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Pseudo-DUBs as allosteric activators and molecular scaffolds of protein complexes

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Abstract

The ubiquitin (Ub) proteasome system and ubiquitin signalling networks are crucial to cell biology and disease development. Deubiquitylases (DUBs) control cell signalling by removing mono-Ub and poly-Ub chains from substrates. DUBs take part in almost all processes that regulate cellular life and are frequently dysregulated in disease. We have catalogued 99 currently known DUBs in the human genome and sequence conservation analyses of catalytic residues suggest that 11 lack enzyme activity and are classed as pseudo-DUBs. These pseudoenzymes play important biological roles by allosterically activating catalytically competent DUBs as well as other active enzymes. Additionally, pseudoenzymes act as assembly scaffolds of macromolecular complexes. We discuss how pseudo-DUBs have lost their catalytic activity, their diverse mechanisms of action, and their potential as therapeutic targets. Many known pseudo-DUBs play crucial roles in cell biology and it is likely that unstudied and overlooked pseudo-DUB genes will have equally important functions.

Introduction

Ubiquitylation of proteins serves as a post-translational signal to regulate virtually all cellular processes through the precise spatial and temporal control of protein stability, activity and/or localisation [1]. Enzymes involved in the ubiquitin system are frequently dysregulated in cancer, neurodegeneration, autoimmunity and other human diseases [2-5]. Ubiquitylation is a versatile post-translational modification aptly suited for a cellular communication system, with the essential "*writer, reader* and *eraser"* core signalling modules (**Fig. 1**).

Ubiquitin processing enzymes (E1, E2 and E3) write the ubiquitin signalling code by adding ubiquitin to substrates *(writer module)*. A single ubiquitin (Ub) can be conjugated to lysine residues on the surface of substrate proteins (this is known as mono-ubiquitylation), or conjugated further to lysine residues on the surface of Ub itself, thus leading to poly-Ub chains. Seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) and the free amino terminus (M1) on Ub can be targeted for poly-ubiquitylation, and thus eight different Ub chain types can be generated in total, all having different topologies and unique signalling properties [3]. Ubiquitin chains can be "read out" by ubiquitin binding domains (UBDs), which allow signal decoding and transmission *(reader module)*. Ubiquitylation is a reversible process, and removal of Ub is carried out by deubiquitylating (DUB) enzymes *(eraser module)*. DUB actions allow the cell to produce monomeric Ub, recycle Ub from chains and reverse signalling events resulting from ubiquitylation [6,7]. To date, 99 DUBs have been identified in the human genome, comprising six major structural families, namely (1) the ubiquitin-specific proteases (USPs), (2) the ubiquitin C-terminal hydrolyses (UCHs), (3) the Machado-Joseph domain proteases (Josephins), (4) the ovarian tumour proteases (OTUs), (5) the Jab1/MPN/Mov34 metalloproteases (JAMM/MPNs) and (6) the newly discovered MIU-containing novel DUB family proteases (MINDYs) (**Fig. 2**) [8,9].

The JAMM/MPN family is unique in using zinc during catalysis, whereas all other DUBs are cysteine proteases. Cysteine-dependent DUBs contain a common catalytic triad with a cysteine, a histidine and an acidic residue, although their structural folds differ markedly from one another [6,10]. Nevertheless, four of the DUB families (USPs, UCHs, Josephins and OTUs) can be assigned to the papain-like structural fold, according to Pfam and SCOP databases. Representative structures for each DUB family have been reported and

our understanding of how the families function as proteases has greatly improved in recent years [10]. However, fundamental questions remain as to how DUB function is regulated (i.e. switched on and off) and how substrate specificity is controlled. For example, while some DUBs display exquisite selectivity for specific ubiquitin chain types *in vitro* and *in vivo*, others appear highly promiscuous, at least *in vitro*. Similarly, the catalytic and non-catalytic functions of some DUBs are highly regulated, while others appear constitutively active or permanently repressed (e.g. pseudo-DUBs). This variability of function is underpinned by diverse regulatory molecular mechanisms, even within the same structural family. As such, DUBs represent a fascinating family of enzymes that require a close inspection of mechanism of action as well as biological function.

Overview of pseudoenzymes and pseudo-DUBs

The past 10-15 years have seen a surge in studies showing how pseudoenzymes (enzymes predicted to lack catalytic activity) employ innovative and unexpected mechanisms to regulate biological functions. Pseudoenzymes perform these important roles by allosterically activating catalytically competent enzymes, acting as crucial scaffolds and as molecular switches. The best studied pseudoenzymes are pseudokinases, which have been covered in numerous reviews [11-15]. Around 50 proteins containing the kinase fold have been classed as pseudokinases, representing approximately 10% of the human kinome [16]. While some predicted pseudokinases have shown vestigial kinase activity *in vitro*, the majority are classed as true pseudokinases with no catalytic activity, and perform important biological roles as pseudoenzymes. It is now clear that almost every enzyme family contains some inactive “rogue” members. The precise fraction may vary depending on the enzyme class, but in the relatively small number of families studied, approximately 5-15% of proteins are inactive pseudoenzymes [17-20]. This is true for pseudokinases, pseudoproteases, pseudo-E2 ubiquitin ligases and pseudo-DUBs [21-23].

The human genome contains 99 known DUBs (**Fig. 2**) and this number is likely to increase as new members and families are discovered [9]. Of the 99 known DUB proteins, 11 are predicted to be catalytically inactive pseudoenzymes and classed as pseudo-DUBs. Interestingly, the number of pseudo-DUBs differs widely for each family, and two families, namely UCH and Josephins, have no pseudo-DUBs. The JAMM/MPN family

contains the largest number (seven pseudo-DUBs), followed by the USP family with two inactive pseudo-DUBs, whilst the OTU and MINDY families each contain one pseudo-DUB (**Fig. 2**). Despite the lack of catalytic residues, pseudo-DUBs perform regulatory roles in many biological functions, such as gene expression and regulation [24-26], DNA damage repair [27-29], protein turnover [30,31], cytokine signalling and immune responses [32].

DUB–pseudo-DUB heterodimers

The JAMM/MPN family of DUBs contains 14 members in humans. They share a common metalloprotease domain architecture, denoted the MPN domain, harbouring a zinc chelating active site [33-35]. Seven out of the 14 family members are predicted to be catalytically active (denoted MPN^+), while the others, which bear substitutions to essential catalytic residues, are predicted to be catalytically dead (denoted MPN^-). MPN family proteases typically function within larger multi-subunit complexes, which imparts great potential for multi-layer regulation.

The MPN family DUBs regulate diverse aspects of cellular biology, most notably DNA repair through BRCC36 (MPN^+) in the BRCA1-A complex (BRCC36-ABRAXAS1-BRCC45-MERIT40-RAP80 complex) [29,36-38], interferon receptor signalling through BRCC36 (MPN^+) in the BRISC-SHMT2 complex (BRCC36-ABRAXAS2-BRCC45-MERIT40 complex) [32,39] (**Fig. 3**), and protein degradation through Rpn11/PSMD14 (MPN^+) in the proteasome lid complex [40]. The CSN5-CSN6 MPN^+–MPN^- pair is part of the Cop9 signallosome (CSN) complex that deneddylates cullin proteins modified with the ubiquitin-like (UBL) small protein NEDD8. Thus, CSN5 is a deneddylase rather than a DUB.

Interestingly, many of the MPN^- pseudo-DUBs directly interact with active MPN^+ domains to form stable heterodimers (**Fig. 4**). In a remarkable analogy to kinase-pseudokinase heterodimers where a pseudokinase allosterically activates a kinase [12,41,42], some pseudo-DUBs also allosterically activate DUBs (**Fig. 4A and 5A**).

BRCC36-ABRAXAS1 and BRCC36-ABRAXAS2 (BRCC36-KIAA0157)

The active DUB BRCC36 (MPN^+) is found in two macromolecular complexes in the cell (**Fig. 3**), depending on which partnering MPN^- pseudo-DUB it interacts with (ABRAXAS1

or ABRAXAS2). The BRCC36-ABRAXAS1 complex translocates to the nucleus via the ABRAXAS1 nuclear localisation signal (NLS) and is part of a larger DUB complex called the BRCA1-A complex. This complex localises to sites of DNA damage (e.g. a double strand break) through RAP80 anchoring to K63-linked poly-Ub chains (**Fig. 3**). Therefore, the BRCA1-A complex plays at least two roles that ultimately lead to DNA damage repair: (1) binding and cleaving K63-linked polyubiquitin chains (poly-Ub) and (2) recruitment of BRCA1 to sites of DNA damage.

In the cytoplasm, BRCC36 interacts with ABRAXAS2 (a.k.a. KIAA0157) and is part of a larger complex called BRISC-SHMT2 (**Fig. 3**). This cytoplasmic DUB complex interacts with and deubiquitylates interferon receptors 1 and 2 (IFNAR1/2) and prevents the receptors from being prematurely endocytosed and entering the lysosomal degradation pathway. Thus, the BRISC-SHMT2 complex regulates interferon-dependent immune response by stabilising the interferon receptors and ensuring their availability at the membrane [32].

BRISC deficiency in mice due to genetic deletion of the BRISC specific pseudo-DUB ABRAXAS2 resulted in resistance to bacterial lipopolysaccharide [32]. Interestingly, the mice did not display any adverse phenotypes or deficiency in the DNA damage response, raising the possibility that inhibitors of pseudo-DUB function in the BRISC may have clinical utility against disease processes stemming from elevated inflammatory cytokine signals.

