# Charting the alternative usage of protein repeated regions in evolution and its implication for protein interactions

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# Highlights

- Robust detection of alternatively used repeated protein regions in evolution
- The approach relies on sequence similarity and identifies conserved signatures
- Mapping of the repeats onto protein isoform 3D models predicted by AlphaFold
- 5 000 repeats detected over the human coding fraction, about one third disordered
- Assessment of the structural coverage of their interactions with protein partners

**Abstract**

Alternative splicing of repeats in proteins provides a mechanism for rewiring and fine-tuning protein interaction networks. In this work, we developed a robust and versatile method, ASPRING, to identify alternatively spliced protein repeats from gene annotations. For this, we leveraged an evolutionary meaningful alternative splicing-aware hierarchical graph structure introduced in our previous work. We re-think the definition of repeats by explicitly accounting for transcript diversity across several genes/species. Using a stringent sequence-based similarity criterion, we detected over 5,000 evolutionary conserved repeats by screening virtually all human protein-coding genes and their orthologs across a dozen species. Through a joint analysis of their sequences and structures, we extracted specificity-determining sequence signatures and assessed their implication in experimentally resolved and modelled protein interactions. Our findings demonstrate the widespread alternative usage of protein repeats in modulating protein interactions and pave the way for targeted design of repeat-mediated interactions.

**Keywords:** protein repeats, alternative splicing, protein evolution, protein interactions, AlphaFold models

# Introduction

Repetitive elements are a pervasive feature of proteins, ranging from simple tandem repeats to complex repeat architectures. These repeated regions often play crucial roles in protein function by mediating protein-protein interactions, facilitating protein folding, or acting as flexible linkers between functional domains (Schüler and Bornberg-Bauer, 2016; Kajava, 2012; Andrade et al., 2001). As such, many efforts have been engaged to detect and classify them, as well as characterize their structural and evolutionary properties (Paladin et al., 2021; Delucchi et al., 2020; Pagès and Grudinin, 2019; Schaper et al., 2015; Heger and Holm, 2000; Björklund et al., 2006; Marcotte et al., 1999). Precisely delineating their boundaries remains however challenging. Should it be based on structure, sequence, or a combination of both? A recent census of adjacently repeated sequence patterns, or *tandem repeats*, revealed a wide variability in the characteristics of the repeated units and the number of repetitions (Delucchi et al., 2020). While tandem repeats show enrichment in intrinsic disorder, some fold into specific structures such as solenoids or have "beads on a string" organizations (Kajava, 2012).

Protein repeats are more prevalent in Eukaryotes and Viruses than in Archaea and Bacteria (Delucchi et al., 2020; Marcotte et al., 1999). Toward understanding their origin and expansion, several studies have investigated the extent to which they agree with or

violate the exon-intron structure of genes (Paladin et al., 2020; Björklund et al., 2006). These studies uncovered a variety of scenarios, and proposed the use of evolutionary patterns inferred from the intron-exon boundaries across species to refine the delineation and classification of the repeats. Tandem repeats mostly originate through duplication (Delucchi et al., 2020), and exon shuffling can explain their expansion in some families albeit not in all (Björklund et al., 2006).

Recent works have highlighted the importance of alternative splicing (AS) in modulating the number of protein repeats and their amino acid composition (Osmanli et al., 2022; Martinez Gomez et al., 2021; Zea et al., 2021). AS, along with alternative promoter usage and alternative polyadenylation can produce multiple mature mRNA transcripts from a single gene (Ast, 2004). These transcripts may lead to different protein isoforms with distinct shapes (Birzele et al., 2008), interaction partners (Yang et al., 2016), and functions (Baralle and Giudice, 2017; Kelemen et al., 2013). Over the human coding fraction, a couple of thousand genes, mainly involved in cell organization, muscle contraction, and inter-cellular communication, show evidence of evolutionary conserved AS patterns modulating the usage of similar exonic sequences (Zea et al., 2021). Alternative transcripts tend to contain less tandem repeats than canonical ones and the associated deletion events do not alter the overall protein 3D structure (Osmanli et al., 2022). Mutually exclusive tandem homologous exons have drawn the most attention, with substantial efforts for manually curating their annotations and verifying them with transcriptomics and proteomics data (Abascal et al., 2015; Martinez Gomez et al., 2021; Abascal et al., 2015). They are prevalent, have clinical importance, and ancient evolutionary origin.

