Electron microscopy structure of human APC/C\textsuperscript{CDH1}–EMI1 reveals multimodal mechanism of E3 ligase shutdown

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The anaphase-promoting complex/cyclosome (APC/C) is a \textasciitilde1.5-MDa multiprotein E3 ligase enzyme that regulates cell division by promoting timely ubiquitin-mediated proteolysis of key cell-cycle regulatory proteins. Inhibition of human APC/C\textsuperscript{CDH1} during interphase by early mitotic inhibitor 1 (EMI1) is essential for accurate coordination of DNA synthesis and mitosis. Here, we report a hybrid structural approach involving NMR, electron microscopy and enzymology, which reveal that EMI1’s 143-residue C-terminal domain inhibits multiple APC/C\textsuperscript{CDH1} functions. The intrinsically disordered D-box, linker and tail elements, together with a structured zinc-binding domain, bind distinct regions of APC/C\textsuperscript{CDH1} to synergistically block the substrate-binding site and inhibit ubiquitin-chain elongation. The functional importance of intrinsic structural disorder is explained by enabling a small inhibitory domain to bind multiple sites to shut down various functions of a ‘molecular machine’ nearly 100 times its size.

UBiquitin (Ub) ligation by the action of E1-E2-E3 enzyme cascades is a widespread mechanism that controls protein function. After E1-mediated formation of a transient, thioester-bonded E2–Ub intermediate, E3s promote Ub ligation to specific protein substrates (“~” denotes covalent complex; “−”, noncovalent complex). The 600 predicted members of the largest human E3 family contain a RING (“~” denotes covalent complex; “−”, noncovalent complex). The 600 predicted members of the largest human E3 family contain a RING domain that binds and activates an E2–Ub intermediate to ligate Ub to a substrate recruited to a distal protein-interaction domain\textsuperscript{1}. APC/C is a behemoth \textasciitilde1.5 MDa E3 that controls cell division by promoting timely Ub-mediated proteolysis of key regulatory proteins. Human APC/C has at least 14 core subunits (APC1, APC2, APC3 (CDC27), APC4, APC5, APC6 (CDC16), APC7, APC8 (CDC23), APC10 (DOC1), APC11, APC13, APC15, APC16 and CDC26), several of which are present in duplicate in each holo APC/C enzyme\textsuperscript{2,3}. APC/C is activated at different stages of the mitotic cell cycle by association with either CDC20 or CDH1, which target proteins for ubiquitination by binding substrate KEN-box motifs directly and recruiting substrate D-box sequences in collaboration with APC10 (refs. 4–7). To prevent chromosome segregation defects such as aneuploidy, CDC20 assemblies with MAD2, MAD3 (BUBR1) and BUB3 to form a mitotic checkpoint complex (MCC) that binds and inhibits APC/C until all chromosomes are properly bioriented on the mitotic spindle\textsuperscript{8,9}. MCC serves as a decoy KEN-box–based substrate–receptor complex, which blocks APC/C association with free CDC20 and bona fide KEN- and D-box–containing substrates\textsuperscript{5,10}.

Following MCC disassembly, APC/C associates with free CDC20 to promote Ub-mediated proteolysis of substrates such as cyclin B and securin to initiate chromosome segregation. Subsequently, APC/C associates with CDH1 to regulate exit from mitosis and during G1 to promote Ub-mediated turnover of regulators of the G1-S transition and DNA replication. In higher eukaryotes, APC/C\textsuperscript{CDH1} is restrained during interphase by the distinctive EMI1 (Rca1 in Drosophila)\textsuperscript{11–14}. APC/C inhibition by EMI1 allows accumulation of substrates such as mitotic cyclins and geminin\textsuperscript{11,15–17}. EMI1 also functions later in a localized manner, inhibiting APC/C\textsuperscript{CDH1}–mediated ubiquitination of cyclin B at spindle poles\textsuperscript{18}. Misregulation of EMI1 leads to endoreduplication and mitotic defects such as abnormal spindles and excess centrosomes\textsuperscript{14,16,17,19,20}.

