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Identification of substrates for human deubiquitinating enzymes (DUBs): An up-to-date review and a case study for neurodevelopmental disorders

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A B S T R A C T

Similar to the reversal of kinase-mediated protein phosphorylation by phosphatases, deubiquitinating enzymes (DUBs) oppose the action of E3 ubiquitin ligases and reverse the ubiquitination of proteins. A total of 99 human DUBs, classified in 7 families, allow in this way for a precise control of cellular function and homeostasis. Ubiquitination regulates a myriad of cellular processes, and is altered in many pathological conditions. Thus, ubiquitination-regulating enzymes are increasingly regarded as potential candidates for therapeutic intervention. In this context, given the predicted easier pharmacological control of DUBs relative to E3 ligases, a significant effort is now being directed to better understand the processes and substrates regulated by each DUB. Classical studies have identified specific DUB substrate candidates by traditional molecular biology techniques in a case-by-case manner. Lately, single experiments can identify thousands of ubiquitinated proteins at a specific cellular context and narrow down which of those are regulated by a given DUB, thanks to the development of new strategies to isolate and enrich ubiquitinated material and to improvements in mass spectrometry detection capabilities. Here we present an overview of both types of studies, discussing the criteria that, in our view, need to be fulfilled for a protein to be considered as a high-confidence substrate of a given DUB. Applying these criteria, we have manually reviewed the relevant literature currently available in a systematic manner, and identified 650 high-confidence substrates of human DUBs. We make this information easily accessible to the research community through an updated version of the DUBase website (https://ehubio.ehu.eus/dubase/). Finally, in order to illustrate how this information can contribute to a better understanding of the physiopathological role of DUBs, we place a special emphasis on a subset of these enzymes that have been associated with neurodevelopmental disorders.

1. Introduction

1.1. Ubiquitin pathways and DUBs

The post-translational modification (PTM) of proteins by the covalent conjugation of one or more ubiquitin moieties, preferentially on lysine residues, modulates the fate of many cellular proteins. Ubiquitin is a 76 amino-acid long protein, which itself contains 7 lysines, as well as the N-terminal methionine, that can be used as targets for the conjugation of further ubiquitin moieties. The formation of either mono-ubiquitin conjugates, multi-monoubiquitin conjugates or poly-ubiquitin chains of multiple conformations has thus the potential to code for a multitude of messages [1]. The messages that such different conformational arrangements confer to ubiquitinated substrates are read by an array of ubiquitin-binding domains and ubiquitin interacting motifs, present in a wide range of cellular proteins [2]. While initial research identified the role of one specific poly-ubiquitin modification (K48-linked chains) as a signal for proteasomal degradation [3–5], it is now well established that all other protein fates (e.g. changes in protein-protein interactions or subcellular localization) conferred by ubiquitination can be at least as predominant and crucial for cell function as the proteasome-directed degradative route [6–9]. The dynamic state of the cellular ubiquitome is determined by the action of enzymes (E1, E2 and E3) that catalyze ubiquitin conjugation and the opposing action of DUBs, which release conjugated ubiquitin from certain ubiquitin linkage types or positions within ubiquitin chains [10]. Hence, it is not surprising that DUBs are involved in the regulation of diverse fundamental cellular processes including DNA repair, receptor sorting, mitophagy, cell cycle and the immune response [10,11].

Ubiquitination is essential for many physiological processes, and its

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importance is stressed by the fact that nearly one thousand human genes code for either enzymes of the ubiquitin conjugation/deconjugation machinery (i.e. activating E1, conjugating E2, ligating E3 and deubiquitinating DUB enzymes) or the different subunits of the proteasome [12,13]. In stark contrast to this large genetic investment in one specific regulatory pathway, the fraction of ubiquitinated molecules for any given protein tends to be rather low, in the range of just a few percent [14]. Thus, taking into account this low stoichiometry, it can be speculated that tissue and time specificity are key factors for the regulation of cellular function by ubiquitination [15]. Certainly, much work is still needed in order to clarify whether ubiquitin signals are indeed regulated in specific tissues and times, and how this spatio-temporal regulation is achieved. In this regard, proper levels of protein ubiquitination are likely to be the result of a tightly controlled balance of E3 and DUB enzymes´ activities.

1.2. Identifying ubiquitinated proteins in cells: native vs denaturing conditions

The identification of the proteins that are ubiquitinated or deubiquitinated in a given tissue or cellular context is essential in order to better understand the role that ubiquitination plays *in vivo*. Nevertheless, there are several challenges in ubiquitin research that have traditionally hampered the expansion of the field. Besides the low stoichiometry at which ubiquitinated proteins are found within cells [16,17], some of these modified proteins are degraded with fast kinetics [18], or rapidly deubiquitinated by the action of DUBs present in the cell lysate [19]. Additionally, it is known that proteins are usually modified with ubiquitin only in well-defined temporal windows [20]. Consequently, the ability to survey ubiquitinated peptides and/or proteins has been intimately associated with the development of strategies to enrich ubiquitinated material.

Classically, ubiquitinated material has been isolated using antibodies that directly recognize ubiquitin [21–24]. In numerous other studies, ubiquitin has been expressed fused with a tag (HIS, HA, FLAG, STREP, Myc…) that has then been used as a bait to purify the ubiquitinated material [25–29]. Tags in the amino terminal end of ubiquitin might block the generation of linear ubiquitin chains, so internally tagged ubiquitin has also been developed with the intention to overcome this limitation [30]. However, this approach might also cause other yet unknown effects.

Using antibodies to purify ubiquitinated material in native conditions can be technically challenging for several reasons. On one hand, a significant amount of ubiquitinated material might be lost (degraded or deubiquitinated) during the purification process due to the presence of proteases and cellular DUBs that remain active in non-denaturing conditions [19]. This loss can be reduced, to some extent, by adding metalloprotease inhibitors such as NEM,[1] or by using Tandem Ubiquitin Binding Entities (TUBEs) for the isolation of the ubiquitinated material, as these protect polyubiquitin chains from DUBs and proteasomal degradation [33,34]. On the other hand, co-purification of proteins that interact with the protein of interest under physiological conditions will obscure further identification analysis , as both ubiquitinated and their associated non-ubiquitinated proteins will co-exist in the same sample.

In an attempt to minimize these potential issues, a number of strategies that rely on the isolation of ubiquitinated material under denaturing conditions have been developed. For instance, poly-histidine (HIS) tagged ubiquitin has proven to be efficient at isolating ubiquitinated proteins using nickel affinity resins [25,35]. However, while HIS-tag provides an unbeatable tool when working with bacteria and yeast cells, it is not that well suited for most eukaryotic organisms, including mammals, as those higher organisms contain a large number of histidine-rich endogenous proteins (as evidenced by performing a simple BLAST search to, for example, a 9His motif, which returns 998 entries from the human proteome) that also attach to nickel beads with high affinity. Alternative strategies, such as BioUb [14,36], or GFP-based pull-down of ubiquitinated proteins [37,38], are far more specific and, thanks to the very high affinity of the reagents employed (avidin and nanobodies, respectively), also allow the use of denaturing conditions to reduce the presence of non-desired co-purifying proteins.