One key question is defining the pertinent entities to assess the alternative usage of protein repeats. Ideally, these entities should make sense across evolution and account for the full protein diversity generated by AS. Understanding the modularity of protein repeats is also essential, as it may shed light on their functional relevance in protein-protein interactions. We recently proposed a solution for matching protein isoforms across species toward comprehensively describing AS-induced diversity and assessing its evolutionary conservation (Zea et al., 2021). To do so, we introduced evolutionary splicing graphs (ESGs) as a generalization of the notion of splicing graph (Heber et al., 2002) to many genes/species. Each node in an ESG is a spliced-exon, or s-exon, defined as a set of aligned exonic sequences coming from several orthologous genes (**Fig. 1**, top left panel). The s-exons are minimal building blocks for transcripts in evolution.

In this work, we report on a robust and versatile method, ASPRING (Alternatively Spliced Pseudo Repeats IN-Gene), to identify protein repeats across a set of genes/species with evidence of evolutionary conserved alternative usage. Our approach relies on sequence-based duplication detection and leverages both evolutionary conservation and AS-generated diversity through the use of ESGs (**Fig. 1**). It first detects pairs of s-exons with similar sequence profiles and then combines them following the topology of the ESG to delineate spliced-repeats, or s-repeats. ASPRING finally groups together similar s-repeats, by transitivity, into alternatively spliced repetitive units (ASRU, **Fig. 1**, top right). An s-repeat can be viewed as an instance of an ASRU and it is linked to other s-repeats within the same ASRU by sequence similarity and also by a set of AS events. The original contribution of our approach is twofold. It explicitly accounts for both evolution and AS right from the beginning by directly operating on s-exons, instead of species-specific protein sequences. And it defines evolutionary meaningful and AS-aware entities, the ASRUs, thereby going beyond repeat enumeration.

