

Perspective

# Never Fold to Fold Continuously: A Conundrum in Ubiquitin–Proteasome System (UPS)-Mediated Protein Quality Control (PQC)

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**Abstract:** In the last few decades, the traditional paradigm of teleonomy, in which the amino acid sequence of a protein is tightly associated with its structure and, in turn, with its function, has been partially undermined. The idea of a protein as a two-state object has been superseded by that of understanding it as a multistate object. Indeed, some proteins, or portions of a protein, display intrinsically disordered regions (IDRs), which means that they lack stable secondary or tertiary structures. While we are aware that IDRs are present in almost half of the total human proteins, we are still quite far away from understanding their contextual-specific functions and figuring out how they mechanistically work. In the present perspective article, we will attempt to summarize the role/s of IDRs in ubiquitin–proteasome system (UPS)-mediated protein quality control (PQC) at different levels, ranging from ubiquitination to protein degradation through the proteasome machinery up to their role in decoding the complex ubiquitin code. Ultimately, we will critically discuss the future challenges we are facing to gain insights into the role of IDRs in regulating UPS-mediated PQC.

**Keywords:** ubiquitin–proteasome system (UPS); protein quality control (PQC); intrinsically disordered regions (IDRs)



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## 1. Introduction

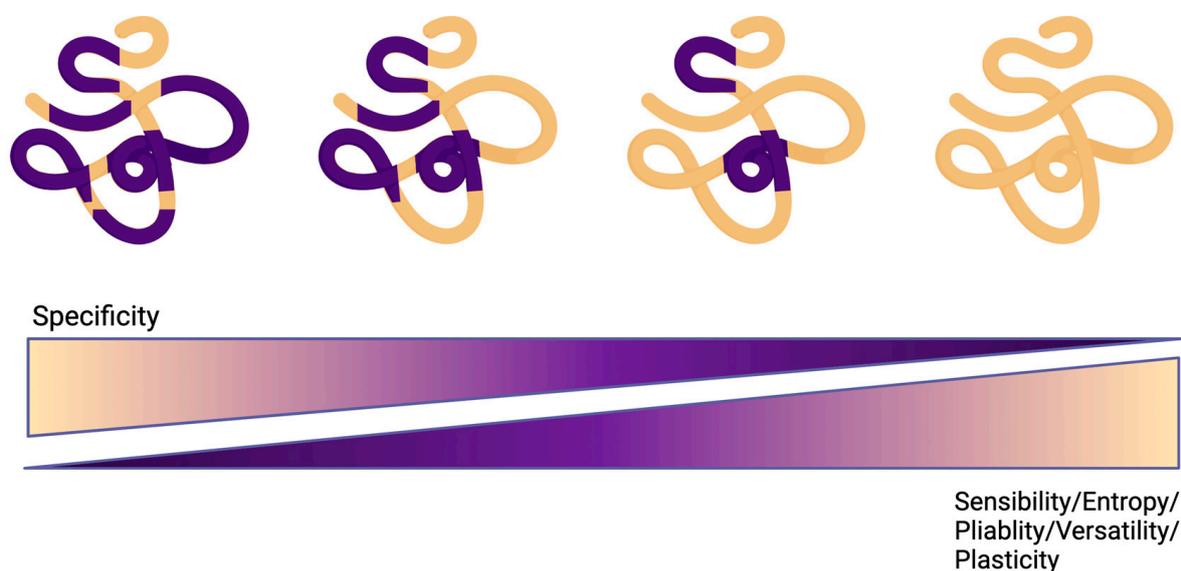
Regardless of the kind of cell, either prokaryote or eukaryote, proteins are the workhorses that accomplish specific tasks with a selective aptitude. Some are structural and thus confer to the cell its shape and motility. Some bind to molecules and transport them to different subcellular compartments, while others catalyze processes that eventually enable cell division and growth [1]. Proteins have seemingly straightforward features allowing for a great deal of variation and specificity in functions. Remarkably, proteins very seldomly act on their own. Usually, they interact with each other to form supramolecular complexes that regulate a plethora of cellular processes. Besides protein–protein interaction, proteins can also interact with other biological macromolecules, including nucleic acids, sugars, and lipids. The complex interactomics network is governed by the physicochemical features and three-dimensional (3D) structures of proteins, which do not necessarily have to be tightly constrained as two-state objects [2].

