed via E2 structure alignments. Structure models include portions of a HECT E3, NEDD4L HECT Domain (3JVZ)32; a RING E3, BRIC7 RING domain (4AUQ); a deubiquitinating enzyme, OTUB1 (4LDT)59; and a prokaryotic inhibitory kinase, OSPG (4BVU)60, which are coloured purple blue, marine, forest green and deepsalmon, respectively. Ub is coloured in red.
Further structural analysis reveals that MUB suppresses E2 activation using the unique LBL (Figs 4 and 5). The LBL disrupts E1 UFD interaction with the E2, providing an explanation for the inhibition of E2 ~ Ub formation (Fig. 6). To our knowledge, the control of E1 access to a Ub E2 subfamily, reported here for MUB, is unique in the current literature.

It is worth noting that unlike plants,12, vertebrates and sea urchins express a second evolutionarily divergent Ub E1 called Uba6 (ref. 45) that has inherent specificity for a Ub E2. While Uba1 and Uba6 both activate the Ube2D subfamily, only Uba6 activates the E2 Use1 (ref. 46). Ube1/Uba6 chimeric enzymes revealed that E2 preference resides in the UFD domain.45 This is reminiscent of MUB manipulation of the UFD interaction surface to block E2 activation. Yet, Ube1 and Uba6 have hard-wired UFD E2 specificities, while MUBs conditionally obscure UFD access for E2. Specifically, MUB inhibition is dependent on subcellular localization and the ability to recognize the BBS. Another difference from Uba6 is that MUBs are more broadly distributed; as they are found in all eukaryotes except for a select few Ascomycota including brewer’s yeast.

Ultimately, examination of downstream reactions will be required to establish how MUB affects the activated E2 population. In large part, the physiological acceptor for a MUB:E2 complex will determine the contribution of Ub discharge rate to the equilibrium of charged Ub on MUB:E2. However, as opposed to the clear structural evidence for MUB impedance of E1 association with E2, we do not detect a conformational change in the E2 active site. Yet, this complex issue has precedents and clearly warrants additional investigation. For example, the G2BR domain of the RING E3 gp78 contacts the BBS of Ub2D2 to effect allosteric changes of the E2 active site,47,48 as does interaction with Ceu1p.49 Furthermore, mutations of E2 UFD-interacting residues alter E2 conjugation activity even when activation occurs via E1 lacking its UFD domain.50

Structurally, it is clear that the prenylated C-terminus of MUB is not obstructed when a MUB is E2-bound, affirming how a MUB:E2 complex localizes to the PM. Furthermore, MUB does not obscure the E2 active-site Cys or downstream ubiquitylation factors (Fig. 6). Thus, MUB:E2 ~ Ub should be available for target protein modification.

Our current working model is that charged E2s bind to MUBs, but then, following Ub discharge, experience slow reactivation due to the competitive inhibition of E1 introduced by MUB:E2 binding. This model is based on the kinetic analysis demonstrating that even with saturating MUB, ~30% E1 activity remains uninhibited (Fig. 5b). Thus, E1 can still activate MUB-bound E2, but at a much reduced rate (K cat'), compared with the native E1 activation rate (K cat) for free E2 (Supplementary Fig. 8). It is also possible that E2 needs to dissociate from MUB before reactivation. This raises the interesting question of how a predicted MUB:E2 target modification cycle would provide energy to eject a spent E2. In any case, it is currently clear that MUB would introduce a severe limit to Ube2D reactivation rate for reactions occurring near the plasma membrane.

MUB:E2 participation in a ubiquitylation reaction would add two unique properties: (i) the unavailability of the E2–BBS, and (ii) the obstruction of the E1-binding site, both forces that suppress chain formation, raising interesting possibilities for Ub chain formation near the cell surface. Here, the current gap in knowledge regarding processive Ub chain formation allows measured speculation.51–52 One proposed framework for the chain-building process is that the difference between mono- and poly-ubiquitylation is largely determined by E2 turnover rates, where slow turnover promotes mono-ubiquitylation. On the basis of our data we predict that MUBs will decrease the turnover rate of the E2s that they recruit to the membrane. Interestingly, in plants there have recently been a number of important monoubiquitylated PM proteins described, including BORON TRANSPORTER1 (ref. 51), IRON-REGULATED TRANSPORTER1 (ref. 52), PRR FLAGELLIN-SENSING2 (ref. 53), PIN-FORMED 2 (ref. 54) and PHOTOTROPIN1 (ref. 55). These and other proteins localize to the PM, where MUBs are found and mono-ubiquitylation is prevalent. The current findings suggest that E2 activation is regulated with subcellular precision, however, key future questions include what substrates are MUB complex-modified, and with what Ub-topology. These questions warrant continued study to better understand selective Ub E2 activation by E1s in eukaryotes.