By applying ASPRING to the human protein coding fraction, we detected over 5,000

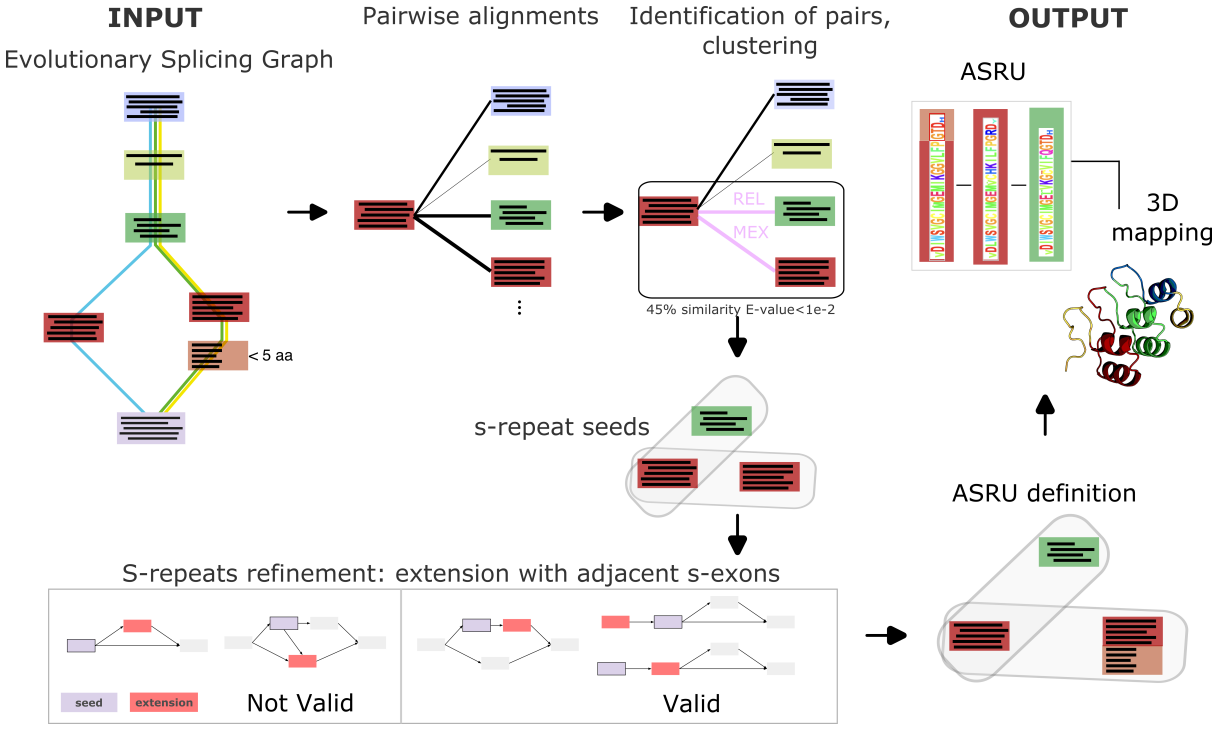


Figure 1: **Overview of ASPRING algorithm.** ASPRING takes as input a Gene Ensembl ID and the ESG computed with ThorAxe for this gene and its one-to-one orthologs in a list of input species. The MSAs defining the s-exons are depicted within the nodes. Three observed transcripts are highlighted as colored paths. The bubble in the graph indicates an AS event of the mutually exclusive type. ASPRING creates the profile HMM for each s-exon of at least 5 residue long, performs all-to-all pairwise profile HMM-HMM alignments and filters the alignments with user-defined parameters values. It infers a set of s-repeat seed and cluster them in ASRUs. The final step consists in extending these seeds with adjacent s-exons in the graph. Valid extensions comply with the graph topology. In this example, ASPRING detected 1 ASRU comprised of two s-repeats. One s-repeat is made of a single s-exon while the other one contains two s-exons. The s-repeats, along with the other s-exons, are mapped onto the 3D structural model of the protein isoforms corresponding to the input transcripts.

repeats defined across a dozen species spanning 800 million years of evolution. We extracted specificity-determining sequence signatures, providing novel insights into the mechanisms of protein-protein interactions. To further elucidate the functional and structural implications of these repeats, we mapped the identified repeats onto experimental 3D structures of monomers and complexes available in the Protein Data Bank (PDB) (Berman et al., 2000), as well as 3D models predicted by AlphaFold2 (Burke et al., 2023; Sommer et al., 2022; Varadi et al., 2022; Jumper et al., 2021). Overall, this comprehensive analysis demonstrates the importance of considering alternative splicing patterns in these regions. By identifying specificity-determining sequence signatures and investigating their 3D structure, this study sheds new light on the mechanisms of protein-protein interactions and paves the way for targeted design of repeat-mediated interactions.

ASPRING is freely available at `https://github.com/PhyloSofS-Team/aspring` and accessible to all as a Python package and a Docker image.

# Methods

## Overview of ASPRING

ASPRING takes as input an evolutionary splicing graph (ESG) computed by ThorAxe (Zea et al., 2021) and sumarizing the transcript diversity observed in a set of orthologous genes (**Fig. 1**, top left panel). Each node in the ESG, called a s-exon, is an alignment of translated exonic sequences coming from different genes/species. An edge between two nodes indicates that the corresponding s-exons co-occur and are consecutive in at least one observed transcript isoform. Hence, the s-exons can be viewed as the minimal building blocks of the isoforms in evolution. Starting from the input ESG, ASPRING extracts a set of ASRUs (**Fig. 1**, top right panel). Each ASRU is defined by a collection of s-repeats, where an s-repeat can be a single s-exon or an ordered list of several s-exons showing sequence similarity and evidence of alternative usage. ASPRING outputs several tables summarizing information about the ASRUs, the s-repeats, the similar pairs of s-exons and the raw alignments, and also annotated 3D models of the protein isoforms corresponding to the input transcripts (**Fig. 1**, top right panel).