EMI1 has three domains. The N-terminal domain mediates regulatory interactions that control localization and EMI1’s own stability\textsuperscript{18,21}. An F-box binds SKP1 but to date has not been shown to function in APC/C inhibition\textsuperscript{12,19}. The C-terminal domain is responsible for APC/C inhibition and contains a D-box, linker sequence, a zinc-binding region (ZBR) and a C-terminal tail\textsuperscript{12,22}. Previous reports concluded that the EMI1 C-terminal domain is a pseudo-substrate inhibitor that binds to CDH1 and competes in a D-box– and ZBR-dependent manner with the substrate cyclin B for binding to APC/C\textsuperscript{CDH1} (refs. 12,22). However, studies of the homologous meiotic APC/C inhibitor EMI2 suggested that a key inhibitory region is the C-terminal tail\textsuperscript{23,24}. Despite its importance, structural mechanisms by which EMI1 inhibits APC/C\textsuperscript{CDH1} are unknown.

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To understand EMI1-mediated inhibition of APC/C, we performed a hybrid structural analysis. NMR studies indicated that other than the 45-residue ZBR, the 143-residue C-terminal EMI1 inhibitory region lacks fixed tertiary structure. Electron microscopy (EM) studies revealed that EMI1 interacts with, approaches and/or alters the conformations of numerous subunits of the ~1.5 MDa APC/C<sup>CDH1</sup> molecular machine. The functional importance of intrinsic structural disorder is explained by the D-box, linker, ZBR and tail synergistically blocking both Ub ligation to a substrate and Ub-chain elongation by APC/C.

**RESULTS**

**Structural dissection of EMI1's APC/C inhibitory domain**

To guide structural studies, we analyzed the human EMI1 protein sequence with the program PONDR (http://www.pondr.com/index) to identify folded domains<sup>25</sup>. The majority of EMI1 is predicted to be intrinsically disordered, with the exception of a short N-terminal region, the F-box and the ZBR (Fig. 1a). In the APC/C-inhibitory C-terminal domain, regions predicted to be unstructured include the D-box, linker region and tail. To test this hypothesis, we characterized structural properties for the EMI1 C-terminal domain (hereafter referred to as EMI1<sub>DLZT</sub> for D-box, linker, ZBR, tail) by NMR. Backbone resonance assignments revealed that the chemical shifts were consistently dispersed for the ZBR residues in the EMI1<sub>DLZT</sub>, EMI1<sub>ZT</sub> and EMI1<sub>C</sub> constructs, indicating that the ZBR is an autonomously folded domain (Fig. 1b). Notably, the remaining resonances in EMI1<sub>DLZT</sub> displayed poor dispersion, low or negative <sup>1</sup>H-<sup>15</sup>N heteronuclear NOE (HetNOE) values and relatively low differences in Cα chemical shifts compared with values predicted for a random coil (Fig. 1c).
Figure 2 EM structures of APC/C^{CDH1} inhibited by EM11–SKP1 and the inhibitory C-terminal domain (EM11^{DLZT}). (a) Three views of human APC/C^{CDH1–EM11–SKP1}, showing the structural superdomains of APC/C (arc lamp, platform and catalytic core) and the CDH1 and APC10 D-box co-receptors. The density attributed to EM11–SKP1 is outlined. (b) Three views of human APC/C^{CDH1–EM11^{DLZT}}, showing the structural superdomains of APC/C (arc lamp, platform and catalytic core) and the CDH1 and APC10 D-box co-receptors. The density attributed to EM11^{DLZT} is outlined.

Previous studies showed that mutation of ZBR cysteines or treatment with a metal chelator decreased EM11’s competition with substrate binding or inhibition of substrate ubiquitination by APC/C^{C9,22}. We found that the addition of a metal chelator, EDTA, to 15N-labeled EM11^{DLZT} eliminated the 15N-1H chemical shift dispersion that is characteristic of the folded structure, which was re-established by ZnSO4 addition (Fig. 1d). To resolve the ambiguity of one potential zinc-coordinating residue19, we ascertained that mutation of Cys409, but not Cys411, eliminated the hallmarks of folding in one-dimensional proton NMR spectra (Fig. 1e).

To further characterize the biophysical properties of EM11^{DLZT}, we measured the hydrodynamic radius ($R_h$) to be 3.1 nm and 2.9 nm by dynamic light scattering (DLS) and analytical ultracentrifugation, respectively (Fig. 1f). A globular protein would have to be ~46 kDa, rather than the 16.0 kDa of EM11^{DLZT}, to yield an $R_h$ value of 3.1 nm by DLS. The best-fit, weight-average anhydrous frictional ratio ($f\text{i}_{\text{w}}$) values of 1.73 and 1.85 obtained from two analytical ultracentrifugation experiments indicate that EM11^{DLZT} is elongated in solution (Supplementary Fig. 1). Overall, the data suggest that EM11^{DLZT} is substantially intrinsically unfolded, with predominantly disordered D-box, linker and tail regions separated by a folded ZBR.