A widespread alternative to the purification of intact ubiquitinated proteins is the isolation of ubiquitinated peptides. Trypsin, commonly used in sample preparation for mass spectrometry analysis, cleaves the peptide bond after positively charged residues, so that resulting tryptic peptides contain an arginine or a lysine at their carboxy-terminal end [39]. Since the isopeptide bonds on lysines conjugated with ubiquitin lose their charge, trypsin does not cleave after ubiquitinated lysines, resulting in a so-called misscleaveage. Importantly, upon trypsin treatment the last two glycine residues at the carboxy-terminal end of ubiquitin get digested due to an Arg residue preceding them, and remain attached to the target lysine. These two glycines attached to the target protein, known as the di-Gly signature, confer a mark indicating the exact position where the ubiquitin was conjugated. Over a decade ago, the first antibodies recognizing this signature were generated, allowing the massive purification of ubiquitinated peptides [40]. Since then, further di-Gly antibodies have been developed, and a number of proteomic studies have relied on them to isolate ubiquitinated peptides and subsequently identify their parent proteins [41–44]. Using di-Gly antibodies to purify modified peptides serves to detect modified proteins and has the added advantage to intrinsically define exact ubiquitination sites. However, due to the trypsin digestion step, this procedure hampers the validation and characterization of ubiquitinated substrates at the protein level, and does neither inform on ubiquitin chains composition. An additional limitation on the use of this strategy stems from the fact that a di-Gly signature is also generated upon trypsin digestion of proteins conjugated with ubiquitin-like proteins such as Nedd8 or ISG15 [45]. Although Nedd8 and ISG15 modifications are not as common as ubiquitination, they are significantly more abundant when the proteasome is blocked, a routinely applied step in numerous ubiquitome studies [46]. Therefore, the sole detection of such remnant does not necessarily imply that the peptide detected was ubiquitinated.

Ubiquitinated proteins might undergo proteasomal degradation. Therefore, assessing changes in whole proteome abundance can be a valuable strategy to better interpret the changes in the ubiquitinated fraction, and to confirm whether degradation is or not the end-point for the identified ubiquitination events. Thus, differential proteomic experiments have been included in a number of studies to help interpreting ubiquitination results [47–51]. Importantly, however, ubiquitination does not always lead to degradation, and protein abundance in the cell can be regulated in many other, ubiquitin-independent, manners. Thus, a correlation between the ubiquitination of a protein and its abundance should not be assumed.

2. Identification of human DUB substrates

2.1. Methodological considerations and criteria to establish a protein as a bona-fide DUB substrate

With the advances in the methods used to purify and detect ubiquitinated material, the number of proteins identified as DUB substrates is rapidly increasing. In order to identify the substrates of a specific DUB,

[1] NEM (N-ethylmaleimide), an alkylating agent used to inactivate DUBs, form stable and covalent tioether bonds with sulfhydryl groups, and in hence, it is suitable for thiol protease inhibition. Iodoacetamide is a sulfhydryl-reactive alkylating reagent, used to inhibit DUBs by alkylating cysteine residues at the DUB active site. It is broadly used in mass spectrometry studies. Given that in gel digestion protocols using high concentrations of iodoacetamide (55 mm) can induce lysine adducts that are identical in atomic composition to the di-glycine remnant [31], exceptional care should be taken when analyzing those results, although this does not seem to occur with lower concentrations [32].

any of the above-mentioned technical approaches (e.g. using TUBEs, di-Gly antibodies or isolating tagged ubiquitin) can be used in combination with overexpression or silencing procedures to modulate the levels of the DUB of interest, relative to the appropriate control. In a typical silencing experiment, for example, ubiquitinated material can be purified from cells transfected with either a DUB-specific siRNA or a control siRNA. More recently, CRISPR/Cas9 technology is also being applied to target specific DUBs. Then, ubiquitinated material in both cell cultures must be quantified either by western blotting or mass spectrometry-based proteomics, as proteins whose ubiquitination levels have increased upon DUB-silencing would be considered as putative DUB substrates. Conversely, if an overexpression approach is used, those proteins found less ubiquitinated upon overexpression of the enzyme can be considered as putative DUB substrates. The importance of using appropriate controls in these experiments needs to be stressed. In silencing approaches, a non-targeting siRNA with a scrambled sequence is routinely used as negative control, and it is crucial to monitor the correct silencing of the DUB in the experimental set up. When increasing the DUB abundance by an induced expression of the WT enzyme, the most appropriate control would be, when possible, the comparable expression of a catalytically inactive version of the DUB enzyme, and the correct expression of the overexpressed enzymes should be tested.

By applying either classical biochemical approaches or by using mass spectrometry (MS)-based experiments, hundreds of proteins have been described as substrates of human DUBs [52–56]. However, the methodologies used in some of these studies present some limitations that do not, in our opinion, allow to fully establish that a protein is a DUB substrate. On one hand, the outdated view that ubiquitination invariably leads to protein degradation has resulted in far too many studies relying on protein abundance changes detected in whole cell extracts to conclude that a change in protein ubiquitination has occurred upon DUB silencing or overexpression. But, as mentioned above, other molecular mechanisms may also be responsible for changes in protein levels. Additionally, this approach explicitly excludes all ubiquitinated proteins that are not targeted for degradation, severely limiting the scope of such studies. On the other hand, in several studies using immunoblot analysis of whole cell extracts it is assumed that protein bands of higher molecular weight, above the band corresponding to the size of the candidate substrate, represent ubiquitinated species. In the absence of further validation, this apparent increase in the size of the protein might be due to other post-translational modification(s), or even to antibody inspecificity. In fact, since less than 5% of a given protein is usually ubiquitinated, when performing immunoblot analyses of a whole cell extract it is actually more likely to observe non-specific bands than a band corresponding to specific ubiquitin-modified version of the protein. Therefore, due to the uncertainties associated with those studies in which only whole cell extracts were analyzed, the putative substrates described in these works have not been incorporated into the DUBase database.

In summary, while most proteins reported to date to be deubiquitinated by human DUBs may represent *bona-fide* substrates, some candidates will require further validation. In this manuscript, we aim to define the criteria to be applied in order to consider that a given protein can be considered with high-confidence to be a DUB substrate. As general criteria, we propose that, in order to demonstrate that a given protein is the substrate of a particular DUB, it is necessary (i) to include a purification step in the procedure, prior to checking the ubiquitination status, and (ii) to monitor the ubiquitinated fraction of that protein, using either western blotting or mass spectrometry. Using these criteria, we have manually reviewed the existing literature for high-confidence DUB substrates, and we make those data available through DUBase (https://ehubio.ehu.eus/dubase), a public Database for human DUB substrates [57].