Biochemical studies demonstrated that BRCC36 is inactive in isolation and that heterodimerisation with the pseudo-DUB ABRAXAS2 was required for DUB activity [43-45]. The crystal structure and biochemical studies of BRCC36-ABRAXAS2 (a.k.a. BRCC36-KIAA0157) provided insights into the mechanism of BRCC36 activation [45]. BRCC36 forms an obligate heterodimer with ABRAXAS2 and interactions are mediated via MPN domains and C-terminal helices from both proteins. The C-terminal helical bundle participates in higher-order interactions to form a dimer of heterodimers (super dimer), which constitutes the minimally active assembly capable of cleaving Lys63-linked poly-Ub chain substrates **(Fig. 4A and 5A)**. A forced BRCC36-BRCC36 homodimer was also observed by X-ray crystallography, however the BRCC36 active site residues in this

crystal structure were not optimally positioned for catalysis (**Fig. 5A**). Instead, BRCC36 requires direct interactions with ABRAXAS2 to attain an active conformation (**Fig. 5A**) [45].

The JAMM motif is typical of MPN domain containing proteases and is required for catalysis. It contains five conserved residues from four key structural motifs, namely:
1) E-loop, which houses a glutamic acid residue (E30, **Fig. 4A**). Its role is to coordinate a catalytic water molecule.
2) H-strand, which houses the His-X-His motif with two histidine residues (H94 and H96, **Fig. 4A**) involved in Zn^{2+} binding.
3) S-loop, which houses a serine residue (S104, **Fig. 4A**) involved in stabilising the transition state intermediate.
4) D-helix, which houses an aspartic acid residue (D107, **Fig. 4A**) involved in Zn^{2+} binding.

ABRAXAS1 and 2 both have several changes in the JAMM motif that preclude function as an active DUB, with changes in the E-loop, H-strand and D-helix (**Supp. Fig. 1**). The structure of BRCC36-ABRAXAS2 complex revealed that ABRAXAS2 is incapable of interacting with Zn^{2+} and is therefore a pseudo-DUB [45]. Structural comparison of active BRCC36 with the pseudo-DUB ABRAXAS2 revealed that while there are non-conservative changes in the active site residues, the overall MPN fold and the position of the JAMM motif residues are the same for both active and inactive MPN domains (**Fig. 4A**). As a result, the MPN fold maintains its structural integrity, despite the loss of Zn^{2+} and several changes to conserved catalytic residues. Interestingly, the positive charge lost by the absence of the Zn^{2+} ion is compensated by a guanidinium group from Arg103 (ABRAXAS2) which replaces the Zn^{2+}-interacting His96 (BRCC36) residue (**Fig. 4A**).

The biochemical and structural data thus far support the notion that ABRAXAS2 and the related pseudo-DUB ABRAXAS1 serve as allosteric activators of BRCC36 and have a non-catalytic role as molecular scaffolds for the correct assembly of BRCA1-A and BRISC-SHMT2 complexes.

Rpn11-Rpn8 (PSMD14-PSMD7 in humans)

Yeast Rpn11 and Rpn8 (PSMD14 and PSMD7 respectively, in humans) are part of the 19S regulatory particle of the proteasome and play a key role in ubiquitin processing and

recycling. The MPN^+ domain protein Rpn11 removes poly-Ub chains from ubiquitylated substrates before they translocate to the 20S proteasome. Similar to BRCC36, Rpn11 partners with the pseudo-DUB Rpn8 (**Fig. 4B**), and is part of a larger macromolecular complex. Rpn8 supports the role of Rpn11 through direct interactions and is required for full Rpn11 catalytic activity [31,46].

The yeast pseudo-DUB Rpn8 contains three notable changes in catalytic residues: E-loop (Glu to Arg), H-strand (His-X-His motif changed to His-X-Gly) and S-loop (Ser to Arg). Changes in the H-X-H motif render Rpn8 incapable of binding the catalytic Zn^{2+} (**Fig. 4B**). Interestingly, the overall fold and integrity of the MPN domain remains unaffected by compensating interactions in the form of a salt bridge between Asp103 (D-loop) and Arg32 (E-loop), and Arg100 (S-loop) hydrogen bonding with a backbone carbonyl group from the E-loop.

Collectively, these active site residue changes abrogate Rpn8 catalytic activity. It is still unclear if Rpn8 allosterically activates Rpn11 or if its main role is to stabilise the Rpn11 fold within the multiprotein complex.

CSN5-CSN6

The COP9 signalosome (CSN complex) has eight subunits amounting to approximately 450 kDa [47]. CSN5, the active protease subunit, removes Nedd8 from cullins and thus regulates the activity of cullin RING E3 ligases [48,49]. CSN5 and CSN6 proteins are overexpressed in many cancers, and the Cop9 signalosome is implicated in Ub-mediated degradation of several cancer related genes: p27, p53, Mdm2, Smad7, Runx3, Id1, Skp2 and HIF1 [50]. Despite the fact that CSN5 is not specific for cleaving ubiquitin, CSN5 (MPN^+) and CSN6 (MPN^-) heterodimers are similar in topology to Rpn11-Rpn8 complexes (**Fig. 4B, C and 5B**) and represent a recurring theme in MPN domain heterodimerisation.

The pseudo-DUB CSN6 contains four notable changes in the E-loop (Glu to Gln), H-strand (His-X-His motif changed to Thr-X-Gly) and S-loop (Ser to Asn). Changes in the H-X-H motif render CSN6 incapable of binding the catalytic Zn^{2+} (**Fig. 4C**). Interestingly, the position of the D-helix remains unchanged by compensating interactions between Asp135 and Lys153 from a neighbouring β-strand. This ensures minimal disruption to the CSN6 MPN fold in the absence of the Zn^{2+} ion.

Collectively, these active site residue changes abrogate CSN6 catalytic activity. It is still unclear if CSN6 allosterically activates CSN5 or if its main role is to stabilise the CSN5 fold within the multiprotein complex.

Heterodimerisation of CSN5-CSN6, Rpn11-Rpn8 and eIF3f-eIF3h (discussed below) are topologically similar to each other, but differ from the heterodimer formed by BRCC36-ABRAXAS2 (**Fig. 4 left panels and Fig. 5**). This may be because BRCC36-ABRAXAS1/2 require further assembly into a dimer of heterodimers (super dimer), which represents the active form of the enzyme (**Fig. 4A and 5A**) [45]. This diverse mode of activation could be exploited to develop selective BRCC36 inhibitors by targeting the dimerisation and higher order super-dimerisation sites, instead of the common MPN^+ active site.

Pseudo-DUBs as scaffolding modules of large complexes

Pseudo-DUBs can also act as molecular scaffolds of many macromolecular complexes, which are central to cellular biology. Examples include the spliceosome, eukaryotic initiation factor 3 (eIF3) complex and the RNA deadenylating complex Pan2-Pan3 (**Fig. 6**).

Prp8 and USP39

Splicing of pre-mRNA is an essential process performed by the spliceosome complex and a crucial step in gene expression and protein translation. Correct processing of introns from pre-mRNAs requires a high degree of accuracy and the spliceosome machinery is highly regulated. The pseudoenzyme Prp8 is central to the assembly of this large multiprotein–nucleic acid complex (**Fig. 6A**). Prp8 is a large protein (human Prp8 has 2335 amino acids) and contains four different inactive pseudoenzyme domains: (1) a restriction endonuclease (RE) homology domain (pseudo-RE), (2) a reverse transcriptase (RT) homology domain (pseudo-RT), (3) an RNaseH homology domain (pseudo-RNAseH) and (4) an MPN^- domain (pseudo-DUB). Each of these domains has a role in binding to either a protein or nucleic acid component of the spliceosome.

The Prp8 MPN^- domain contains two active site residue changes, one in the E-loop (Glu changed to Gln) and the other in the H-strand (His-X-His motif changed to His-X-Gln). It was shown that the single His to Gln change in the H-X-H motif of yeast Prp8p is sufficient

to completely abolish Zn^{2+} binding [51,52] and potentially catalysis, however the Prp8 MPN^- domain retains the ability to interact with Ub [53]. It is as yet unclear if the Prp8 pseudo-DUB domain has other roles in the spliceosome beyond acting as a macromolecular scaffold.

Interestingly, the spliceosome complex also contains the pseudo-DUB USP39 (Sad1 in yeast) [54], although the role of this pseudoenzyme in the splicing reaction is not clear. Mutations in the active site of key catalytic residues (Cys to Asp and His to Ser) render USP39 inactive (**Supp. Fig. 2**). Similarly, yeast Sad1 is devoid of catalytic activity, although Sad1 is still capable of interacting with ubiquitin [55]. As with the Prp8 MPN^- domain the exact biological role of Sad1 ubiquitin interaction is not clear, although they could serve as readers of ubiquitin signalling by interacting with ubiquitin and/or ubiquitin chains.

eIF3f-eIF3h complex

eIF3f and eIF3h are MPN^- domain pseudo-DUBs that are part of the eukaryotic initiation factor 3 (eIF3) complex. Binding of initiation factors is required to initiate protein synthesis and recruit transfer and messenger RNAs to the 40S subunit before assembly of active ribosomes. eIF3 is involved in both ribosome biogenesis and protein synthesis [56]. The full eIF3 complex consists of 13 subunits and amounts to approximately 800 kDa [57]. Interestingly, eIF3 displays a significant degree of similarity with the CSN complex and both contain an MPN–MPN heterodimeric core. In the eIF3 complex, eIF3f and eIF3h are pseudoenzymes with their main role thought to be of a scaffolding nature (**Fig. 6B**). Both eIF3f and eIF3h are involved in direct binding to other members of the eIF3 complex and although they are devoid of catalytic activity, their scaffolding roles are essential [57,58].