ASPRING algorithm unfolds into four main steps (**Fig. 1**, see also *Supplementary Methods*). It first performs an all-to-all comparison of the s-exons in the input ESG using profile HMMs with the HH-suite3 (Steinegger et al., 2019) (**Fig. 1**, step 1). Secondly, it identifies similar s-exon pairs by considering four criteria, namely the significance of the p-value associated with the profile HMM-HMM alignment, the sequence identity and the coverage shared between the s-exons, and their evolutionary conservation (**Fig. 1**, step 2). By default, we require a high level of shared sequence identity (45%) to allow for contrasting amino acid divergence across isoforms versus species. All filtering criteria can be changed by the users depending on their system of interest. As this point, since our motivation is to assess the AS-induced modulation of the composition and number of protein repeats, we need to verify the existence of some alternative s-exon usage. For this, ASPRING explicitly accounts for the AS events encoded in the topology of the ESG.

Following (Zea et al., 2021), we define as AS event as a variation from a reference *canonical* transcript chosen for its high conservation and length (**Fig. S1A**). Given a pair of s-exons, ASPRING considers four types of AS-mediated relationships, namely MEX, ALT, REL and UNREL, inferred from the role of each s-exon in the AS events (**Fig. 1**, see labels on the pink edges, and **Fig. S1B**). We introduced these four relationships in (Zea et al., 2021). Briefly, MEX s-exons are mutually exclusive and ALT s-exons are interchangeable but not in an exclusive fashion. These two relationships correspond to AS events of the types mutually exclusive and alternative (**Fig. S1**). In a REL pair, one s-exon is located inside the bubble representing an AS event and is thus alternatively used, while the other serves as an anchor for the event and is thus always expressed. The fourth relationship, UNREL, is the weakest one: one s-exon is part of an AS event while the other is located outside the event. The REL and UNREL pairs may be inferred from different types of AS events (**Fig. S1**). In terms of protein sequence proximity, the MEX s-exons are exactly at the same location (and consecutive in the genome). The ALT s-exons can be at the same place or next to each other. The REL s-exons are typically also close to each other in the protein, whereas the UNREL s-exons may be located in remote and completely different contexts. ASPRING determines for each pair of similar s-exons whether it is of type MEX, ALT, REL or UNREL, in that priority order in case multiple relationships exist. It filters out the pairs that do not fit any category, and thus that do not have any evidence of alternative usage.

Next, ASPRING algorithm's third step clusters the selected s-exons using the transitivity principle (**Fig. 1**, step 3). For any two valid pairs of s-exons sharing an s-exon in common, *e.g. (A,B)* and *(A,C)*, ASPRING will group the three s-exons $A$, $B$ and $C$ in the same cluster. The procedure amounts to detecting connected components in a graph where the nodes are the s-exons and the edges indicate valid pairs of similar s-exons. The resulting clusters can be viewed as prototypes of the ASRUs and the s-exons composing them are s-repeat seeds. In the final fourth step, the ASPRING algorithm aims at extending these seeds with adjacent s-exons, according to the topology of the ESG, to obtain more homogeneous ASRUs in terms of length (in aa) of their s-repeats (**Fig. 1**, step 4). We ensure that the s-exons used for extending the seeds comply with the same sequence similarity constraints as the seeds themselves.

On top of the ASRU detection, ASPRING maps the s-exons and s-repeats onto the 3D models of the input isoforms available from the AlphaFold database (Varadi et al., 2022) (**Fig. 1**, top right panel). This functionality allows users to easily visualise the sequential and spatial localisation of the s-exons and s-repeats.