2.2. Identification of human DUB substrates: reported studies based on individual candidates

We searched the Pubmed database (NCBI) for scientific papers reporting the identification of specific DUB substrates on a case-by-case basis. We typed the leading gene name according to Uniprot for each of the 99 human DUBs, and checked all the available literature, with no other extra filters. Only the results for DUBs containing more than 600 entries (CYLD, UCHL1, BAP1, TNFAIP3, ATXN3 and COPS5) were subsequently filtered with the additional terms “ubiquitination” or “deubiquitination”. We manually examined a total of 7276 papers (Fig. 1), and focused on the ones reporting the identification of DUB substrates, or describing a “stabilization”, “regulation”, “interaction” or “modulation” relationship between the DUB and the candidate protein.

We next filtered one by one each of those papers, so that they all fulfilled the general criteria described above. In addition, as illustrated in Fig. 2 and Table 1, other specific criteria were applied for our literature review. Firstly, in this work we have limited our analysis to studies on which both the DUB and the corresponding substrate are human proteins. Secondly, we have chosen to consider only *in vivo* studies (including those performed in cell culture). The reason is that, although *in vitro* reconstituted assays might be useful to elucidate if a DUB is able to deubiquitinate a protein when both are present at high concentrations, substrate identification may be overestimated by *in vitro* studies, as simply by mass action law, enzymes will incur into non-specific reactions that may not occur in cells. Finally, we have examined in detail the experimental evidence provided in each study to support the identification of a DUB substrate. In this regard, when studying protein ubiquitination, the protein of interest, ubiquitin, or both are typically tagged. If a candidate protein is purified, its ubiquitinated fraction is usually checked by immunoblot against ubiquitin (Fig. 2). Conversely, if ubiquitinated material is purified, an antibody against the protein of interest is used for detection. This orthologous approach ensures that the ubiquitinated fraction of a substrate is monitored, and therefore, DUB substrates identified by these means have been included in DUBase. However, some studies use the same antibody for both purification and detection, which may result in the presence of higher or lower molecular weight bands that do not necessarily correspond to ubiquitinated versions of the protein. Candidate substrates reported in these studies have been excluded from DUBase. Also excluded were those candidates reported in studies that, while using different antibodies for purification and detection, describe substrate ubiquitination based on bands below the size of the non-modified substrate.

The selected papers were then grouped on a file, together with their corresponding information about: DUB name, DOI number, PMID number, Gene name, Uniprot code, cell line used, figure number and link to the figure in which the DUB-substrate relationship is experimentally proved, and whether proteasomal inhibitors were employed or not. Papers in which a substrate was validated in different cell lines, were included as different entries. This process was performed for each of the seven DUB families (USP, UCH, MINDY, OTU, JAMM, ZUP1 and MJD) and the final file, containing a total of 772 entries was uploaded to DUBase. The number of substrates identified for each DUB, and the number of papers uploaded in each case, are visualized on the DUB chart in DUBase (Fig. 1B). Papers reporting proteomic studies are further described within the following section.

2.3. Identification of DUB substrates: reported studies using proteomics

Mass spectrometry-based proteomics has revolutionized our knowledge about PTMs and their regulation. The detection of modified proteins and peptides had been largely hampered mainly due to their substoichiometric nature. But during the last two decades, thanks to the development of numerous tools to enrich modified protein/peptides and more robust and sensitive mass spectrometers, it is relatively easy, for instance, to detect and quantify thousands of site-specific modifications