The pseudo-DUB eIF3f contains five notable residue changes in the catalytic motifs: E-loop (Glu to Arg), H-strand (His-X-His motif changed to Ala-X-Gly), S-loop (Ser to Thr) and D-Helix (Asp to Ser). Changes in the H-X-H motif and D-helix render eIF3f incapable of binding the catalytic Zn^{2+} and therefore inactive.

The pseudo-DUB eIF3h also contains five notable residue changes in the catalytic motifs: E-loop (Glu to Val), H-strand (His-X-His motif changed to Gln-X-Thr), S-loop (Ser changed

to Thr) and D-Helix (Asp to Leu). Similar to eIF3f, changes in the H-X-H motif and D-helix render eIF3h incapable of binding the catalytic Zn^{2+} and inactive.

Taken together, active site amino acid substitutions in eIF3f and eIF3h, which collectively abrogate catalytic function, suggest their primary function is to serve as molecular scaffolds for assembly of the eIF3 complex.

USP52/Pan2

The yeast Pan2-Pan3 deadenylating complex takes part in shortening of poly(A) tails of mRNA [59]. The Pan2 orthologue in humans is the pseudo-DUB USP52 and many of the functions are evolutionarily conserved. USP52 was recently described as an important regulator of the HIF1A-mediated hypoxic response, by regulating Hif1A mRNA stability [24].

USP52/Pan2 lack the catalytic cysteine (Cys mutated to Ala) and histidine (His mutated to Ser) residues required for DUB activity and are classed as pseudo-DUBs. However, Pan2 contains a catalytically active RNAse domain fused to the pseudo-DUB domain (**Fig. 6C**). Interestingly, Pan3 contains a pseudokinase domain that dimerises, resulting in a Pan2-Pan3 complex with 1:2 stoichiometry (**Fig. 6C**). Dimerisation of the Pan3 pseudokinase and the interactions of the Pan2 pseudo-DUB domain are both required for RNAse activity and biological function [60,61]. Furthermore, it is possible that USP52 binds Ub and acts as a Ub binding domain and a reader of Ub signalling.

Predicted Pseudo-DUBs with unknown functions

FAM105A

FAM105A is an OTU domain containing protein that is closest to OTULIN (a.k.a. FAM105B or Gumby) (**Fig. 2**). The latter is an active DUB, which specifically cleaves Met1-linked linear Ub chains involved in cellular signalling [62,63]. FAM105A lacks the catalytic cysteine residue, which has been mutated to an Asp (**Supp. Fig. 3**). FAM105A is conserved as an inactive pseudo-DUB in vertebrate species, including mammals, birds, frogs and a turtle species (*Pelodiscus sinensis*), where the active site Cys is replaced by Asp or Ser (data not shown). Therefore, FAM105A is a predicted pseudo-DUB, the function of which remains to be identified. Interestingly, the *Drosophila* genome contains

two OTU domain pseudo-DUBs, although these are related to OTUD4 and involved in regulation of apoptosis [64]. Similar pseudo-OTUD4 proteins are found in the tunicate *Ciona* and in the brachiopod *Lingula*.

MINDY4B

The MINDY DUB family was only recently identified and contains four members, MINDY1-4 [9]. MINDY DUBs are active against K48-linked poly-Ub chains, displaying exquisite specificity [9]. Interestingly, a previously unannotated member (MINDY4B) is predicted to have the same fold, but lacks all three catalytic residues (**Supp. Fig. 4**). MINDY4B appears conserved as an inactive pseudo-DUB across vertebrates as well as in some invertebrates, for example in the tunicate *Ciona intestinalis*, in the aphid *Acyrthosiphon pisum* and in the crab *Limulus polyphemus*. The function of MINDY4B predicted pseudo-DUBs remains unknown.

Evolution of pseudo-DUBs

One striking observation is that 50% of JAMM/MPN DUBs are classed as pseudo-DUBs, leading to questions as to how these evolved. A possible explanation is that most JAMMs have a propensity to function as dimers with examples of JAMM/MPN functional dimers observed in many eukaryotes (e.g. yeast, ants, rodents, humans), as well as archaea [65]. It is possible that after gene duplication events, accumulation of mutations in active site residues of one protomer are tolerated, since dimerisation ensures at least 50% enzyme activity is always present. Over time, the supporting role as an allosteric activator or as a scaffolding protein may be more important than catalytic activity, thus leading to the evolution of pseudoenzymes. This would represent a gain in evolutionary terms, since repurposing an established fold may be more advantageous than creating a new one.

Pseudo-DUBs as drug targets

Given that many pseudo-DUBs take part in influencing major cellular functions, including activity of the proteasome (Rpn11-Rpn8), protein synthesis (Prp8 and USP52), Cop9 signallosome (CSN5-CSN6), DNA damage repair (ABRAXAS1) and interferon signalling (ABRAXAS2), they present an opportunity for therapeutic intervention in disease conditions. Developing inhibitors of active DUBs represents challenges, since compounds that target JAMM domain DUBs have so far only targeted the active site Zn^{2+} moiety [66,67],

rendering them relatively non-specific. Similarly, many compounds that target other DUB families react with the active site cysteine [4,68]. Therefore, developing small molecule inhibitors against pseudo-DUBs by exploiting their allosteric mode of action rather than their active site, may be a more selective way of inhibiting active DUB complexes such as Rpn11-Rpn8, CSN5-CSN6 and BRCC36-ABRAXAS1/2.

Concluding remarks

The DUB families, like most other enzyme families, contain a fraction of pseudoenzyme members that lack catalytic activity. These pseudo-DUBs have important biological functions involved in cell signalling, protein turnover and Ub recycling. Despite their lack of catalytic activity, they have evolved different ways to influence cell signalling and cell biology. One key observation is the various mechanisms of action that pseudo-DUBs employ, in many cases still influencing ubiquitin signalling systems by partnering with active DUBs. Pseudo-DUBs are often found as part of large macromolecular complexes where they act as allosteric activators of other enzymes, but also as molecular scaffolds required for complex assembly.

While the roles of most pseudo-DUBs are known, many questions still remain. For instance, what is the functional relevance of Prp8 and USP39/Sad1 interaction with ubiquitin? Do other pseudo-DUBs retain ubiquitin binding ability and ubiquitin chain selectivity, thereby acting as readers of ubiquitin signalling? Pseudo-DUBs that interact with mono-ubiquitin or poly-Ub chains may still have the same effect as active DUBs through a *"substrate trapping"* mechanism. For instance, recent studies show that alanine substitutions of active site residues can convert DUBs into avid ubiquitin-binding domains [69]**.** By interacting with Ub or poly-Ub, pseudo-DUBs can sterically hinder binding of other proteins that act as readers of ubiquitin signals, thus reversing the signal in the same way that an active DUB can by removing Ub.

Finally, some pseudo-DUBs like FAM105A and MINDY4B remain unstudied with no assigned functional roles, despite their evolutionary conservation. As new DUBs continue to be discovered, the pseudo-DUB field is also likely to see additional new members. These have the potential to reveal new mechanisms of action of how pseudoenzymes work as well as important functional and biological insights.

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FIGURE LEGENDS

Figure 1. Overview of ubiquitin signalling
Ubiquitin signalling can employ the *writer*, *reader* and *eraser* modules as communication tools in a signalling network.

Figure 2. Catalogue of known human DUBs and pseudo-DUBs
Known DUB families are shown as phylogenetic trees (PhyML) [70] with pseudo-DUBs highlighted in red. USP17 is a highly polymorphic gene with around 30 copies as a result of a megasatellite repeat in chromosomes four and eight. Since these genes are almost identical, they are represented by USP17-L2. USP, ubiquitin-specific protease; OTU, ovarian tumour protease; JAMM, JAB1/MPN/MOV34 (MPN, Mpr1/Pad1 N-terminal); UCH, ubiquitin C-terminal hydrolase; Josephin (a.k.a. Machado-Joseph disease proteases); MINDY, MIU-containing novel DUB family. Gene symbols from the HUGO Gene Nomenclature Committee database (http://www.genenames.org/) are shown where possible. Trees were visualized using the Interactive Tree of Life (iTOL v3) tool accessible online at: https://itol.embl.de).

Figure 3. BRCC36 functions as part of two distinct protein complexes
Illustration of the BRCC36 deubiquitylase (DUB) functions. Cytoplasmic BRCC36 regulates interferon receptor signalling as part of the BRISC complex. Nuclear BRCC36 takes part in DNA damage repair as the active DUB in the BRCA1-A complex. Pseudo-DUBs ABRAXAS1 and ABRAXAS2 are the main determinants for BRCC36 complex formation and subcellular localisation.

Figure 4. Heterodimerisation of DUBs with inactive pseudo-DUBs
Cartoon representation of active DUBs (blue) in complex with inactive pseudo-DUBs (pink).
Left panels: Comparison of MPN^{+}–MPN^{-} modes of heterodimerisation between **(A)** BRCC36-ABRAXAS2(KIAA0157) (PDBid 5CW3), **(B)** Rpn11-Rpn8 (PDBid 4O8X) and (C) CSN5-CSN6 (PDBid 4D10).
Right panels: Zoom in view of the superimposed MPN^{+} active site regions with the same region of MPN^{-} counterparts. Amino acid side chains are shown as sticks, except glycine residues which are depicted as spheres. Atom colours are as follows: nitrogen = blue, oxygen = red, sulphur = yellow.