Consistently, a protein’s shape determines its function, and when protein production goes wrong, the resulting incorrect proteins produce a variety of troubles from the unpleasant (when they form a sticky, clumpy mess inside of cells) to the detrimental (when proteins neglect their crucial role). Errors frequently occur during the process of protein synthesis, or throughout their “life”, thus leading to proteins misfolding, which has been connected to a variety of human illnesses [3].

For these reasons, cells have developed alleviatory mechanisms that are aimed at removing misfolded and damaged proteins. The latter, known as protein quality control (PQC) pathways, aim to either fix (i.e., refold) the proteins or degrade the recalcitrant clients [4]. Degradative PQC displays high selectivity toward the misfolded proteins and broad inclusivity of the many substrates that can be handled. Remarkably, the detection of quality control substrates requires recognition of the common structural hallmarks of misfolding that are found in a wide variety of unrelated proteins but are absent in their normal counterparts.

## 2. Intrinsically Disordered Regions (IDRs)

The intrinsically disordered regions (IDRs) within proteins are ubiquitous across all kingdoms of life [5], and they are usually referred to as biologically active peptide stretches, or proteins (IDPs), which do not possess stable secondary or tertiary structures; in addition, they are highly dynamic and pliable [6], as depicted in Figure 1.



**Figure 1.** A diagram distinguishing the hallmarks of fully structurally stable proteins and intrinsically disordered regions that contain the proteins involved in UPS-mediated PQC. Progressing from left to right, a gradual decrease in the structurally stable regions (purple) was found to be consistent with a reduction in specificity (i.e., the ability to recognize a unique substrate based on its 3D conformation). Conversely, as the number of intrinsically disordered regions (IDRs) increases, so too does the sensitivity—i.e., the capacity to recognize multiple substrates sharing similar but not identical features—at the expense of specificity. Accordingly, the disordered motifs are characterized by higher entropy, pliability, flexibility, and versatility.

In addition, IDRs are characterized by peculiar biophysical and structural properties, namely their amino acid (aa) composition heterogeneity; low content of hydrophobic aa residues; the high net charges associated with a lack of significantly ordered secondary structures; flat energy landscapes; their involvement in biomolecular condensates; their remarkable binding promiscuity; and their ability to gain different structures upon binding to different partners. Eventually, they maintain an essential amount of disorder even in their bound form [7].

Previously, IDRs were merely understood as no more than passive pieces in protein sequences, wherein they simply “linked” well-structured domains. Later, the opinion was profoundly revised, and, currently, the notion that IDRs are robustly engaged in the diverse functions mediated by protein/s is fully recognized [6,8,9]. Overall, these regions broaden the classical notion of the structure–function paradigm, and thus that of the protein function itself.

Remarkably, IDRs frequently undergo post-translational modifications (PTMs), thus increasing the functional states in which a protein occurs [10,11]. Furthermore, IDRs can expose short linear peptide motifs (3 to 10 residues), thus enabling the interaction with structure domains in other proteins [12,13].

These features, either alone or in combination, allow for the spatio-temporal interaction and recruitment of various proteins, thereby potentially facilitating the regulation of nearly all cellular processes [12].

Based on their functionality, the IDRs of proteins have been proposed as falling into the following categories: (I) Entropic chains, which perform biological functions without ever becoming a real structured entity (e.g., flexible linkers and spacers) [14,15]. (II) Modification or display sites, which are classified as such due to their predisposition to be post-translationally modified because of the conformational flexibility of the disordered regions providing advantages when compared to well-structured and constrained domains. Flexible regions facilitate the attachment of different chemical moieties and, upon being post-translationally modified, can result in a disordered region losing conformational freedom. Proteins like histones, p53, E-cadherin, p130Cas, and glucocorticoid receptors are a few examples of proteins where the PTMs within their IDPRs play a critical role in their regulation and functioning [16–19]. (III) Chaperones, which are classified as assisting biomolecules (i.e., proteins and RNAs) in folding. Notably, over 50% of the sequences of RNA chaperones and approximately one third of that of protein chaperones are made by disordered segments. Disordered chaperones, which are formed by binding and solubilizing misfolded proteins, prevent the formation of toxic proteinaceous aggregates. To this category belong members such as GroEL,  $\alpha$ -crystallin, Hsp33, and hnRNP1 [20–22]. (IV) Molecular recognition effectors, which are understood to, upon binding to other proteins, modify their actions. Such IDPRs often undergo disorder-to-order conformational transition upon binding to their interacting partners' coupled folding and binding (i.e., WASP, p21, p27, DisUBM containing proteins, and adenovirus E1A oncoproteins) [23–25]. (V) Assemblers, which, as suggested by the name, interact with multiple binding partners, thereby bringing them together and thus encouraging and promoting the formation of higher-order protein complexes, such as members of the transcription preinitiation complex, ribosomes, and RIP1/RIP3 necrosomes [26–28]. Lastly, we have (VI) scavengers and sponges, which are classified as such as they are capable of storing and neutralizing small ligands and heavy metals (i.e., chromogranin A, caseins, and other calcium-binding proteins) [29–31].