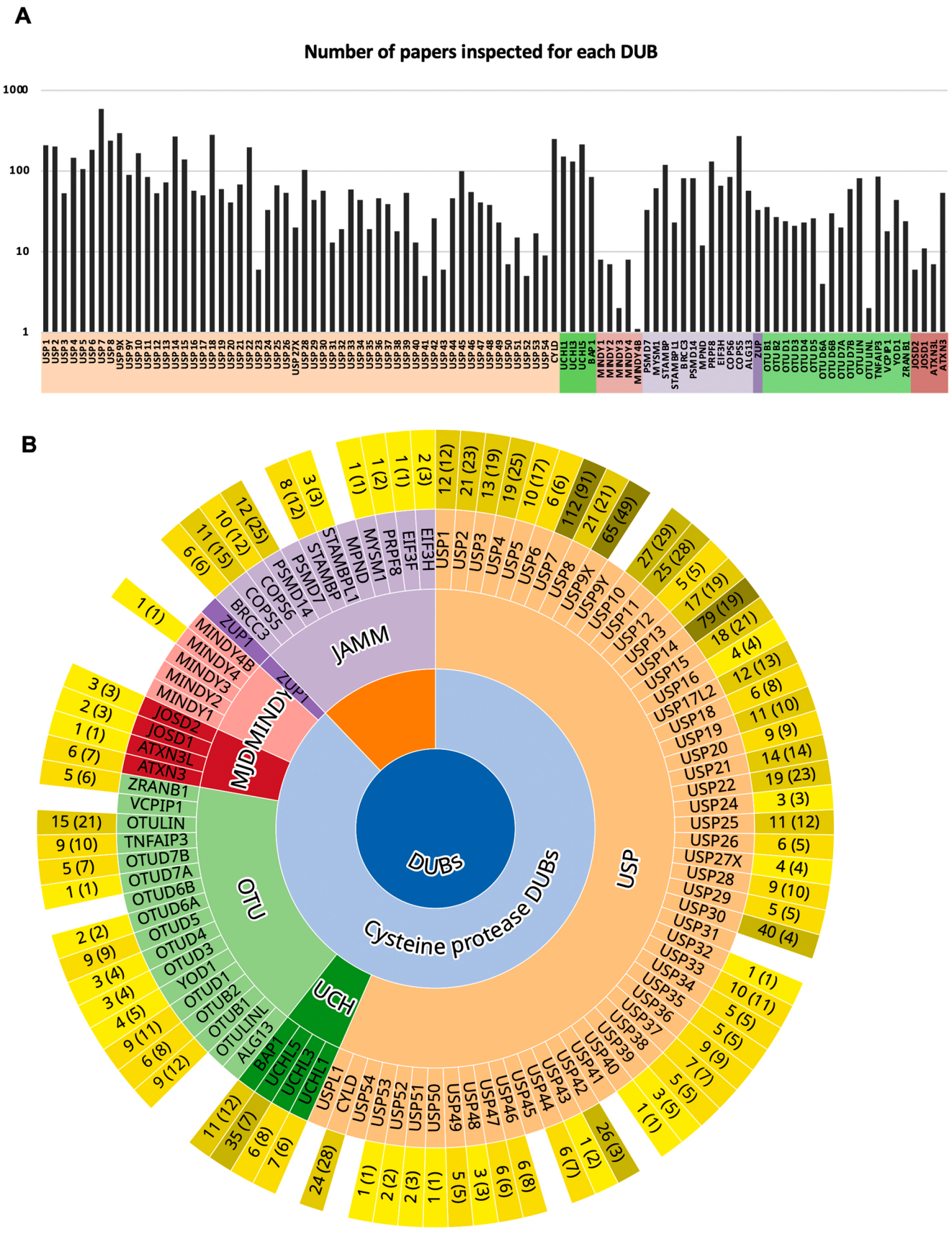

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Fig. 1. The human DUB ubiquitome. A. Overview of the review process. Available literature as of 30th September 2021 manually curated for all 99 human DUB enzymes. In the Y axis, the number of papers found in Pubmed for each of the human DUBs is indicated. Different families are highlighted with distinct colors, according to the DUBase chart (see Fig. 3): USP (orange), UCH (dark green), OTU (light green), MJD (dark red), MINDY (light red), ZUP (dark purple) and JAMM (light purple). Only the most common name has been included for the following DUBs: UCHL5/UCH37, ZUP/ZUPSP MINDY1/FAM63A, MINDY2/FAM63B, MINDY3/FAM188A, MINDY4/FAM188B, MINDY4B/FAM188B2, STAMBP/AMSH, PRPF8/PRPC8, COPS6/CSN6, COPS5/CSN5/JAB1. B. DUBase browsing chart. This chart shows for each DUB the number of substrates curated and the number of experiments (in brackets) fulfilling the criteria described in section 2.1 and Fig. 2. The number of substrates can vary depending on the proteomics thresholds defined by the user. By default, each proteomic dataset is loaded with the original thresholds selected by the authors of each experiment.

in a single run [58–60].

As mentioned above, it can be anticipated that the ubiquitination of a DUB substrate will be increased when the corresponding DUB is silenced, while it will be reduced when the same DUB is overexpressed. Bearing this in mind, the most efficient strategy to uncover DUB substrates is to either silence, inactivate or induce a DUB, and quantify ubiquitinated peptides or proteins using mass spectrometry-based methods. Indeed, a number of research groups, included ours, have successfully applied distinct MS-based quantitative strategies to disclose a significant number of putative DUB substrates that have been included in our DUBase database as proteomics or manual (MS-based) type data.

In order to distinguish candidate substrates from all the proteins detected by mass spectrometry it is necessary to set a fold change threshold and/or a statistical significance threshold. While in most proteomics studies the statistical significance threshold is set at p-value $< 0,05$, the fold change threshold varies substantially. The lower the threshold, the more chance to detect false positives. So far, there is no rule to set such threshold, and it is up to the researcher to be more or less restrictive.

Bingol and colleagues are pioneers in using quantitative proteomics to unveil substrates of a DUB, more specifically of USP30 [61]. Using a label-free approach, they compared the ubiquitinome of control and siUSP30-treated HEK293 cells combining immunoaffinity enrichment of ubiquitinated peptides using anti-K-ε-GG antibodies with LC-MS/MS analysis. In order to consider a protein as putative USP30 substrate, they set the fold change threshold at 1,3 and p-value $< 0,05$. 319 proteins fulfilled these criteria, and two of them, MIRO and TOM20, were confirmed by western blot to be direct USP30 substrates [61]. More recently, they have applied a TMT-based quantitative approach to compare K-ε-GG enriched ubiquitinated peptides in wild type and USP30 knockout HEK293T cells treated or not with a USP30 inhibitor. By setting the threshold of adjusted p-value < 0.1 and no specific thresholds for fold-changes they detected 80 proteins that were differentially ubiquitinated by USP30 inhibitor only in WT cells, of which 30 proteins -annotated as mitochondrial- showed increased ubiquitination upon USP30i. In brief, they demonstrated that both, pharmacological inhibition or genetic ablation of UPS30 results in accumulation of substrates that are normally localized in the mitochondria. Further characterization revealed that mitochondrial import is dynamically regulated by the ubiquitin system through USP30 and the E3 ligase March5 [9].

A number of USP32 substrates have also been detected by quantifying enriched ubiquitinated peptides in MelJuSo and HeLa cells, this time using SILAC [62]. Nevertheless, in the absence of data regarding protein ratios and significance p-values, only RAB7, which was further confirmed by immunoblot, has been included in DUBase. Liu and colleagues followed a similar strategy to reveal USP14 substrates. They used anti-K-ε-GG antibodies to enrich ubiquitinated peptides obtained from USP14-KD and control HeLa cells, that were SILAC-labeled. The study resulted in the detection of 299 candidate proteins displaying a SILAC ratio above 1,2 and p-value $< 0,05$. Moreover, they carried out SILAC-based differential proteomic analysis to identify putative degradation substrates of USP14 and a label-free interactome study aiming to detect direct USP14 interactors [63]. According to the authors, direct USP14 substrates should interact with the DUB, as well as be downregulated in the USP14–KD condition, in addition to contain upregulated ubiquitination sites. Free ubiquitin and FASN, which was further confirmed by immunoblotting, were the only two proteins fulfilling these three criteria. But a further 42 proteins fulfilled at least two of the three MS-based criteria (downregulated in the proteome, upregulated in the ubiquitinome and present in the interactome) and were also described as potential USP14 substrates. Accordingly, these 44 substrates were included in DUBase, but given that distinct MS-based experiments were combined to obtain the final list of candidates, they were included as manual (MS-based) type data.

Following the observation that USP14 regulates K48-linked ubiquitin chains in HEK293T cells, further work was performed to isolate these K48-based polyubiquitinated proteins by immunoprecipitation [64]. MS data were compared using the Sum Intensity parameter to determine which proteins appeared less abundant (fold change ratio < 0.83) in HEK293T cells when expressing the WT USP14 relative to the inactive mutant C114A. This resulted in 22 putative USP14 substrates, of which two (PSMC1 and PSMC3) had also been detected in the SILAC study by Liu and colleagues [63], and a total of three (alpha-synuclein, PSMC1 and PSMD4) were validated by immunobloting. After showing that UCHL5 also regulates K48-linked ubiquitin chains, the same strategy was deployed to identify 29 putative UCHL5 substrates, including PSMC3. It should be noted that, although the study aiming to detect USP14 and UCHL5 target proteins was performed in triplicate, authors do not report a significance p-value. Consequently, the list of putative substrates was not included in DUBase as proteomics type data, but as manual (MS-based) entry.