Figure 5. Mechanism of MPN domain heterodimerisation
A) Cartoon representation of BRCC36 allosteric activation by ABRAXAS2. In the absence of ABRAXAS2, BRCC36 exists in a forced homodimer (left panel) with a disordered active site (depicted as dashed lines) and unstructured C-terminus (not shown). Binding of ABRAXAS2 orders the active site regions and ensures catalytic residues are in optimal position. ABRAXAS2 C-terminus interacts with BRCC36 C-terminus leading to a helical bundle which causes the formation of an active super-dimer.

B) Cartoon representation of various MPN domain heterodimerisation. Rpn11-Rpn8, CSN5-CSN6 and eIF3f-eIF3h heterodimers are similar to each other but differ from the BRCC36-ABRAXAS2 heterodimer and are not known to form higher order super-dimers.

Figure 6. Pseudo-DUBs as molecular scaffolds

A) *S. cerevisiae* spliceosome structure (U4/U6.U5 tri-snRNP complex; PDBid 5GAN). Prp8 is represented as ribbons and transparent surface. Prp8 contains four pseudoenzyme domains: MPN^{-} domain (pseudo-DUB), restriction endonuclease (RE) homology domain (pseudo-RE); reverse transcriptase (RT) homology domain (pseudo-RT) and RNaseH homology domain (pseudo-RNAseH).
B) Structure of eIF3 complex (PDBid 5A5T). Pseudo-DUBs eIF3f and eIF3h contain an MPN^{-} domain and are represented as ribbons and transparent surface.
C) Structure of Pan2-Pan3 deadenylase complex (PDBid 4XR7). Pan2 contains a UCH-like domain as a pseudo-DUB and is represented as ribbons and transparent surface. The pseudokinase Pan3 forms a homodimer.

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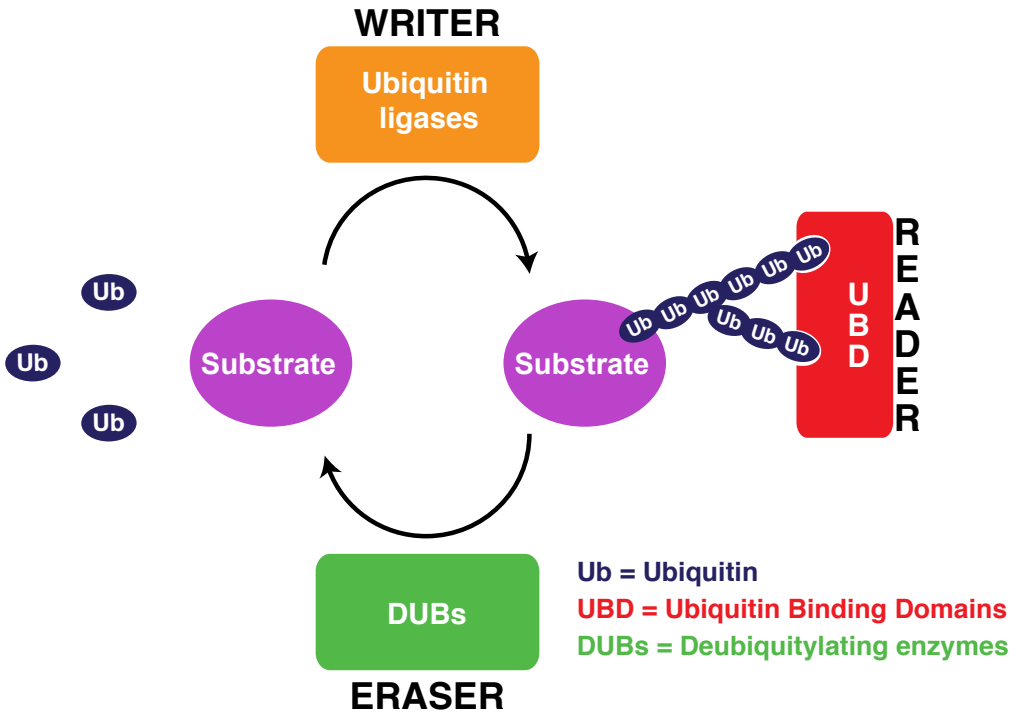
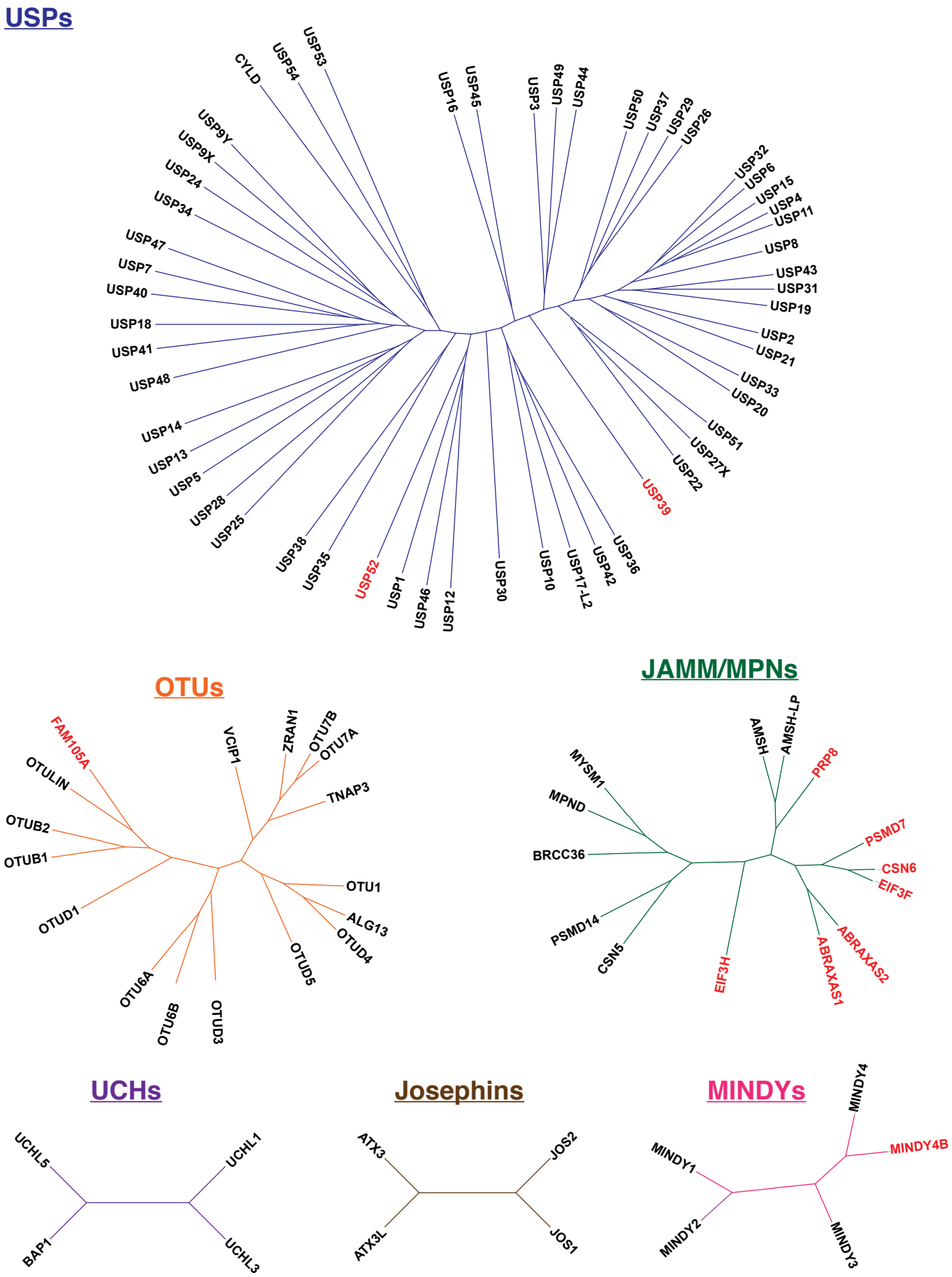

WRITER
Ubiquitin ligases
Ub
Ub
Ub
Substrate
Substrate
Ub Ub Ub Ub Ub Ub Ub Ub Ub
UBD
READER
DUBs
ERASER
Ub = Ubiquitin
UBD = Ubiquitin Binding Domains
DUBs = Deubiquitylating enzymes

FIGURE 1


USPs
USP53
USP54
CYLD
USP9Y
USP9X
USP24
USP34
USP47
USP7
USP40
USP18
USP41
USP48
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USP5
USP28
USP25
USP38
USP35
USP52
USP1
USP46
USP12
USP30
USP10
USP17-L2
USP42
USP36
USP39
USP22
USP27X
USP51
USP20
USP33
USP21
USP2
USP19
USP31
USP43
USP8
USP11
USP4
USP15
USP6
USP32
USP26
USP29
USP37
USP50
USP44
USP49
USP3
USP45
USP16
OTUs
FAM105A
OTULIN
OTUB2
OTUB1
OTUD1
OTU6A
OTU6B
OTUD3
OTUD5
OTUD4
ALG13
OTU1
TNAP3
OTU7A
OTU7B
ZRAN1
VCIP1
JAMM/MPNs
MYSM1
MPND
BRCC36
PSMD14
CSN5
EIF3H
ABRAXAS1
ABRAXAS2
EIF3F
CSN6
PSMD7
PRP8
AMSH-LP
AMSH
UCHs
UCHL5
BAP1
UCHL3
UCHL1
Josephins
ATX3
ATX3L
JOS1
JOS2
MINDYs
MINDY1
MINDY2
MINDY3
MINDY4B
MINDY4