### 3. The Ubiquitin Proteasome System—UPS

Among the eukaryotic reversible PTMs, ubiquitination (or ubiquitylation) is one of the most abundant and evolutionary conserved [32,33]. Ubiquitination utterly reshapes a protein's functions, thus influencing nearly all the cellular processes [34,35]. Ubiquitination entails the synthesis of a covalent isopeptide bond between the C-terminal residue (Gly) of the short (76-aminoacids) ubiquitin (Ub) and protein substrates [34]. The prevalent ubiquitination sites are Lys residues. Nonetheless, Ub can also, less frequently, be attached to N-terminal Met, Ser/Thr hydroxyl, and Cys thiol groups. Remarkably, ubiquitylation rules the fate of proteins. Indeed, in conjunction with protein synthesis, it contributes considerably to cellular proteostasis and PQC [36]. Hence, ubiquitination exerts its action on proteins by either inducing them to proteolysis through the ubiquitin–proteasome system (UPS), thus shortening its half-life, or by affecting its interactions with other proteins. The fate guidance is assigned to a complex and detailed code [37], which is deciphered with the help of “decoders”, as represented by Ubiquitin-Binding Domain (UBD)-containing proteins [38]. The Ub monomer can be directly coupled to a target protein or to itself via one of the seven Lys residues, or through the N-terminal Met residue in the case of linear head-to-tail Ub-chains, which is achieved with an isopeptide bond [39].

The ubiquitination process necessitates the serial activity of Ub-activating (E1), -conjugating (E2), and -ligating (E3) enzymes. The hierarchical cascade is energy-dependent. The E3 ligase

superfamily encompasses the following four distinct families based on their catalytic features and 3D structure: RING (Really Interesting New Gene), U-box, HECT (Homologous to the E6AP Carboxyl Terminus), and RBR (Ring Between Ring) [40]. Differently from the RING and U-box families, HECT and RBR family members (which are a kind of RING-Hect hybrid) directly catalyze the Ub-transfer to the substrate through a two-step reaction. Specifically, Ub is first transferred to a catalytic Cys residue on the E3 ligase, and it is then covalently linked to the substrate. Common to all the E3 ubiquitin ligases, is the interaction with both the target substrate and E2 ubiquitin ligase, which then leads to the direct or indirect transfer of ubiquitin from the E2 to the target substrate.

By binding to the target substrate, the large E3 ligase members determine the selectivity and specificity recognition of the aforementioned families. There are many hundreds of E3 members that recognize a plethora of diverse substrate proteins; occasionally, these can be a single protein and, sometimes, they can be whole families with conserved structural features [41,42]. While the selective substrate identification and recognition of most of the RING members require the assembly of multi-subunit complexes, the N-terminal region appears to be sufficient for the HECT and RBR members. Notably, a degree of redundancy and multiplicity is crucial in the UPS, namely that individual protein substrates may be targeted by multiple E3 members and that a single E3 may have multiple protein substrates.

The collaboration of the E1, E2, and E3 members mediates the following three different types of ubiquitination: (I) when a single passage through an E1–E2–E3 enzymatic cascade leads to a monoubiquitinated substrate (i.e., mono-ubiquitination); (II) when additional passages, where several single Ub are attached to the target proteins, yield multi-ubiquitinated substrates (i.e., multi-, polymono-, or multi mono-ubiquitination); and (III) when poly-Ub chain/s are ligated to the substrates (poly-ubiquitination) [43].

The reversibility of the process is guaranteed by a special class of deubiquitin hydrolase (DUB) enzymes [44].