Our research group has recently published a work reporting quantitative proteomics data of the putative substrates of 5 DUBs; USP1, USP7, USP9X, USP11 and USP42. Each DUB was separately silenced in HEK293 cells that were simultaneously transfected with the bioUb system. Ubiquitinated substrates were enriched at the protein level from siDUB and control cells by biotin pulldown and resulting peptides subjected to label-free quantitative proteomic analysis. The total amount of proteins quantified in USP1, USP7, USP9X, USP11 and USP42 experiment was 1472, 2672, 4285, 857 and 3056, respectively. We considered a putative substrate should display a fold change above 2, and p-value $< 0,05$, and we initially obtained a list of 16 candidates for USP1, 17 for USP7, 44 for USP9X, 9 for USP11 and 40 for USP42. In order to reduce the number of false positive, we only considered those candidates that were detected with at least two unique peptides and repeatedly quantified. Hence, our final list of putative DUB substrates was comprised of 3 proteins for USP1, 6 for USP7, 20 for USP9X, 3 for USP11 and 24 for USP42 [57]. Given that this strategy does not specifically enrich the ubiquitinated peptides, those were present at the rate usually observed in other bioUb experiments (just ~10–20%) and are not used as a criterion for neither detection nor quantification of these ubiquitinated peptides, but can be informative nevertheless.

As we were completing this review, USP7 targets have also been screened by quantifying the ubiquitomes and proteomes of control and HCT116 cells treated with the USP7 inhibitor FT671 [65]. Ubiquitinated peptides were isolated using di-Gly antibodies in this study, and data-independent acquisition (DIA)-MS method with a neural network-based data processing were combined to identify the modified proteome [65]. Interestingly, already after 15 min of USP7 inhibition, they detected 1243 peptides, corresponding to 552 proteins, displaying increased ubiquitination (fold change > 2; p-value<0.05). Of those, 42 proteins were significantly downregulated after 6 h of treatment, suggesting they might be direct USP7 substrates targeted for proteasomal

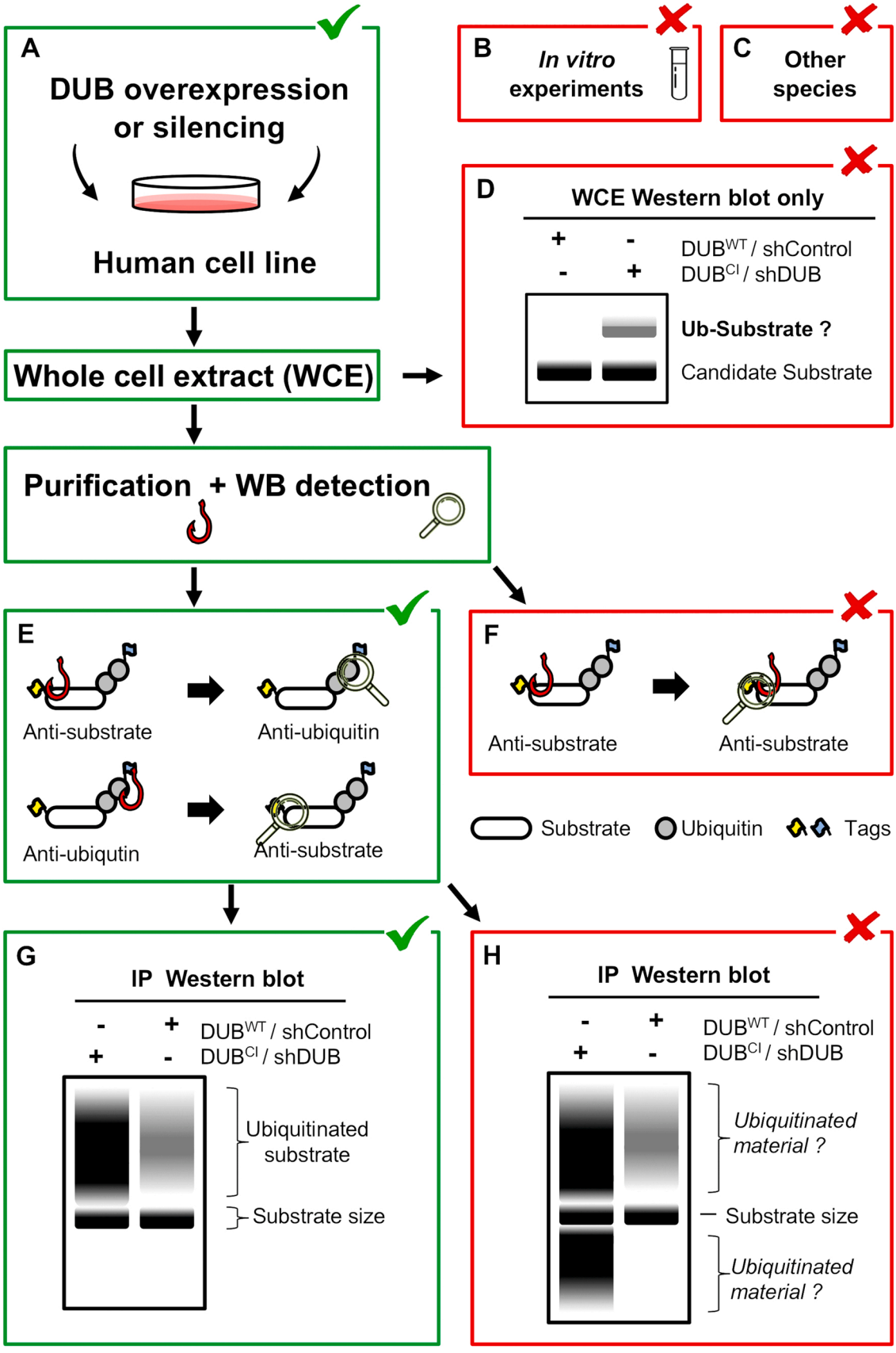

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Fig. 2. Workflow of DUB substrate identification and accepted/excluded scenarios. Experiments eligible to be included in the DUBase are highlighted in ticked green boxes, while excluded scenarios are shown in crossed red boxes (Table 1: Criteria to determine whether a protein is a DUB substrate). Experiments overexpressing or silencing the DUB in human cell lines were considered (A), excluding *in vitro* experiments (B) as well as studies using non-human DUBs or candidate proteins (C). Many studies that directly analyzed whole cell extracts (WCE) by western blot were discarded (D), since in WCE the detection of bands above the molecular weight of the candidate substrate does not necessarily indicate them to represent its ubiquitinated fraction. A purification step is stablished to be necessary to confidently identify substrates, and purified material needs to be detected by western blot analysis. Experiments using different antibodies for purification and detection were only included (E). When a substrate is purified, the ubiquitinated fraction needs to be detected, and if ubiquitinated material is purified, the substrate needs to be detected. Studies using substrate targeting antibodies both for purification and detection, were excluded (F). The antibodies used for purification and detection are different in each study, specific for the protein they are investigating, or for the tags they are using, so general terms were used in this figure (anti-substrate, anti-ubiquitin). Finally, detection of IP material by western blotting (G) needs to display ubiquitination smears (Substrate-Ub) only above the molecular weight of the substrate (Substrate). If "ubiquitinated" smears appear below the substrate size (H), the bands above become also suspect of being non-specific, and the study was excluded. The expected substrate size can be visualized on the same membrane by dual western blotting, where antibodies to the substrate and to the conjugated ubiquitin chains from different species are employed in the same membrane, complemented with different fluorophores for each antibody. Alternatively, a similar result can be obtained by reblotting the membrane to the substrate after probing for the ubiquitin conjugates. Finally, ubiquitination smears need to show an intensity reduction on wild-type (DUB^{WT}) vs ligase-dead (DUB^{LD}) DUB overexpression, or an increase in siDUB vs control siRNA treated conditions (G), while the non-modified form of the substrate needs to be constant. Apart from the western blot for the IP material, it is recommended to show the western blots of input samples to ensure similar ubiquitination levels in all conditions (not shown).