EM reveals multisite EM11 binding to APC/C^{CDH1}

EM analysis further reveals that despite its small size, the inhibitory domain, EM11^{DLZT}, retains key features to obstruct APC/C^{CDH1}.

Figure 3 EM11 is a tight binding inhibitor of APC/C Ub ligation and Ub-chain formation. (a) Fluorescence detection of cycB-NT* ubiquitination by APC/C^{CDH1} with UBC10, alone or in combination with UBE2S, in the absence or presence of EM11–SKP1 or EM11^{DLZT}. (b) Fluorescence detection of APC/C-dependent UbcycB-NT* ubiquitination by UBE2S in the absence of UBC10 and in the absence or presence of CDH1. The KOUbcycB-NT* substrate has all lysines in the Ub moiety mutated to arginines. (c) Sequence alignment of the EM11 D-box region with EM11 and Hsl1 D-box peptides. (d) Fluorescence detection of cycB-NT* ubiquitination by APC/C^{CDH1} and UBC10 in the absence or presence of the indicated D-box peptides at 100 µM.
activity (Fig. 2b and Supplementary Fig. 2c). For the complexes with both EMI1–SKP1 and EMI1DLZT, there are differences in the vicinity of the D-box co-receptors CDH1 and APC10 and additional density linking this region and the catalytic core and platform regions. Substantial additional density is also observed emanating from the platform. Notably, relative to prior apo-APC/C and APC/C<sub>CDH1</sub> structures, in both EMI1-inhibited complexes the catalytic core forms a prominent contact with platform regions assigned to APC1 and APC4, which are repositioned. The density assigned to APC1 and APC4 has lost contact with APC5, is no longer in the position previously observed for apo-APC/C<sup>26,27</sup> and is instead bent toward the catalytic core.

A difference map comparing the full APC/C<sub>CDH1</sub>–EMI1–SKP1 complex with APC/C<sub>CDH1</sub>–EMI1DLZT reveals additional exposed density more distal from the APC/C central cavity and catalytic core, which we attribute to the N-terminal domain and SKP1-bound F-box (Supplementary Fig. 2d). This agrees with the location of another E2 (UBE2S in humans<sup>32–35</sup>) transfers Ub to a substrate-ligated Ub to mediate chain elongation<sup>28,36</sup>.

**Table 1** Apparent <i>K<sub>i</sub></i> values for EMI1 variants in the indicated APC/C-dependent ubiquitination reactions

| E2 substrate | UBC10 i app (nM) | UBC10 <i>K<sub>i</sub></i> (nM) | UBE2S i app (nM) | UBE2S <i>K<sub>i</sub></i> (nM) | No CDH1 i app (nM) | No CDH1 <i>K<sub>i</sub></i> (nM) |
|-------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| EM1–SKP1    | ≤2.5            | ≤2.5            | ≤2.5            | ≤2.5            | 690 ± 130       | 230 ± 35        |
| EM1FDLZT    | ≤2.5            | ≤2.5            | ≤2.5            | ≤2.5            | NT              | NT              |
| EM1DLZT     | ≤2.5            | ≤2.5            | ≤2.5            | ≤2.5            | NT              | NT              |
| EM1DIM4DLZT | >10,000         | >10,000         | 810 ± 230       | NT              | 220 ± 37        |
| EM1NFDL     | 440 ± 60        | 360 ± 47        | 940 ± 220       | NT              | >6,000          |
| EM1NFDL     | 510 ± 120       | 330 ± 74        | 1,500 ± 340     | NT              | NT              |
| EM1Z        | >10,000         | >10,000         | 520 ± 86        | NT              | 2,000 ± 400     |
| EM1Z        | >80,000         | >80,000         | >80,000         | NT              | >80,000         |
| EM1DLZT     | >1 mM           | ≥1 mM           | 42,000 ± 12,000 | 48,000 ± 12,000 | 13,000 ± 2,400  |