Severely damaged or misfolded proteins that cannot be amended with the aid of chaperones are usually tagged with poly-Ub chains, which are linked through K11 or K48, and they then address the proteasome for degradation [45,46]. The 26S proteasome is a 2.5 MDa complex composed of different protein subunits that are arranged into an elongated structure composed of a central 20S core particle (CP) with one or two terminal 19S regulatory particle(s) (RP(s)) [47]. Ubiquitinated proteins are recognized by the RP and unfolded through an energy-consuming, ATP-dependent mechanism. Ultimately, the unfolded proteins are then channeled to the catalytic CP, where the inner beta subunits, thanks to their different proteolytic activities, degrade them.

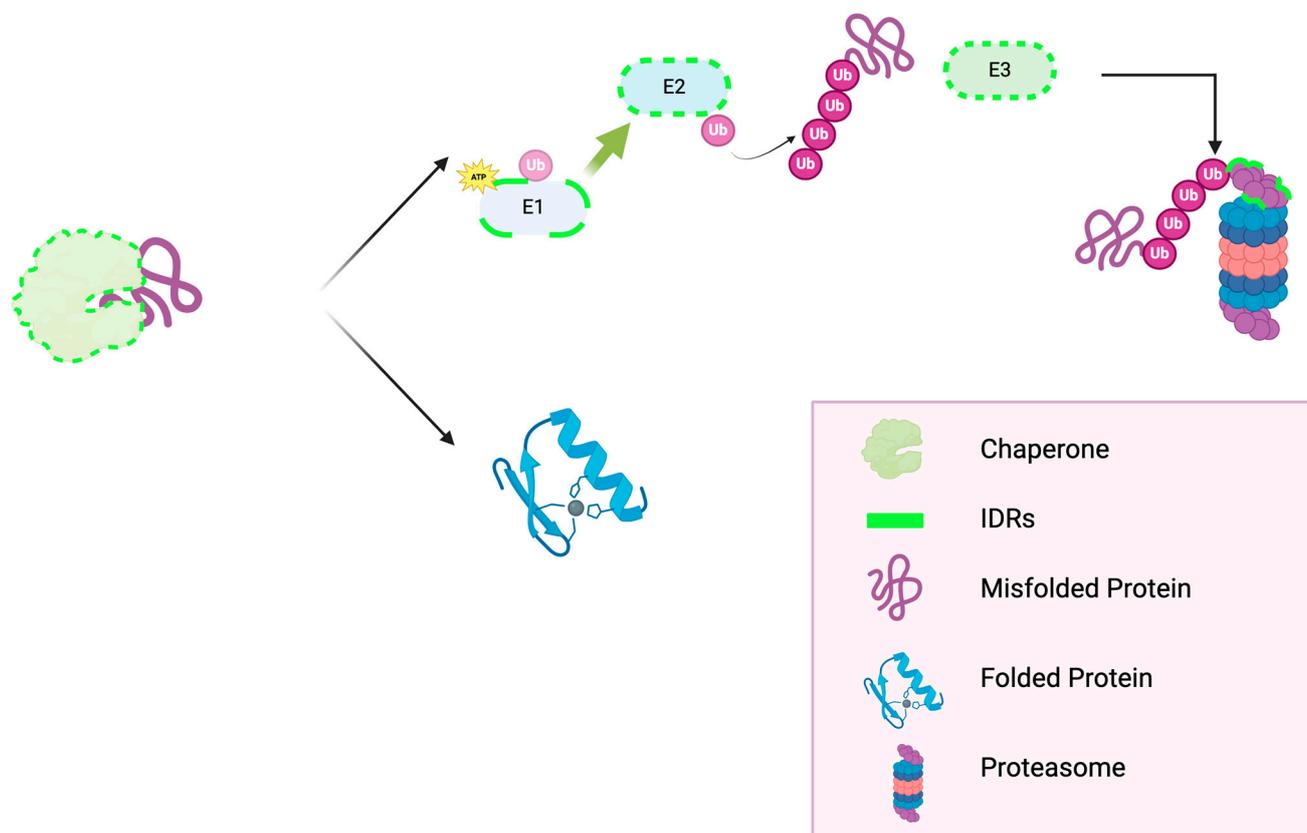
#### 4. The IDRs in UPS- and Chaperone-Mediated PQC

The functions and regulations of proteins, including those of several PQC players, are governed by a combination of (a) ordered and (b) intrinsically disordered regions. The experimental, coupled with computational, evidence indicates that IDRs are crucial components of different PQC components at different levels (e.g., protein–protein recognition, protein ubiquitination, and degradation), either in proteasome-mediated or in lysosome-mediated (i.e., autophagy)—which will not be discussed here—protein degradation.