FIGURE 2

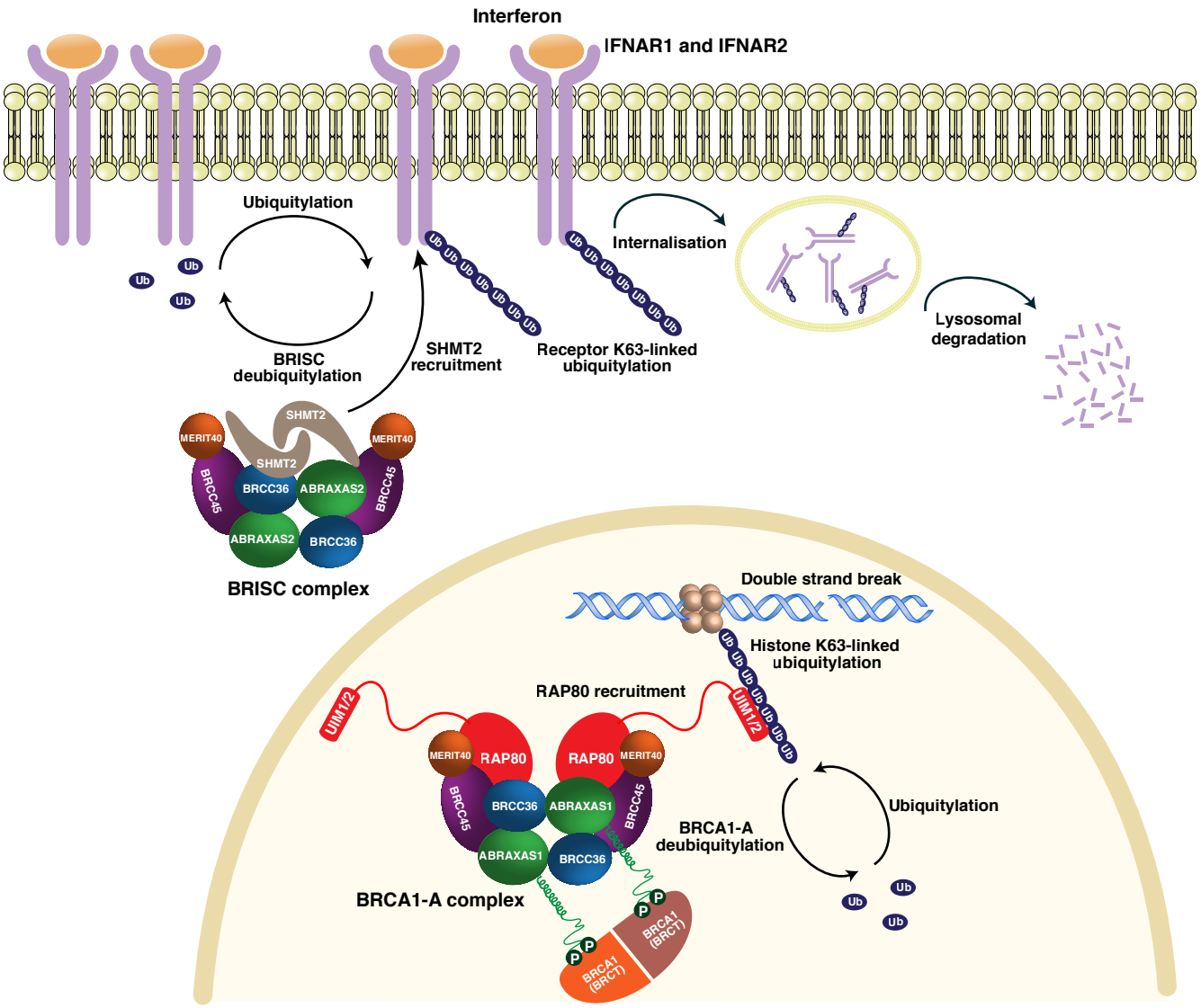

Interferon
IFNAR1 and IFNAR2
Ubiquitylation
Ub
Ub
BRISC
deubiquitylation
SHMT2
recruitment
Receptor K63-linked
ubiquitylation
Internalisation
Lysosomal
degradation
SHMT2
SHMT2
MERIT40
MERIT40
BRCC45
BRCC45
BRCC36
ABRAXAS2
ABRAXAS2
BRCC36
BRISC complex
Double strand break
Histone K63-linked
ubiquitylation
RAP80 recruitment
UIM1/2
UIM1/2
MERIT40
RAP80
RAP80
MERIT40
BRCC45
BRCC36
ABRAXAS1
BRCC45
ABRAXAS1
BRCC36
BRCA1-A
deubiquitylation
Ubiquitylation
BRCA1-A complex
P
P
P
P
BRCA1
(BRCT)
BRCA1
(BRCT)

FIGURE 3

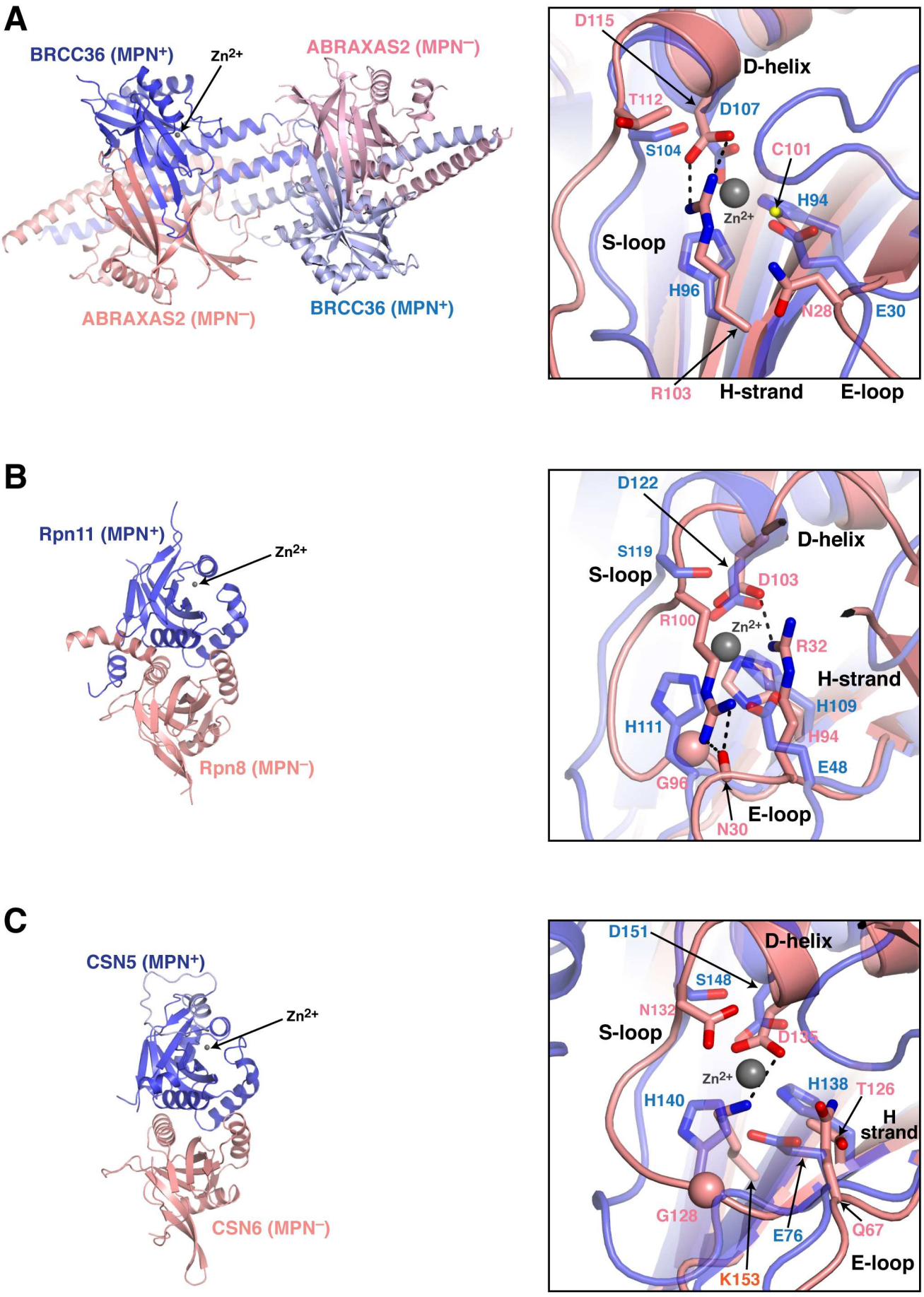
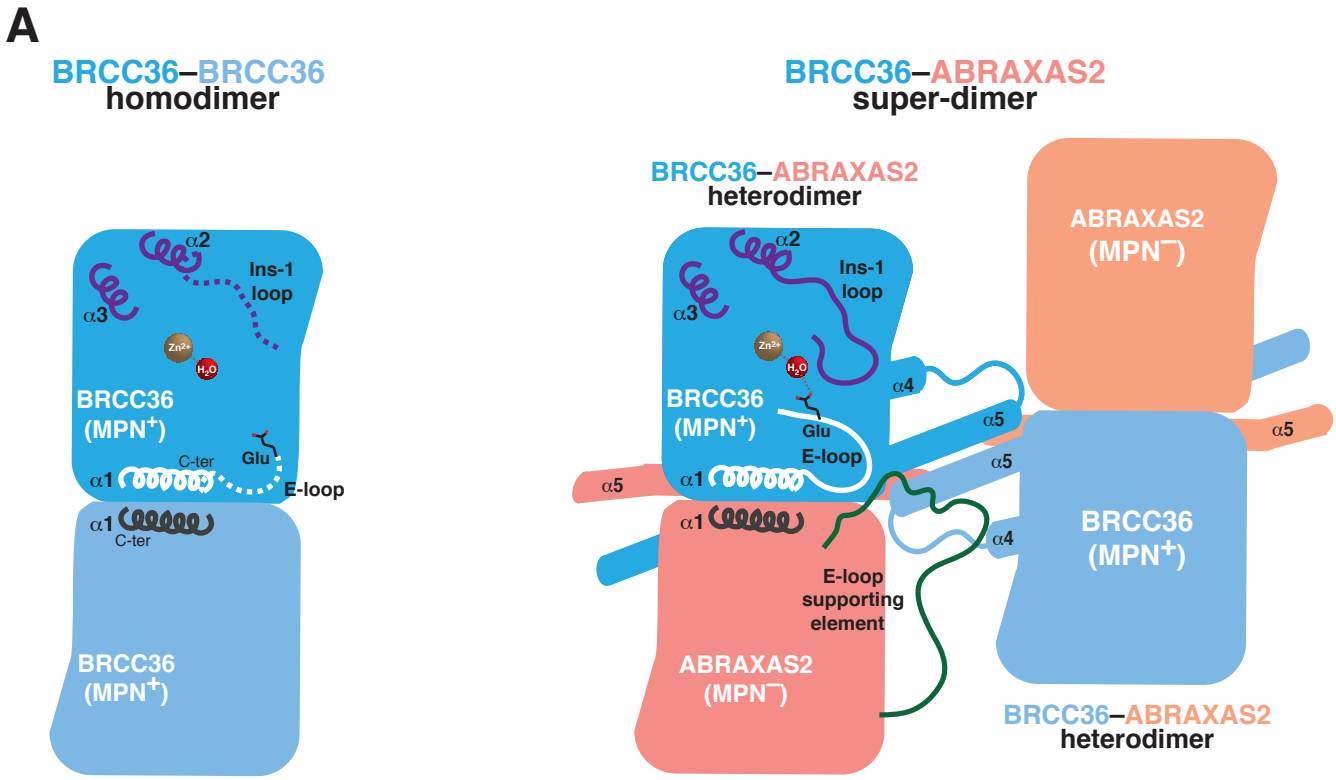