<sup>25 mM peptide changes pH. NT, not tested.</sup>

**Figure 4** EMI1DLZT elements synergize to mediate optimal inhibition. (a) Localization of EMI1 linker and C terminus. A WD40 propeller was inserted into the linker and at the C terminus of EMI1DLZT in complex with SKP1. The three-dimensional structures calculated contained additional densities based on the calculation of difference density maps, although the extra densities do not fully occupy the expected volume for a propeller, probably because of flexible connections. Shown are the strongest peaks in the difference density maps for the linker insertion in blue and the C-terminal insertion in purple, relative to APC/C<sub>CDH1</sub>–EMI1–SKP1. Conformational variability was distinguished from additional densities by the fact that negative and positive difference densities are located next to each other in areas with substantial conformational flexibility, whereas no negative difference density can be observed for the WD40 propeller insertions. (b) Human APC/C<sub>CDH1</sub>–EMI1DLZT, showing density attributed to the D-box and associated CDH1 in yellow, to the linker in green and to the ZBR and tail integrated with portions of APC1 and APC4 in purple. (c) Sequence alignment of the EMI1 D-box and linker region indicating mutations. (d) Fluorescence detection of APC/C<sub>CDH1</sub>–UBCH10-dependent cycB-NT<sup>+</sup> ubiquitination either in the absence or presence of 50 nM of the indicated variant of EMI1DLZT.
Figure 5  NMR structure of the ZBR and identification of a surface required for inhibition. (a) Solution structure of the ZBR portion of EMI1 as cartoon with zins as spheres on left, and as ribbons superimposed with the RNF31 (PDB 2CT7, gold) IBR on right. (b) The crystal structure of the EM1 ZBR is shown with approximate location next to EM structure of APC/C CDH1–EMI1DLZT at the same scale. (c) Alanine scan testing roles of indicated ZBR side chains on EM1DLZT-mediated inhibition of cycB-NT* ubiquitination by APC/C/CDH1 and UBC110. Inhibition by wild-type EM1DLZT and EM1FDL–SKP1 (lacking the ZBR and tail) are shown for comparison. (X) denotes unfolded mutants. Mutants displaying the greatest decrease in inhibitory activity are marked with purple dots; those with a lesser decrease in inhibitory activity are marked with gold dots. (d) Comparison of the effects of the indicated surface alanine mutants and the unfolding C409A mutant on EM1DLZT-mediated inhibition of cycB-NT* ubiquitination by APC/C/CDH1 and UBC110. (e) Structure of EM1 ZBR showing all side chains mutated in alanine scan as sticks, with those causing the greatest decrease in inhibitory activity in purple and a lesser decrease in inhibitory activity in gold. (f) Location of Ser383 (side chain shown as green stick with oxygen in red) cyclin-dependent kinase–mediated phosphorylation site on cartoon representation of EM1 structure with the two zinc atoms shown as spheres, the sites of Ala mutations that impair EM1DLZT-mediated inhibition colored as in panel e. Ser383 is among three cyclin-dependent kinase sites that when phosphorylated impair EM1 inhibition of APC/C.

with previous measurements of 228 and 63 nM for similar substrates with immunopurified endogenous Xenopus laevis and Saccharomyces cerevisiae APC/C, respectively.38,39

Experiments with EM1–SKP1 revealed tight binding inhibition, which we quantified using the Morrison equation.40 Performing experiments under initial rate conditions required our use of 5 nM APC/C/CDH1, for which the tight binding nature of EM1 and data quality at the stoichiometric point (1:1 APC/C/CDH1:EM1) restricted our accurate quantification of apparent Ki (K_{iapp}) values to a lower limit of 2.5 nM (Supplementary Fig. 5d). Titration of EM1–SKP1 revealed K_{iapp} values of ≤2.5 nM, and potentially substantially lower, for APC/C/CDH1-mediated substrate ubiquitination with both UBC110 and UBE2S, as approximately 5 nM of EM1–SKP1 completely inhibits these reactions (Table 1). Similar results were obtained for a version lacking the EM1 N terminus or the entire N-terminal domain and F-box–SKP1, which consisted exclusively of the C-terminal D-box, linker, ZBR and tail regions. Below we describe how the distinct elements within EM1DLZT contribute to a tight binding mode of APC/C/CDH1 inhibition.

EM1 has an essential but relatively weak D-box

An obvious key functional element is EM1’s D-box. In examining the EM data, there were differences between APC/C/CDH1 and the EM1–SKP1– and EM1DLZT-bound complexes in the vicinity of CDH1 and APC10, consistent with EM1’s D-box binding the co-receptors (Supplementary Fig. 2). We were unable to generate stoichiometric complexes for mutants lacking EM1’s D-box. Furthermore, alanine substitutions in place of Arg322 and Leu325 in EM1DLZT’s D-box RxxL motif decreased inhibition of cycB-NT* in reactions with UBC110 to the point that we could not measure a K_{i} under the initial rate conditions (Table 1). The K_{iapp} increased by over two orders of magnitude for Ub-cycB-NT* ubiquitination by APC/C/CDH1 and UBE2S.