Table 1
Criteria to include/exclude studies identifying DUB substrates.

	Criteria to include/exclude studies identifying DUB substrates	
	Included	**Excluded**
1.	Use of human cell lines.	Use of other species, or non-human DUBs/substrates.
2.	*In vivo* cell culture experiments.	*In vitro* experiments.
3.	Silencing (siDUB vs siControl) or Overexpression (WT vs LD) of the DUB.	
4.	Purification step before detection by Western blot.	Whole cell extract analysis only.
5.	Use of different antibodies to purify the substrate and detect its ubiquitinated fraction.	Using the same antibody to purify the substrate and detect by Western blot its ubiquitinated fraction.
6.	Western blots showing substrate ubiquitinated fraction only above its non-modified molecular size.	Western blots referring to ubiquitinated substrate when the smear reaches below its non-modified molecular size.

degradation [65]. As for the SILAC-based investigation searching for USP14 substrates [63], in this study ubiquitinome and proteome analyses were combined to detect potential proteasome-targeted USP7 substrates, and thus, the data were included in DUBase as manual (MS-based) type.

3. Updating the DUBase repository

DUBase [57] is an interactive web-based database of human substrates of DUB enzymes (https://ehubio.ehu.eus/dubase/). This database was created by the authors of this paper in order to provide easy access to our data, and with the scope to grow by integrating data from other groups. In this regard, we have now updated DUBase to accommodate the results of the manual and proteomics experiments curated in this review (Fig. 1). The database schema has been updated (Fig. S1) to support importing results of different types of experiments. Besides the original bulk proteomics data import, now it also possible to include individual data supported by external resources like, for example, a link to a western blot figure in the original paper. These experiments must be linked to one or more publications, although the same publication can result into different DUBase experiments if different DUB enzymes or cellular types have been studied within it. Other minor changes have been newly integrated in the database in order to store additional information, like protein modification scores in proteomics experiments, or gene name aliases useful for the substrate queries.

The website has also been updated (Fig. 3) in concordance to the database schema changes already highlighted. Now the search tab displays a more compact and general view of the search results, although the detailed proteomics view is still available by clicking a button. The details tab has been extended to show information specific to each type of experiment. A summary of the experiment and direct access to supporting files (e.g., western blot figure) has also been included in this tab. Other features specific to the proteomics experiments, like the user defined thresholds (p-value, fold change, etc.) or the volcano plots, are now available in a per experiment bases instead of being common to all the experiments. This is mandatory given the variability of proteomics experimental designs being reported. Finally, we have included a submission form in order to ease the growth of DUBase with external contributions. The first section of this form comprises several fields that establish whether the submission complies with our inclusion criteria,