A
BRCC36 (MPN+)
Zn2+
ABRAXAS2 (MPN−)
ABRAXAS2 (MPN−)
BRCC36 (MPN+)
D115
D-helix
T112
D107
S104
C101
H94
Zn2+
S-loop
H96
N28
E30
R103
H-strand
E-loop
B
Rpn11 (MPN+)
Zn2+
Rpn8 (MPN−)
D122
D-helix
S119
S-loop
D103
R100
Zn2+
R32
H-strand
H109
H111
H94
E48
G96
E-loop
N30
C
CSN5 (MPN+)
Zn2+
CSN6 (MPN−)
D151
D-helix
S148
N132
S-loop
D135
Zn2+
H138
T126
H140
H
strand
G128
E76
Q67
E-loop
K153

FIGURE 4


A
BRCC36–BRCC36
homodimer
BRCC36–ABRAXAS2
super-dimer
BRCC36–ABRAXAS2
heterodimer
α2
Ins-1
loop
α3
Zn2+
H2O
BRCC36
(MPN+)
Glu
C-ter
α1
E-loop
BRCC36
(MPN+)
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(MPN−)
α4
α5
E-loop
supporting
element
BRCC36–ABRAXAS2
heterodimer

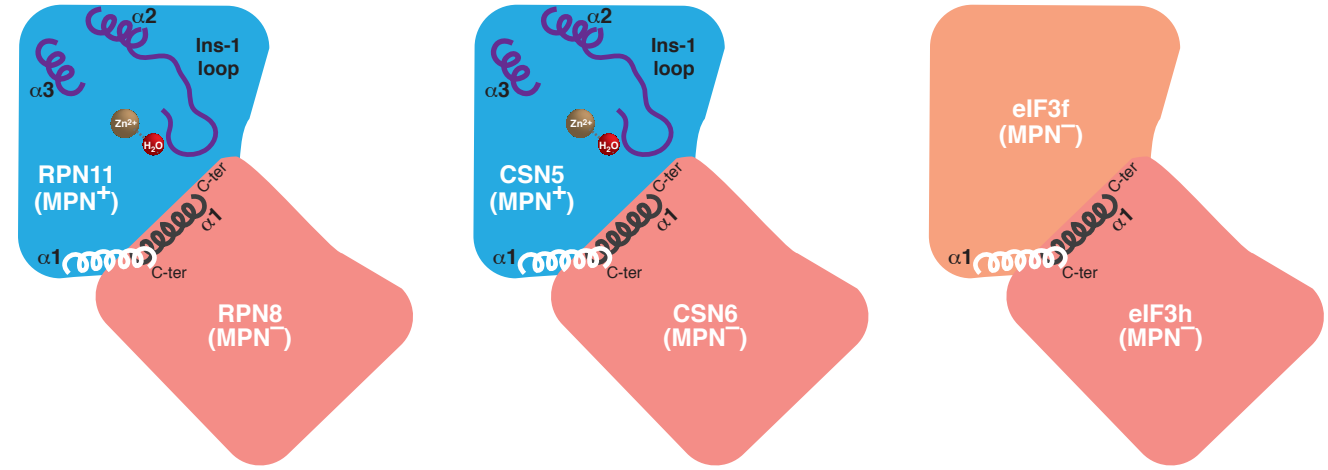
B
RPN11–RPN8
heterodimer
CSN5–CSN6
heterodimer
eIF3f–eIF3h
heterodimer
α2
Ins-1
loop
α3
Zn2+
H2O
RPN11
(MPN+)
C-ter
α1
RPN8
(MPN−)
CSN5
(MPN+)
CSN6
(MPN−)
eIF3f
(MPN−)
eIF3h
(MPN−)

FIGURE 5

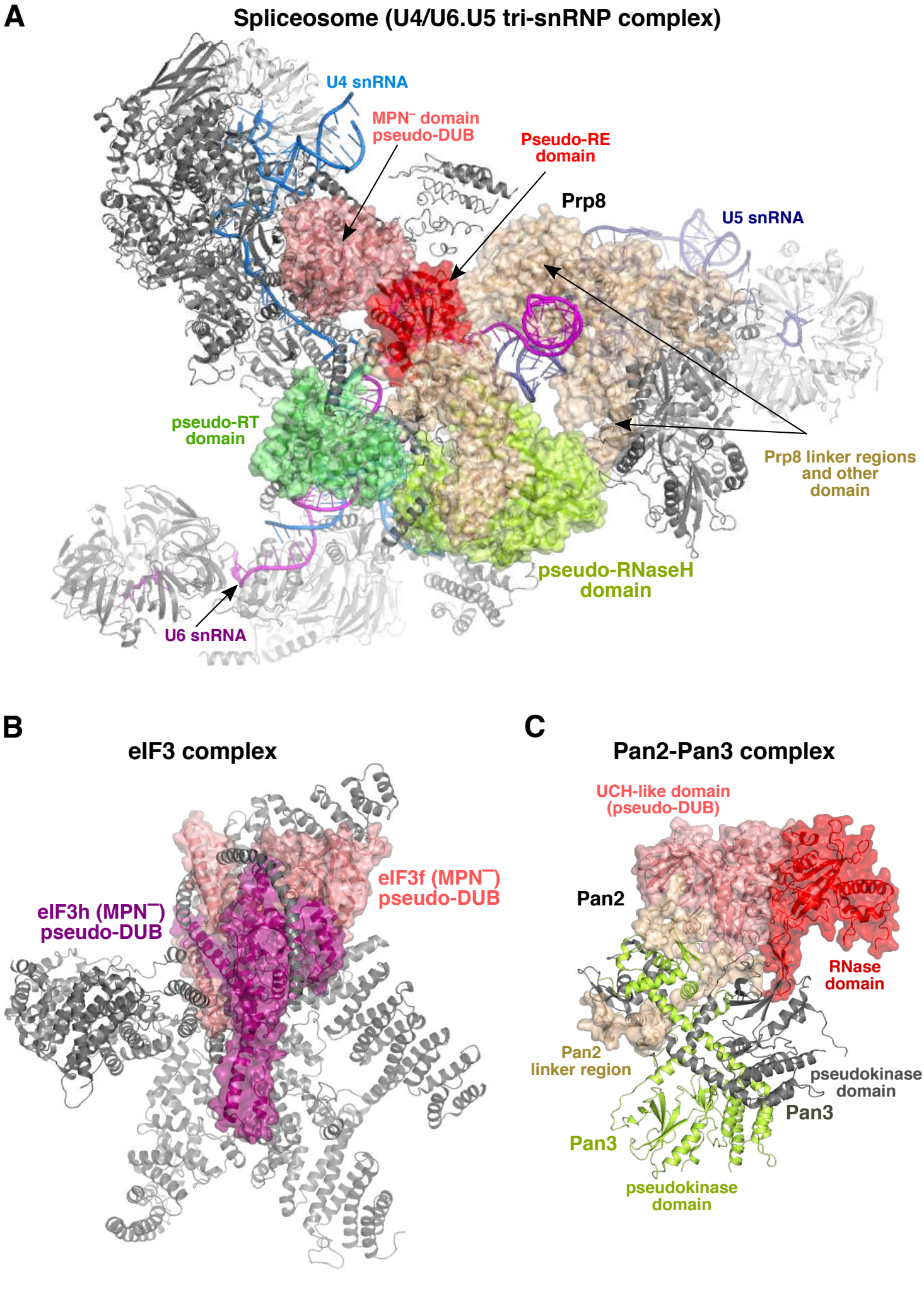
A
Spliceosome (U4/U6.U5 tri-snRNP complex)
U4 snRNA
MPN⁻ domain
pseudo-DUB
Pseudo-RE
domain
Prp8
U5 snRNA
pseudo-RT
domain
Prp8 linker regions
and other
domain
pseudo-RNaseH
domain
U6 snRNA
B
eIF3 complex
eIF3f (MPN⁻)
pseudo-DUB
eIF3h (MPN⁻)
pseudo-DUB
C
Pan2-Pan3 complex
UCH-like domain
(pseudo-DUB)
Pan2
RNase
domain
Pan2
linker region
pseudokinase
domain
Pan3
Pan3
pseudokinase
domain