Despite its requirement, the EM1 D-box on its own is relatively weak: an isolated EM1 D peptide is insufficient as an inhibitor even at the 100 μM concentration we could achieve in our assays. This contrasts with an isolated D-box peptide from the S. cerevisiae APC/C substrate Hsl1 (ref. 41), which is a more potent inhibitor (Fig. 3c,d).

EM1 elements synergize to inhibit substrate ubiquitination

In order to map the locations of the remaining portions of EM1, we generated single-particle EM reconstructions for mutants in EM1FDLZT–SKP1 with insertions between residues 354 and 355 in the linker upstream of the ZBR and at the C terminus of EM1 (Fig. 4a). In this latter case, it was necessary to replace the EM1 D-box with that from Hsl1 to enhance complex formation. The insertions contained the β-propeller from S. cerevisiae Doa1, which has N and C termini in close proximity.42 Although we cannot be certain that the insertions do not alter native interactions, the data are consistent with the linker projecting away from the D-box bound to CDH1 and APC10 and toward the APC/C platform and catalytic core and with the ZBR and tail extending across the platform (Fig. 4a,b).

Promoted by the structural data showing that an insertion maps to the central location between the D-box co-receptor APC10, the
arc lamp and the platform (Fig. 4a), we considered that the linker might be functionally important. Indeed, the linker is not simply a spatial connector joining the D-box and ZBR effectors: deleting 20 linker residues impairs EMI1D^DLZT-mediated inhibition, which is not restored by replacing them with a 20-residue, glycine-rich sequence (Fig. 4c,d). As a first attempt to address whether this sequence or structure may be important, we tested two 10-residue deletions and found only one to cause severely impaired inhibition. Triply mutating conserved Leu345, Tyr356 and Arg358 within the essential sequence to alamines, either in the context of the benign 10-residue deletion or in EMI1D^DLZT, was sufficient to substantially impair inhibition. Thus, specific side chains within the linker contribute to inhibition (Fig. 4d).

One reason the linker or other C-terminal elements may be important would be to compensate for EMI1’s relatively weak D-box. Inhibitory roles of regions other than the D-box are reflected by the NMR analysis of 15N-labeled samples confirming folding of the "Super" D-box into EMI1 DLZ largely overcame the defects resulting from deleting the linker, even in combination with the ZBR-unfolding C409A mutation (Fig. 4d). Thus, it appears that EMI1’s weak but essential D-box must function combinatorially with additional elements for optimal inhibition. C-terminal deletions, which retained the D-box and linker (EMI1D^DLZ and EMI1D^DEL) but lacked the tail or both the ZBR and tail, inhibited the reactions with both E2-substrate pairs, with K_{iapp} values increased by more than two orders of magnitude. Notably, deleting the ZBR had little effect in the absence of the EMI1 C-terminal tail; this underscores the importance of the tail in functioning with the D-box and/or linker to maximally inhibit APC/C^CDH1 activity with both E2s (Table 1). Taken together, the data suggest that the D-box, linker, ZBR and tail bind multiple sites on APC/C^CDH1 to synergistically antagonize ubiquitination activity.

### NMR structure and inhibitory surface of the EMI1 ZBR

To gain insights into potential contributions of the ZBR in APC/C inhibition, we assigned backbone and side-chain resonances of 13C, 15N-labeled EMI1ZT and determined the solution structure (Fig. 5a,b and Table 2). Although the EMI1ZT construct spans residues 363–447, only residues 375–420 exhibited the features of a folded domain, with a central, twisted, four-stranded β-sheet and two zinc ions on opposite ends of the sheet. One zinc is chelated by the β1-β2 and β3-β4 loops, and the other by the β2-β3 loop and a loop following β4. The ZBR displays an In-Between-RING (IBR) domain topology, aligning with the IBR of RNF31 with 5.5 Å r.m.s. deviation.

The NMR structure enabled identification of a functionally important surface on the ZBR through alanine-scanning mutagenesis of EMI1D^DLZT. Under conditions in which APC/C^CDH1 is insensitive to a version of EMI1 lacking the ZBR and tail (EMI1^NDFTD) but is inhibited by wild-type EMI1D^DLZT, several mutants impaired EMI1D^DLZT-mediated inhibition of APC/C^CDH1 ubiquitination with either UBCH10 or UBE2S (not shown) (Fig. 5c,d). The majority of defective mutants (L375A, K376A, R380A N382A, K386 D388A, Y387A, P384A) mapped to a single surface (Fig. 5e,f). The diminished inhibition caused by the three most deleterious individual alanine mutants—at positions Leu375, Lys376 and Arg393—reminded that caused by unfolding the ZBR through mutation of the Cys409 zinc ligand (Fig. 5d). Although we found the R393A mutant to also be unfolded, NMR analysis of 15N-labeled samples confirmed folding of the L375A and K376A mutants (Supplementary Fig. 7), suggesting that the ZBR is not solely a structured tether between the linker and tail. Rather, the surface harboring these residues is important for inhibition. Leu375 and Lys376 are at the N terminus of the ZBR domain, suggesting spatial proximity between the inhibitory ZBR surface and the upstream linker sequence in EMI1.