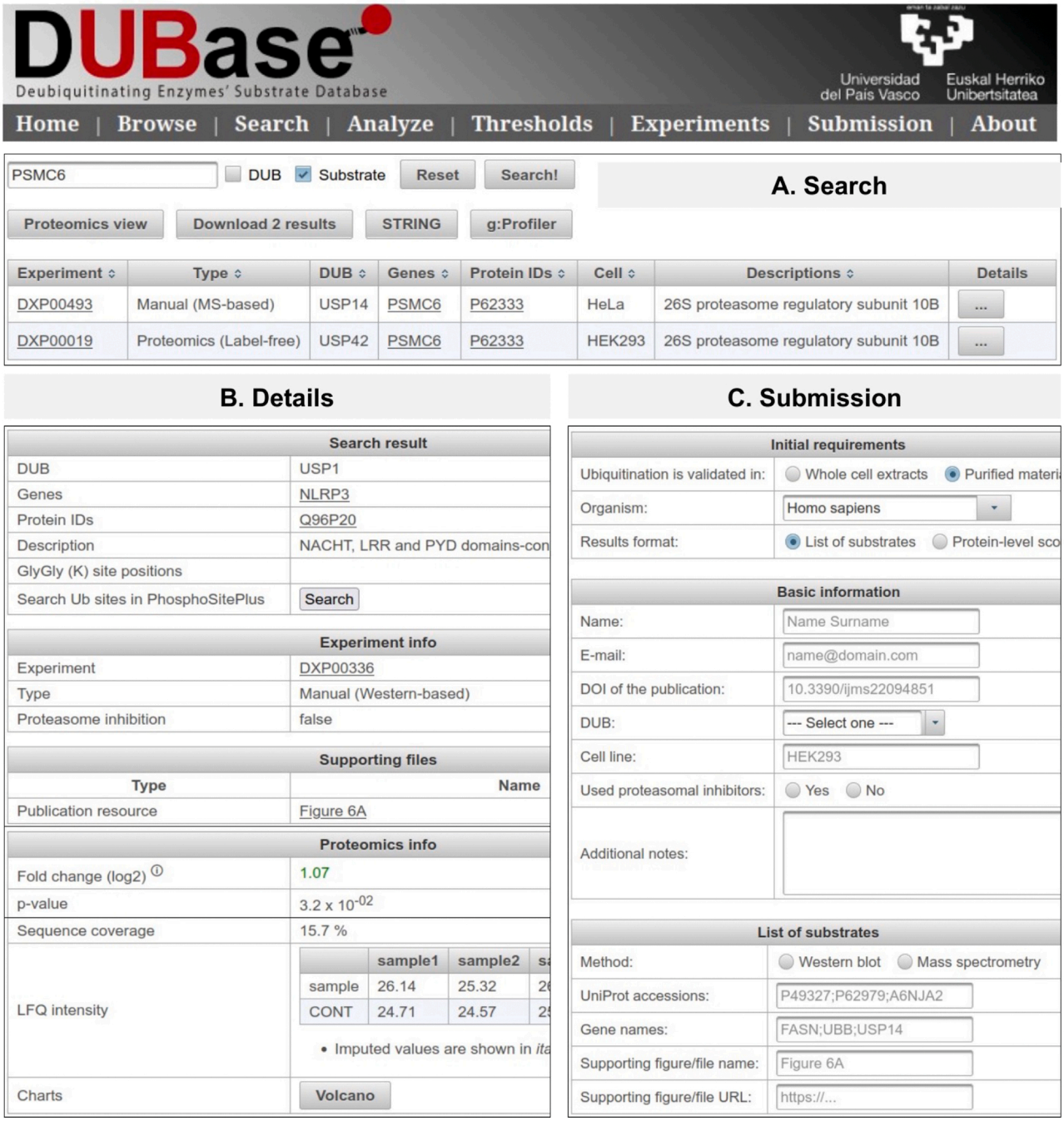


Fig. 3. Newly integrated changes in DUBase. A. *Search tab*. This page has been updated to accommodate different types of experiments. The original proteomics view is still available by clicking a button. B. *Details tab*. This page has been updated to show information specific to each type of experiment. A summary of the experiment and direct access to supporting files (e.g., western blot figure) has also been included. C. *Submission tab*. A submission form has been included to ease the contribution of external researchers. Fields at the beginning of the form determine whether inclusion criteria are fulfilled and which other fields will be shown next.

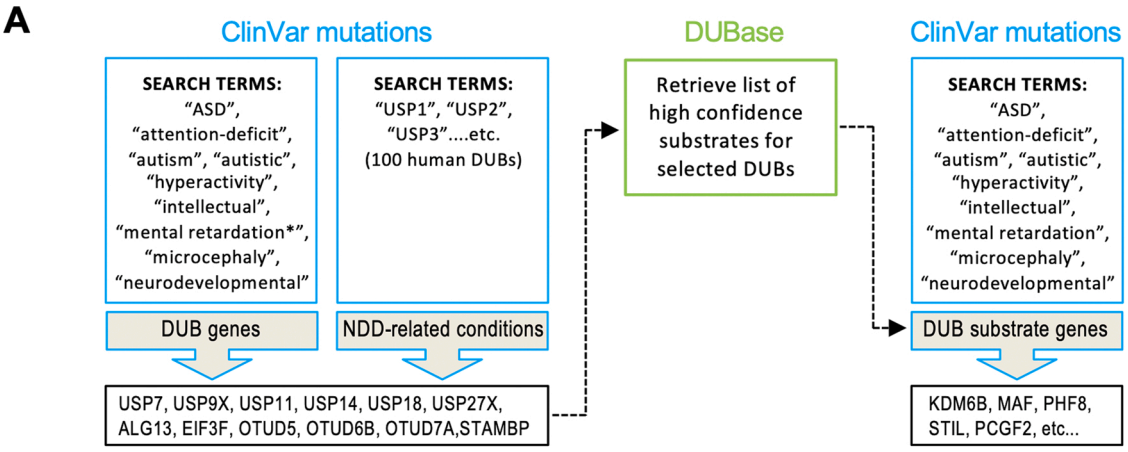
and will determine which other fields will be shown next. Once fulfilled, a submission file is generated and sent by e-mail to us in order to manually review all the information. After reviewing the information provided, we will reply by e-mail to the submitter either to gather more information or to acknowledge the inclusion of the results in the database.

4. Mutation of DUBs and their substrates in neurodevelopmental disorders

Altered DUB function is known to contribute to several human pathologies, including cancer and neurological diseases [66,67]. Neurological diseases, which are characterized by defects in central nervous system (CNS) development and/or function, comprise a number of pathological entities often classified into two broad categories: late onset neurodegenerative disorders, and early-onset neurodevelopmental disorders (NDDs). Nearly one third of human DUBs could be involved to some extent in one or both types of disorders [68–71]. Here, we will limit our discussion to those DUBs with a described link to NDD pathogenesis. In particular, we will focus on a subset of DUBs encoded by genes bearing mutations that result in NDD-related conditions, as reported in the ClinVar mutation database (https://www.ncbi.nlm.nih.gov/clinvar/) [72].

NDDs are a heterogeneous group of disorders whose classification

can still be a matter of some debate [73–75]. For the purpose of this review, NDDs will be broadly considered to encompass medical conditions such as intellectual disability (ID), autism-spectrum disorder (ASD), attention-deficit and hyperactivity (ADH) and microcephaly. Mutations in several genes encoding DUBs, such as USP7, USP9X, USP11, USP18, USP27X, OTUD5, OTUD6B, OTUD7A, ALG13, STAMBP/AMSH, and EIF3F have been linked to NDDs [68,70,76–78]. These genetic defects might contribute to NDD pathogenesis by disrupting ubiquitin-mediated regulation of crucial processes and signaling pathways related to brain development, such as chromatin remodeling, and