Upon damage compromising its 3D structure, and depending on the damage severity, a protein is not immediately degraded via UPS, but the cell will attempt to refold it with the aid of molecular chaperones [20,22]. How chaperones mechanistically recognize the different clients, how they discriminate between the native/folded state and the denatured/misfolded state, and how they handle the different misfolded states (i.e., the misfolding that occurs due to physical agents versus that which is due to different point mutations occurring on different protein regions) remain mostly unknown. Remarkably, any protein can potentially become misfolded, and it will do so in ways that are distinct from other proteins. Consistently, chaperones must display unique features that enable them a wide degree of “degeneracy” and “plasticity”, which represents the prerequisite of selectively recognizing the “damaged” proteins. IDRs have been reported in chaper-

ones [48,49], whereby they contribute to substrate recognition and interactions [50–52] by distinguishing abnormal proteins from normal ones. Thus, chaperones robustly rely on IDRs to discriminate and recognize the wide palette of distinctly shaped misfolded proteins they encounter.

On the other hand, though still a matter of debate, several studies have documented the importance of a disordered region for efficient proteasomal degradation [53–56]. To some extent, it seems that the IDRs embedded in a protein condemned to proteasomal degradation will facilitate energy saving for RPs during its unfolding duty. Over the past decade, quite a few bioinformatic approaches, which have employed multiple different protein disorder predictors, have been undertaken to survey the presence of the IDRs in UPS components. Based on those findings, it appears that IDRs are present in each component of the UPS (i.e., E1, E2, E3, proteasome, DUB, and Ub-binding domain-containing proteins). By surveying the whole human repertoire of E1-activating, E2-conjugating, and E3 Ub-ligases, a steep increase in IDRs emerges from E1 to E2. In addition, it has also been noted that there is a further, but to a lesser extent, rise from E2 to E3 with a trend of  $E1 < E2 < E3$  [57] (Figure 2), as is shortly summarized in Table S1.



**Figure 2.** The potential fates of a misfolded protein. Chaperones rely on intrinsically disordered regions (IDRs), which are depicted as a bright green dashed border here, to recognize misfolded proteins. Recalcitrant unfolded proteins are targeted for degradation via the ubiquitin–proteasome system (UPS). Notably, a significant increase in IDRs was observed in the E1-activating enzymes and E2-conjugating enzymes, with a moderate yet discernible rise also noted between the E2 and E3-ligases, which are depicted as bright green dashed borders. In some cases, E3 Ub-ligases recognized the substrate thanks to the structural pliability conferred by the IDRs. The recognition of ubiquitinated proteins, as well as the Ub-chain removal before channeling the protein into the core, by the proteasome was due to the proteasome cap, which hosts quite a few IDR-containing proteins (created with the help of BioRender).