### ZBR and C-terminal tail synergize to block chain elongation

A distinctive pattern of inhibition was observed for the EMI1 fragment consisting only of the ZBR and tail, which on its own inhibited APC/C^CDH1–UBE2S–mediated ubiquitination of UbcycB-NT*, although relatively weakly, with a K_{iapp} of 520 nM (Table 1). The ZBR and tail synergize, as we could not measure inhibition with the isolated ZBR at the highest concentration we could achieve, and the K_{iapp} increased to 42 µM for a synthetic peptide corresponding to the isolated EMI1 (Table 1). We did not observe obvious effects of EMI1ZT on the initial rate of Ub ligation by UBCH10 (Table 1).

Because the EM data indicated that the EMI1ZT region interacts with the APC/C catalytic core and/or platform regions (Fig. 4a), we tested whether EMI1ZT inhibits APC/C^CDH1–UBE2S–mediated Ub ligation independently of counteracting substrates recruited to the distal D-box binding site. Even though UBE2S binds coactivator proteins and generates Ub chains even in the absence of an E3 (ref. 44), we identified conditions in which APC/C stimulated UBE2S-mediated ubiquitination of UbcycB-NT* even in the absence of CDH1 (Fig. 3b and Fig. 6a). Although the K_{iapp} for full-length EMI1 increased substantially, presumably due to lack of its D-box recruitment by CDH1, the isolated EMI1 increased APC/C–UBE2S–mediated ubiquitination of UbcycB-NT* equally well in the presence or absence of CDH1 (Table 1 and Fig. 6a,b).

### Table 2 NMR and refinement statistics

| Parameter | Value | r.m.s. deviation (Å) | Hydrogen bonds | Percentage violations |
|-----------|-------|----------------------|----------------|-----------------------|
| Distance constraints (Å) | 0.0093 ± 0.001 | 0.1833 ± 0.061 | 0.8600 ± 0.300 | 0.2100 ± 0.060 |
| Deviations from ideal geometry | Bond lengths (Å) | 0.0 | Bond angles (°) | 0.0 | Improper (°) | 0.015 |
| | Average pairwise r.m.s. deviation (Å) | 0.554 | Backbone | 0.954 |

*Backbone and heavy atom r.m.s. deviations were calculated by superimposing residues 375–420 of the 20 lowest-energy conformers of EMI1ZT.
The indifference to CDH1 implied that the isolated EMIT targets a catalytic function of APC/C–UBE2S independently of blocking APC/C binding to a D-box substrate. We developed an assay for APC/C- and UBE2S-dependent synthesis of di-Ub chains, using as a substrate a Ub variant labeled with fluorescein on a C-terminal cysteine (Fig. 6c,d). Full-length EMII remained a superior inhibitor, with an order-of-magnitude lower $K_i$ than that measured for EMII$^T$ even for this substrate-independent Ub chain formation, suggesting that the structurally observed multisite APC/C binding enhances EMII inhibition of catalysis. Full-length EMII and EMII$^{DLZT}$ displayed similar degrees of inhibition, consistent with the notion that the D-box is recruited to CDH1. Inhibitory effects of EMII$^T$, EMII$^Z$ and EMII$^T$ roughly paralleled those observed in CDH1-dependent ubiquitination of UbcycB-NT$^*$ (Table 1). Thus, EMII$^T$ inhibits APC/C–UBE2S-catalyzed Ub-chain formation. Furthermore, this appears to be the case even in assays containing both UBE2S and UBC10H, as upon adding high concentrations of the shorter EMII$^Z$, we observed a selective decrease in the HMW products corresponding to those generated when UBE2S was included in the reactions (Fig. 6e). Comparing EMII and UBE2S sequences provides a rationale for the E2 selectivity, as the conserved residues in their C-terminal sequences are identical (Fig. 6f). Previous studies showed that the unique UBE2S C-terminal sequence is essential for APC/C recruitment$^{34,35}$.
Alternatively, a key EMI1 function may be to block Ub-chain elongation. UBE2S is apparently required for normal mitosis only in a subset of cell types but in other cells may be important for recovery from the spindle checkpoint. UBE2S-mediated Ub-chain elongation is thought to be particularly important for substrates with few lysines serving as sites of initial Ub ligation and may also be important for substrates whose turnover is regulated by deubiquitinating enzymes. Thus, by extension, such substrates might also be especially sensitive to regulation by EMI1.