### Methods

**Cloning and mutagenesis.** The coding regions of AtMUB3 and HsMUB were PCR-cloned to remove stop codons, enable pET28b ligation and append C-terminal 6x HIS tags. C-terminal 6x His plus FLAG-tagged AtMUB3 were generated by DNA 2.0. Generation of N-terminal 6x HIS AtMUB3, GST-AtUBC8 and AtUBC8Ser22Arg was as described30. AtUBC8Cys85Ser and all AtMUB3 mutants were generated by bridging-PCR and PCR-cloned into pET28a. PCR-cloning of AtUBCs 4, 10, 28 and 36 with intact stop codons, and AtUBC8, AtUBC8Ser22Arg and Ube2D3 lacking stop codons were moved into pENTR-D-TOPO for subsequent LR cloning. AtUBC8, AtUBC8Ser22Arg and Ube2D3 were transferred to pGWA56 to generate N-terminal 6x HIS E2 constructs, while AtUBCs 4, 8, 10, 28 and 36 were transferred to pGWA56 to generate N-terminal 6x HIS E2 constructs. All constructs were sequence verified and catalogued as glycerol stocks for distribution. Primers for construct assemblies are listed in Supplementary Table 2.

**Protein expression and purification.** Throughout, protein concentrations refer to total protein. All proteins were expressed in Rosetta 2 (DE3) pLacI E. coli (Novagen). Cultures in LB medium were induced by addition of 0.2 mM isopropyl-β-D-1-thio-galactoside at 16 °C for 16h with vigorous shaking. For activity assays, AtMUB3 and UBC preparation cell pellets were sonicated in 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% Glyceral and 10 mM Imidazole. The soluble lysate fraction was purified on TALON metal affinity resin (Clontech) washed sequentially in 7.5, 300 mM NaCl, 10% Glycerol and 10 mM Imidazole. The soluble extract fraction was immobilized on GSH resin made in lab according to ref. 57. Hydroxylapatite flow-through was incubated 10 mM Imidazole, washed and eluted as above, and then applied to hydroxylapatite resin made in lab according to ref. 58. Hydroxylapatite flow-through was incubated at 70 °C for 10 min and centrifuged to remove heat-labile contaminants. For crystallization, AtUBC8 crystallography, activity-grade elutions were diluted 10-fold and re-purified using metal affinity.

**In vitro interaction studies.** GST–tagged AtUBC8 was induced as described above, and the soluble extract fraction was immobilized on GSH resin (Thermo Scientific) for 20 min, followed by three washes with sonication buffer. Equimolar His-tagged...
AtMUB3, AtUBC8 mutants and Ub were incubated separately with AtUBC8-loaded GST resin for 15 min, washed three times using sonication buffer and boiled in Laemmli buffer. MUB recovery was determined by immunoblotting and chemiluminescent detection using Fujifilm LAS4000 imaging.

For FLAG pull-down assays, His-tagged AtUBC8/Cys59Ser–O–Ub was generated. Flag-tagged AtUBC8 was immobilized on FLAG resin (Thermo Scientific) for 20 min, followed by three washes with sonication buffer, AtUBC8/Cys59Ser–O–Ub protein or Ub were incubated separately with AtMUB3-loaded FLAG resin for 15 min, washed three times using sonication buffer and boiled in Laemmli buffer. For immunoblotting, antibodies against Group VI E2s, MUBs and GST were custom made and used at 1:5,000. Anti-FLAG (Sigma) and anti-Ub (Boston Biochem) were used at 1:12,000. Immunoblot HRP luminescence was imaged using a Fujifilm LAS4000.

I TC was performed on 8 μM Ub with 110 μM MUB as the titrant using a MicroCal ITC200 (GE). Proteins were purified as described for crystalization and dialyzed extensively against a buffer containing 25 mM HEPS, pH 7.5, 200 mM NaCl, 1 mM TCEP. Data were processed with MicroCal Origin 7 software, and normalized against the dialysis buffer’s heat of dilution.

Crystallization. An equinominal mixture of AtMUB3 and AtUBC8 was gently mixed, concentrated to 12 mg ml⁻¹, and exchanged to 25 mM HEPS, pH 7.5, 500 mM NaCl, 1 mM TCEP and 0.1 mM CHAPS using Amicon Ultra centrifugal filter (Millipore). Crystals grew using hanging-drop vapour diffusion at 4°C. ITC measurements were used to determine average 2.8 Å was collected on the GM/CA-CAT 23D beam line at APS, ANL, Scicuca (1QCQ; PubMed: 8268156) and ubiquitin structure (SNOB/A; PubMed: 20655260) were used for molecular recognition using Phaser (PMID: 19461840). Model building and refinement were completed using the Refmac and Coot programs (PMID: 21460454; PMID: 15572765). Final model include AtUBC8 amino acids 1–147 in chain A and 1–148 in chain C, and AtMUB3 amino acids 2–94 in chain B and 3–94 in chain D as alanines due to poor density. Data collection and refinement statistics are shown in Table 1.

Data availability. Coordinates and structure factors have been submitted to the Protein Data Bank under accession codes 4X57 for the AtMUB3:AtUBC8 complex. All additional experimental data are available from the corresponding author on request.

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