More recently, an updated inquiry has been carried out on E1 and E2 proteins, which displayed slight discrepancies, likely due to the different and upgraded predictor algorithms [58]. Notably, E3 Ub-ligases often act as multimeric complexes and require the aid of “professional folding assistants”, like chaperones, to discern and selectively recognize their substrates. How monomeric E3 mechanistically discriminate the different substrates by themselves has long been a debated and rather thorny issue. In this framework, a few years ago, experimental evidence was provided to prove that the nuclear yeast E3 ligase, San1, selectively and autonomously detects several misfolded proteins for nuclear PQC, as well as brokers the transfer of Ub [59]. Elegantly, the authors uncovered that San1 harbors IDRs by flanking the RING domain, as well as that the aforementioned proteins are pivotal in selectively and autonomously recognizing the substrates by combining different motifs, namely short peptide sequences, which are embedded within IDRs. Thanks to those sequences, the E3 ubiquitin ligase discriminates between dozens of different substrates. In other words, the combination of the motifs leads to a sort of “dynamic code”, which is used for the recognition of the diverse substrate without the assistance of chaperones. Several proteasome-associated proteins display IDRs within them, including the proteasome-associated poly-Ub receptors PSMD4 and ADRM1, as well as the DUBs USP14 and ATXN3 [58]. Ubiquitination has been proven to be present among the most complex PTMs due to the enormous complexity of the Ub-moieties attached to the substrate [35]. Indeed, Ub can be attached as monomers or as chains. The latter can be homo- or hetero-typic, thereby giving rise to a complex code. A fact that is not to be missed is that unanchored free Ub-chains have been also reported and their role/s has/have not been fully explored; in addition, their biological significance is currently being debated upon, and in many other respects the understanding of them is rather vague [60]. Thus, Ub—besides its function in addressing a protein to the proteasome, as well as to its destruction—delivers an array of signals that require to decoding. This job is executed by several so-called “readers”, which, by selectively recognizing the different alternative Ub-moieties (i.e., monomeric Ub versus Ub-chains), transduce the input signals into specific cellular outputs. This is accomplished thanks to a palette of Ub-binding proteins [61]. Very recently, two independent groups have provided experimental evidence that IDRs play a crucial role in recognizing Ub moieties [25,62]. Among the variety of Ub-binding domains (UBDs), or motifs (UBMs), the Ub-interacting motif (UIM) is very common. They are quite short peptides, i.e., they are approximately 20 amino acid residues in length, and they are present in several proteins, including the proteasomal subunit PSMD4 [63]. Interestingly, they are quite often found organized in tandem or as triplets [64], and their binding partners are not restricted to Ub [65]. Human ATXN3, whose mutated form is associated with the neurodegenerative Machado–Joseph disease, harbors 3 UIM. Recent findings have indicated that the C-terminal displays unique features, whereby it accounts for both a stable structured conformation and more disordered ones [62]. Remarkably, the latter confers to the region a higher pliability, and thus a higher versatility, toward a broader range of interactors that are beyond the Ub itself. Yet, recent computational inquiries suggest that some protein regions can recognize Ub via a process of folding upon binding [25]. Based on this premise, by combining peptide binding arrays, NMR spectroscopy, and bioinformatics, a novel disordered Ub-binding motif (DisUBM) was freshly identified and characterized. Astonishingly, DisUBM likely remains disordered even upon Ub binding. These findings further, potentially, and enormously expand the repertoire of Ub-binding proteins.

## 5. Outlook and Outstanding Questions

In the recent past, we have witnessed a growing body of evidence indicating that the different UPS components implied in PQC rely on disordered motifs to properly fulfill their job. To what extent, if any, the IDRs’ physicochemical properties influence the affinity/selectivity toward the Ub-moiety (i.e., monomeric versus chains, linear chains versus branched chains, and homotypic chains versus heterotypic chains) is currently

unknown. At a superior level, Ub-PTMs contribute to extending the complexity of the Ub-code. Due to their plasticity and versatility, could IDRs be suitable for discriminating between different Ub-PTMs? Conversely, because of the IDRs' pliability, could they be involved in discriminating between the recognition of free-unanchored versus anchored Ub-chains? When present in E3 Ub-ligases, is their role restricted to substrate recognition, or do they, by binding to Ub, contribute to elongating the chain/s? Eventually, do they act as simple flexible linkers or do they allosterically regulate the enzymatic activity? Currently, it cannot be ruled out that the roles of the IDRs in the UPS-mediated PQC framework may not be related to any of the previously questioned features. Perhaps they simply act as "docker" regions, thus controlling the subcellular localization of the E3 members. Ultimately, should we expect the UBDs family to be replenished with novel members whose peculiar feature is not attributable to an aminoacidic consensus sequence, or a constrained 3D shape, but rather to a variety of regions that simply share disordered and pliable organization? These are just a few questions that currently await answers. Currently, the experimental evidence, which has been aimed at physicochemically, mechanistically, and functionally characterizing the IDRs implied in the UPS-mediated PQC, is still rather restricted and requires robust implementation in the future. Genetic approaches aimed at dissecting the context-specific role/s of IDRs (i.e., by chimerically combining them, using a knock-in approach, or via mutagenesis) would be of great benefit for unraveling their functional behavior. Furthermore, proteomics would enable us to improve our current, but so far still rather scant, knowledge of their binding promiscuity. Ultimately, based on the existing scientific community projects (i.e., ELIXIR) [66], as well as on the freshly developed deep- and machine-learning approaches [67], the prediction of IDRs should be greatly facilitated and boosted.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biophysica4020011/s1>, Table S1: Summary of IDRs in the human E1, E2, E3 repertoire (adapted from Bhowmick P. et al. [57]).

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