Notably, we found that EMI1’s APC/C inhibitory domain is substantially natively disordered, thus defining a distinct structural class of E3 inhibitor and differing not only from the crystallographically characterized MCC but also from several other RING E3 inhibitors. Intrinsically disordered enables a small sequence to have multiple discrete interaction motifs that mediate binding to multiple sites. Consistent with prior studies, we detected inhibition by isolated D-box and EMI1 C-terminal tail peptides. Our data also indicate that the linker and ZBR do not simply serve as spacers between the EMI1 D-box and C-terminal tail. We defined, for the first time, the linker as an effector of EMI1 function and identified surfaces on the linker and ZBR important for inhibiting APC/C. Each element is weak on its own, and the combination of the multiple motifs is required for maximal inhibition.

The structural data provide a rationale for many previously described features of EMI1 regulation. In addition to inhibition by the C-terminal domain, EMI1 also plays a role in localizing a fraction of APC/C to spindle poles. EM structures reveal that EMI1’s N-terminal domain, which binds NuMA–dynein–dynein to mediate localization, is exposed on the surface and available for interaction without disrupting contacts anchoring the C-terminal domain to APC/C.”

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. APC/C<sup>CDH1</sup>–EMI1–SKP1, EMD-2354; APC/C<sup>CDH1</sup>–EMI1<sup>DLZT</sup>, EMD-2353; EMI1<sup>ZT</sup>, 2M6N.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.-M.P., H.S. and B.A.S. planned and supervised the project. J.J.F., N.G.B., G.P., E.R.W., C.R.R.G., A.N. and M.A.J. designed the experiments. G.P. prepared samples for and contributed to EM experiments. J.J.F., N.G.B. and E.R.W. performed biochemical and biophysical analyses. A.N. performed analytical ultracentrifugation. C.R.R.G. and R.W.K. performed NM reader input with input from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Protein purification. EMI1 variants correspond to: EMI1, residues 1–363; EMI1NFDL, residues 236–447; EMI1DZT, residues 305–447; EMI1ZT, residues 363–447; EMI1NDL, residues 1–432. EMI1, ubiquitin variants, cycb-NT* and Ubycb-NT* were expressed as GST fusions, either from pGEX4T1 or pFlashbacGST modified to contain a TEV proteolytic site, and were purified by glutathione affinity chromatography, followed by His-TEV treatment. EMI1 and EMI1NFDL were expressed in High Five cells. All other EMI1 variants were expressed in Escherichia coli BL21-Gold cells. All EMI1 variants containing a F-box were co-purified with SKP1. EMI1 wild-type and its variants were purified by ion exchange chromatography before sizing. GST-cycb-NT* and Ubycb-NT* were expressed in High Five cells treated with His-TEV, with GST and His-TEV removal by glutathione and nickel affinity chromatography. After sizing, cycb-NT* and Ubycb-NT* were FPLA labeled using a C-terminal tetra-cysteine site. Other proteins were described previously37.

APC/CDH1-EMI1 purification for electron microscopy. HeLa cells were grown in DMEM including 10% FBS (Invitrogen), 2 mM l-glutamine and 100 µg ml−1 penicillin/streptomycin (both from Sigma) and plated on 245 × 245 cm tissue culture dishes (NUNC). Cell extracts were prepared by lysis of frozen log-phase HeLa cells in extract buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 0.1% (w/v) octyl-β-d-glucopyranoside) using a Dounce homogenizer followed by centrifugation. APC/C was immunoprecipitated from the soluble fraction by incubation with an APC3 polyclonal antibody (produced in rabbit) cross-linked to Affi-prep protein A beads from Bio-Rad at 1.33 mg per 1 µl beads38. Beads were washed four times (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) octyl-β-d-glucopyranoside), and the APC/CDH1-EMI1 complexes were reconstituted in vitro by mixing 3.5 µM PreScission-cleaved CDH1 and 150 nM EMI1–SKP1 with APC/C-bound APC3 antibody beads in binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) octyl-β-d-glucopyranoside, 4 mg ml−1 BSA). After a 1-h incubation, the beads were washed four times, and APC/C complexes were recovered by elution with antigenic peptides. APC/CDH1-FlagEMI1 complexes were enriched by re-immunoprecipitation experiments using anti-Flag M2 Agarose (Sigma) and subsequent elution with antigenic peptides. APC/C specimens were subjected to GraFix to further purify and stabilize the complexes.