FIGURE 6

SUPPLEMENTARY MATERIAL TO:
Pseudo-DUBs as allosteric activators and molecular scaffolds of protein complexes

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Multiple sequence alignment of JAMM/MPN DUB family
Multiple sequence alignment (black = conserved, white = not conserved) of human JAMM/MPN domain DUBs. Alignments were performed using Promals3D with use of selected 3D structures [1] and displayed using ALINE [2]. The secondary structure of AMSH (PDBid 2ZNV) was determined by the program DSSP [3] and is depicted in magenta. Large insertions and deletions are not included in the alignment. Residues important for catalysis are highlighted in cyan and mutated active site residues in red.

Supplementary Figure 2. Multiple sequence alignment of a subset of USP DUB family
Multiple sequence alignment (black = conserved, white = not conserved) of a selected subset of human USP domain DUBs. Alignments were performed using Promals3D with use of selected 3D structures [1] and displayed using ALINE [2]. Large insertions and deletions are not included in the alignment. Residues important for catalysis are highlighted in cyan and mutated active site residues in red.

Supplementary Figure 3. Multiple sequence alignment of OTU DUB family
Multiple sequence alignment (black = conserved, white = not conserved) of human OTU domain DUBs. Alignments were performed using Promals3D with use of selected 3D structures [1] and displayed using ALINE [2]. The secondary structure of OTULIN (PDBid 4KSJ) was determined by the program DSSP [3] and is depicted in magenta. Large insertions and deletions are not included in the alignment. Residues important for catalysis are highlighted in cyan and mutated active site residues in red.

Supplementary Figure 4. Multiple sequence alignment of MINDY DUB family
Multiple sequence alignment (black = conserved, white = not conserved) of human MINDY DUBs. Alignments were performed using Promals3D with use of selected 3D structures [1] and displayed using ALINE [2]. The secondary structure of MINDY1 (PDBid 5JQS) was determined by the program DSSP [3] and is depicted in magenta. Large insertions and deletions are not included in the alignment. Residues important for catalysis are highlighted in cyan and mutated active site residues in red.

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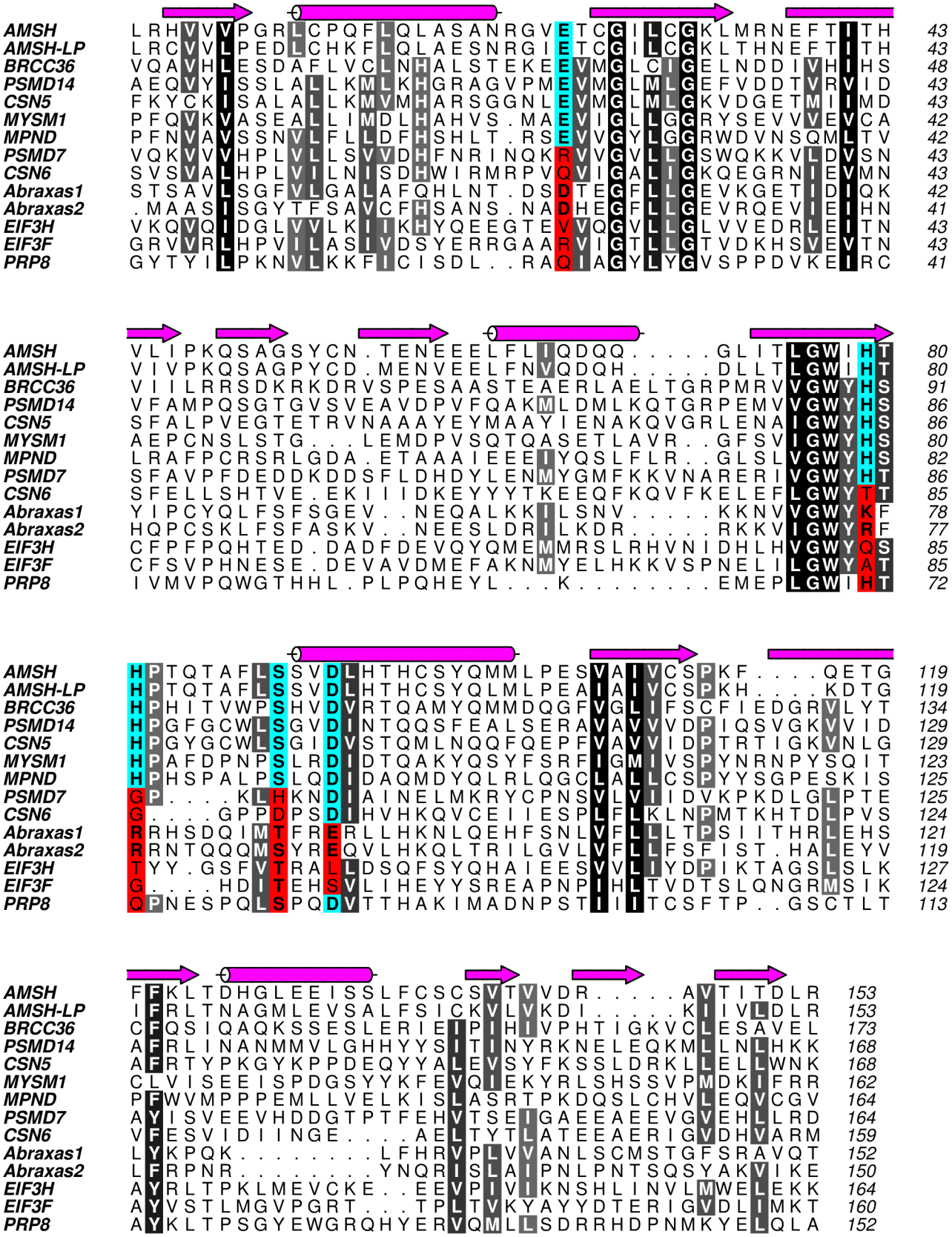
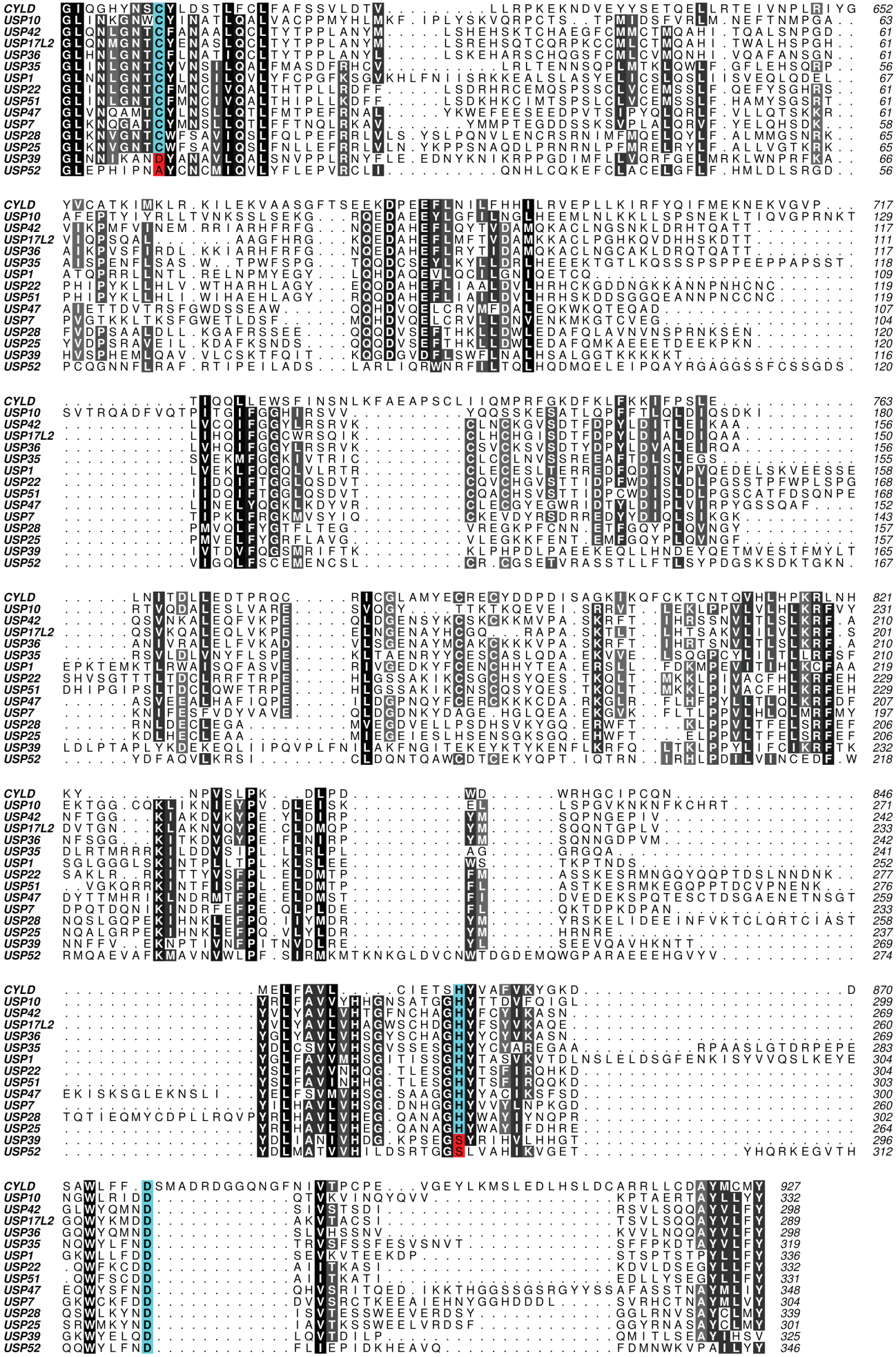
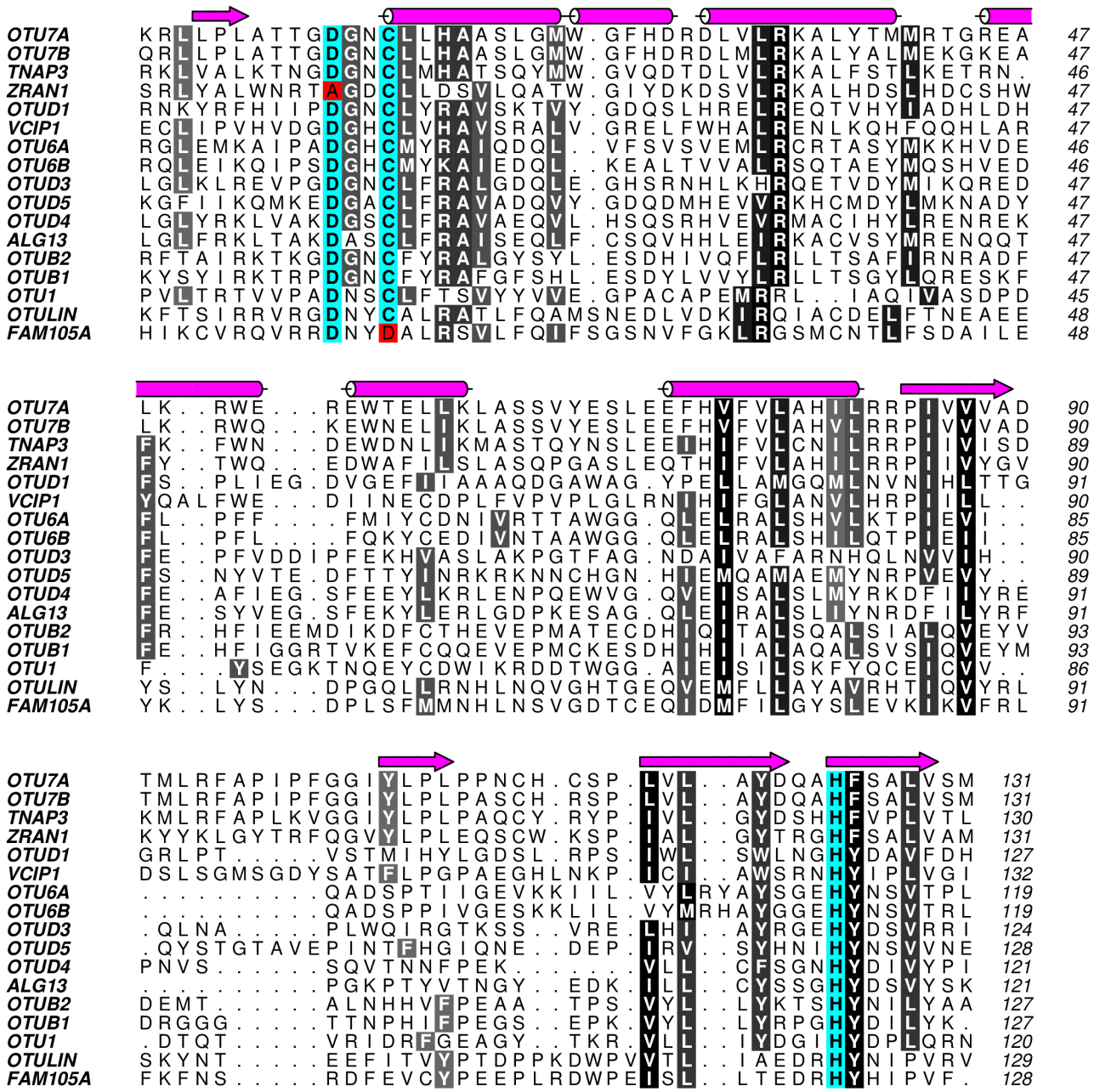