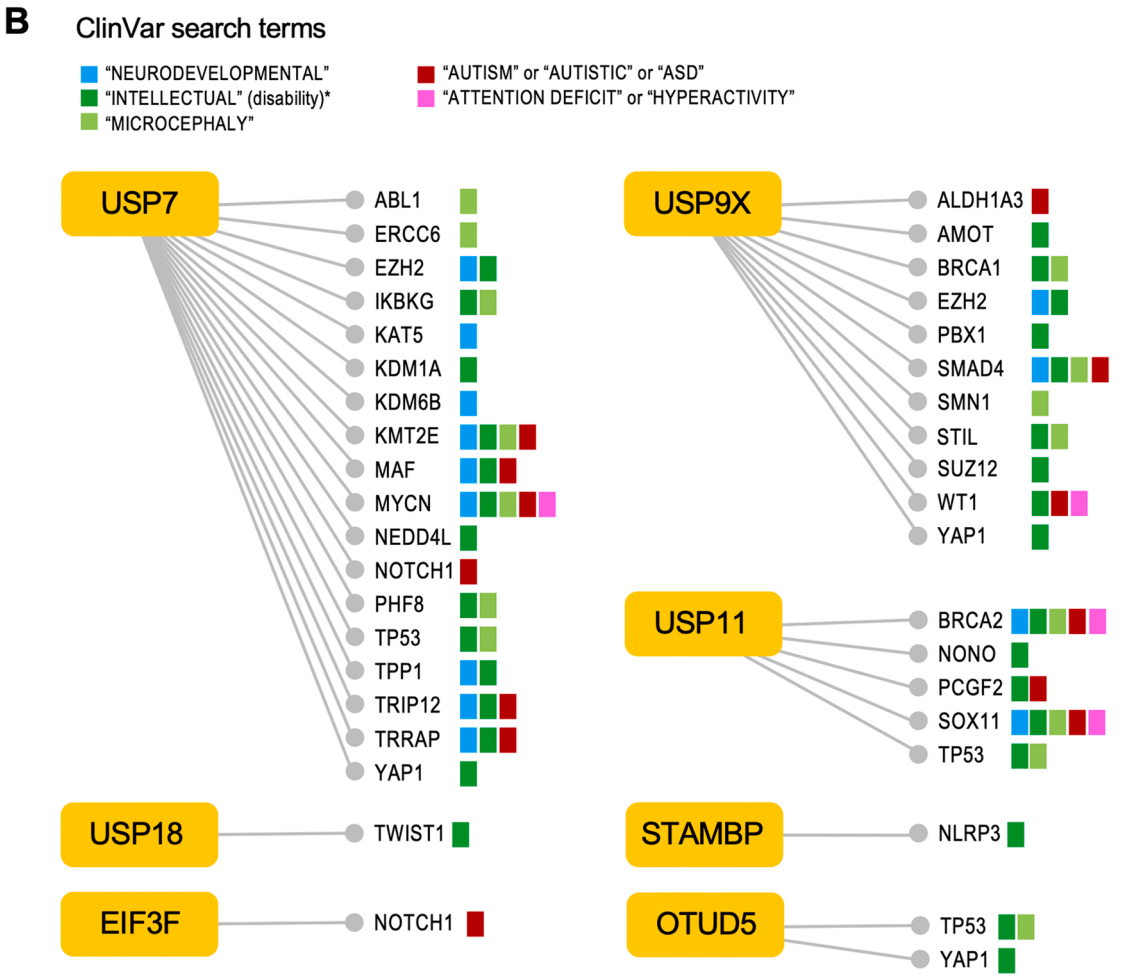


Fig. 4. Using DUBase and ClinVar to search for potential phenotypic overlaps between mutations in DUBs and their substrates. A. Flowchart illustrating the approach. In all ClinVar searches the following Filters were applied: "Clinical significance" = *Likely pathogenic* or *Pathogenic*, "Molecular consequence" = *Missense*, *Frameshift*, *Nonsense* or *Splice site*, and "Variant-gene relationship" = *Single gene*. *The term "mental retardation" was used in the past to refer to intellectual disability. Although it is now disused, it was also included in the search, as it is still present in the ClinVar database to describe certain conditions. B. DUB substrate-encoding genes retrieved by searching ClinVar with the indicated search terms, and structured according to their corresponding indicated DUBs.

the mTOR, WNT, or TGFβ pathways [68,70], or to brain function, such as glutamate receptor signaling in synaptic plasticity [79].

The contribution of mutations in specific DUBS, such as USP9X or USP7, to the molecular pathology of NDDs has been, at least partially, elucidated over the last years, as recently reviewed in detail [68]. For example, USP9X mutations might lead to syndromic and non-syndromic ID by interfering with USP9X-mediated regulation of the TGFβ signaling pathway [80] or with processes such as ciliogenesis [81], centriolar duplication [82], or dendritic spine development [83]. On the other hand, the pathogenic effect of NDD-related USP7 mutations has been shown to be related to their interference with proper endosomal protein trafficking mediated by the MAGE-L2-TRIM27 complex [84].

Most recently, the mechanism that underlies the pathogenic consequences of a hemizygous missense mutation in USP11 (R241Q) has been described [85]. This USP11 mutation had been previously detected in a male patient with ID and brain malformations [78]. Using a mouse model, Chiang et al. show that Usp11 regulates several aspects of brain development by promoting deubiquitination, stabilization and upregulation of Sox11 during cortical neurogenesis. Usp11 deficiency impairs cortical neuron production and migration, leading to a phenotype that includes cognitive and behavioral disturbances related to human NDDs. Importantly, the R241Q mutation was shown to disrupt the ability of Usp11 to deubiquitinate and stabilize Sox11, and the mutant protein was unable to support cortical neurogenesis and neuronal migration [85].

In contrast to the three examples described above, however, the biological mechanisms underpinning the phenotypic consequences of NDD-related DUB mutations are, in most cases, still poorly characterized. A key element in elucidating the causal links between DUB mutations and NDDs is a better understanding of DUB function, which requires a more complete identification of the substrates of these enzymes. In this regard, we believe that, by providing extensive and easily accessible information on DUB substrates, DUBase may represent a useful resource to explore the pathogenic role of DUB mutations in NDDs and other human diseases. To illustrate this point, we have combined DUBase and the ClinVar mutation database to collect information on the phenotypic overlap between mutations in DUBs and their substrates, which might point to a possible mechanistic link (Fig. 4).

First, ClinVar was searched for mutations causing NDD-related conditions (Fig. 4A). To this end, a set of separate searches was carried out using the following terms: “ASD”, “attention-deficit”, “autism”, “autistic”, “hyperactivity”, “intellectual”, “mental retardation”, “microcephaly” and “neurodevelopmental”. The following DUB-coding genes were retrieved in one or more of these searches: ALG13, EIF3F, OTUD5, OTUD6B, STAMBP, USP7, USP9X and USP27X. We also searched ClinVar for mutations reported in each human DUB gene, and manually examined the conditions associated with each mutation. This search led to identification of mutations that result in NDD-related conditions in four additional human DUBs: USP11 (abnormality of brain morphology), USP14 (arthrogryposis with CNS abnormalities), USP18 (pseudo TORCH syndrome 2, including brain malformations) and OTUD7A (specific learning disability, severe global developmental delay). Next, DUBase was used to obtain a list of high-confidence substrates for each of these 12 DUBs. Finally, ClinVar was used again to determine which DUB substrate-encoding genes were retrieved in searches for mutations associated to NDD-related conditions.

Initially, a total of 35 genes encoding substrates for 8 different DUBs were retrieved. Next, we used PubMed, OMIM, and the PanelApp knowledgebase [86] to obtain further information on the strength of the evidence linking mutations in these 35 genes to NDDs. After this manual curation step, the 33 genes encoding substrates for 7 different DUBs shown in Fig. 4B were retained. Of note, some of these genes, such as EZH2, have been reported as substrates for more than one DUB.

Supporting the validity of this approach, some expected phenotypic overlaps were retrieved, for example between mutations in USP9x and mutations in STIL [82] or SMAD4, an important regulator of TGFβ signaling [87]. It was also expected to find an overlap between the phenotype caused by mutations in USP11 and SOX11 [85]. It must be noted that the purpose of the analysis presented here is to exemplify a potential use of DUBase, but does not pretend to be an exhaustive search for NDD-related DUB substrates. Nevertheless, some of the phenotypic overlaps revealed in this search may provide the rationale for potential lines of future experimental investigation.

5. Conclusions and future directions

The case-study on NDD-related DUBs and substrates presented here illustrates how, besides contributing to a better understanding of the basic physiological functions of human deubiquitinases, DUBase may also facilitate exploring the link between these enzymes and different human pathologies. Similarly, investigators with other research interests will be able to employ the data collected in DUBase to explore how DUB enzymes regulate other pathways. Additionally, DUBase will provide an easy access point to the growing literature on DUB substrates. This has been achieved by establishing some simple objective criteria, which we hope will provide a clear guidance for future studies.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.semcdb.2022.01.001.

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