Electron microscopy. Purified APC/CDH1-EMI1 complexes were adsorbed to a thin film of carbon and then transferred to an electron microscopic grid covered with a perforated carbon film. The bound APC/C particles were stained with 2% (w/v) uranyl formate, blotted and air-dried for ~1 min at room temperature. Images were recorded at a magnification of 119,000× or 155,000× on a 4×4 CCD camera (TVIPS GmbH) using two-fold pixel binning (2.5 Å or 1.8 Å per pixel) in a Philips CM200 FEG electron microscope (Philips/FEI) operated at 160 kV acceleration voltage. APC/CDH1-EMI1 complexes were analyzed as described14.

Generation of recombinant human APC/C. Recombinant human APC/C was purified as described, using a C-terminal 2×Strep tag on APC6 and an N-terminal GST tag on APC4 with the following modifications37. Multibacs were constructed using donor plasmids pFL, pIDC, pIDK and pIDS43, except the p10 baculoviral promoter was replaced with the polyhedron promoter and an additional polyadenylation site (SV40). Three Multibacs were generated by Cre recombination of (i) pIDS CDC26/APC6-2×Strep-tagII, pFL APC11/APC2, pIDK APC10/APCS and pIDC APC16/APC13; (ii) pIDC APC15/APC7 and pIDK APC8; and (iii) pFb APC1 with a loxP site from pIDS and pIDS APC3. Cre recombination of (i) pIDS CDC26/APC6-2xStrep-tagII, pFL APC11/APC2, pIDK APC10/APCS and pIDC APC16/APC13; (ii) pIDC APC15/APC7 and pIDK APC8; and (iii) pFb APC1 with a loxP site from pIDS and pIDS APC3. pIDS CDC26/APC6-2xStrep-tagII, pFL APC11/APC2, pIDK APC10/APCS and pIDC APC16/APC13; (ii) pIDC APC15/APC7 and pIDK APC8; and (iii) pFb APC1 with a loxP site from pIDS and pIDS APC3. Cre recombination of (i) pIDS CDC26/APC6-2xStrep-tagII, pFL APC11/APC2, pIDK APC10/APCS and pIDC APC16/APC13; (ii) pIDC APC15/APC7 and pIDK APC8; and (iii) pFb APC1 with a loxP site from pIDS and pIDS APC3. Cre recombination of (i) pIDS CDC26/APC6-2xStrep-tagII, pFL APC11/APC2, pIDK APC10/APCS and pIDC APC16/APC13; (ii) pIDC APC15/APC7 and pIDK APC8; and (iii) pFb APC1 with a loxP site from pIDS and pIDS APC3. Cre recombination of (i) pIDS CDC26/APC6-2xStrep-tagII, pFL APC11/APC2, pIDK APC10/APCS and pIDC APC16/APC13; (ii) pIDC APC15/APC7 and pIDK APC8; and (iii) pFb APC1 with a loxP site from pIDS and pIDS APC3.
and 32 hydrogen bond restraints based on exchange cross-peaks with water in the
$^{15}$N-NOESY spectrum were used in the structure calculation of EMI1$^{ZT}$ (Table 2).
Seven iterations of refinement for 100 structures per cycle were completed after proper distance calibrations. After the initial fold of the protein was determined, a CYANA amino acid library using a modified zinc-ligated cysteine residue was used to incorporate the two zinc ions into the structures.

*Generation of metal-free EMI1$^{ZT}$ and zinc reconstitution*. $^{15}$N-labeled EMI1$^{ZT}$ was incubated in 10 mM sodium phosphate (pH 6.5), 50 mM NaCl, 1 mM DTT and 20 mM EDTA and then dialyzed against 10 mM sodium phosphate (pH 6.5), 50 mM NaCl, 1 mM DTT. Unfolding was confirmed by measuring one- and two-dimensional $^1$H-$^{15}$N HSQC spectra post-dialysis. Six rounds of dialysis resulted in removal of EDTA. Dialysis against 10 mM sodium phosphate (pH 6.5), 50 mM NaCl, 1 mM DTT, 200 µM ZnSO$_4$ restored zinc.

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