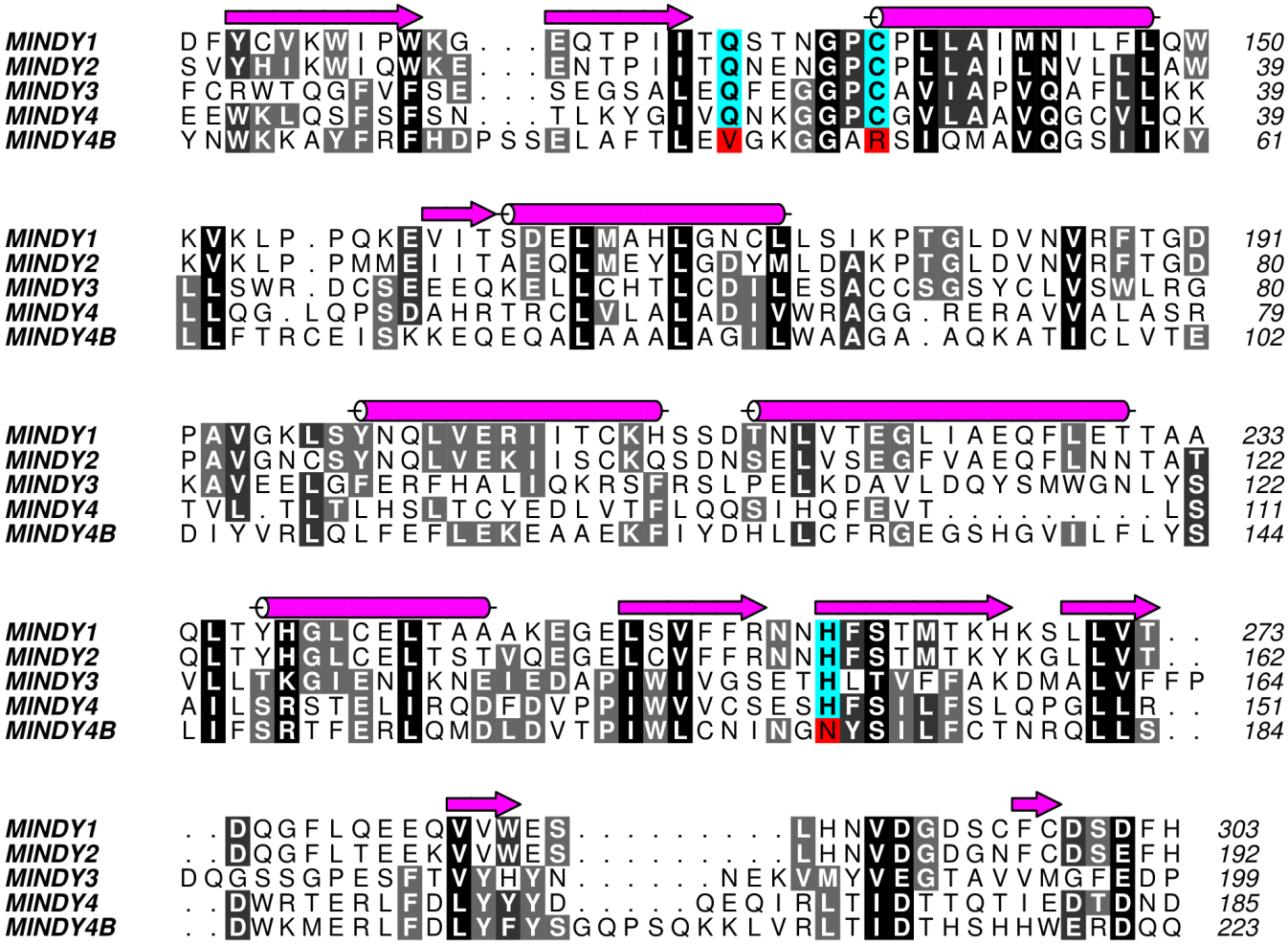
SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 2



SUPPLEMENTARY FIGURE 3



SUPPLEMENTARY FIGURE 4