## Application over the human coding fraction across a dozen species

We ran ThorAxe (Zea et al., 2021) on the ensemble of 18 226 human protein-coding genes and their one-to-one orthologs across 12 species, namely three primates (*Homo sapiens*, *Gorilla gorilla*, *Macaca mulatta*), two rodents (*Rattus norvegicus*, *Mus musculus*), four other mammals (*Bos taurus*, *Sus crofa*, *Ornithorhynchus anatinus*, *Monodelphis domestica*), one amphibian (*Xenopus tropicalis*), one fish (*Danio rerio*), and one nematode (*Caenorhabditis elegans*). We downloaded the corresponding gene annotations from Ensembl release 105 (December 2021) (Cunningham et al., 2022). We used the default parameters except that we did not apply any filtering of the transcript based on Transcript Support Level. We obtained exploitable results for a vast number of 17 920 genes. We then ran ASPRING on the computed ESGs using the default parameters (see *Supplementary Methods*). The calculation took approximately 4-5 days on 100 CPUs Intel Xeon Silver 4210 2.2GH. The most computationally expensive step was the generation of the profile HMM alignments with the HH-suite 3 (Steinegger et al., 2019). Once these alignments have been computed for a given gene, the users can re-run ASPRING with different filtering criteria within a few minutes.

We performed a comprehensive analysis of the evolutionary signals encoded in the detected repeats, their 3D structural properties and interactions. For the evolutionary analysis, we focused on the information contained in the alignments generated by the HH-suite. For the structural analysis, we predicted disorder using the protein Language Model-based method SETH (Ilzhoefer et al., 2022). To characterize interactions, we looked at all the physiologically relevant experimental 3D complex structures involving the ASRU-containing proteins or their close homologs. We detected interfaces based on a distance criterion and we mapped them to the sequences of the s-repeats. Experimental PDB structures are often partial and may contain modifications, which makes the mapping sometimes difficult. To circumvent this issue, we used the full-length transcript 3D models available from the isoform.io database (Sommer et al., 2022). In addition, we explored a ressource of 3D complex models predicted by AlphaFold2 Burke et al. (2023). See Supplementary methods for detailed protocols.

# Results and Discussion

We used ASPRING to uncover the alternative usage of protein repeats on the human proteome scale. We analysed 17 920 human protein-coding genes and their orthologs across 12 species spanning 800 million years of evolution, from primates to nematodes. In total, ASPRING screened over 8 million s-exon pairs, among which about 26 500 passed all four filters for p-value, identity, coverage and conservation (**Fig. S2**). We applied a stringent sequence identity threshold of 45% to allow for contrasting the divergence between repeats coming from the same species versus the cross-species divergence of orthologous repeats. Among the retained s-exon pairs, about 11 000 are linked by some alternative splicing event. ASPRING grouped these s-exon pairs in 1 469 alternatively spliced repetitive units (ASRUs) coming from 1 039 genes. An ASRU is made of at least two s-repeats, each one corresponding to a single s-exon or several consecutive s-exons (see *Methods*). In general, the longer the gene the higher the number of detected ASRUs (**Fig. S3**).

## Overall statistics

ASPRING detected exactly one ASRU made of two s-repeats in about two thirds of the 1 039 genes with evidence of alternatively spliced repeats (**Fig. 2A**), in line with previous observations (Osmanli et al., 2022). Examples include the actinin $\alpha$ chains, the integrin subunit $\beta$1, the Ras-related protein Rab-37, and the synaptosome associated protein 25, where ASPRING automatically detected pairs of alternatively spliced tandem duplicated exons previously reported through manual curation (Martinez Gomez et al., 2021). These pairs have proteomic evidence, clinical importance and ancient evolutionary origin (Martinez Gomez et al., 2021).

In a couple of hundred genes, the detected ASRUs contain many s-repeats (**Fig. 2A**), up to 127 for Nebulin for instance. They often correspond to known tandem repeats detected in a row (**Fig. 2B**, on top, in green). ASPRING is however not limited to detecting tandem repeats and some ASRUs contain repeats lying at distant locations in the protein (**Fig. 2B**, on top, in orange). Among the proteins with the most populated ASRUs, we found giant chains playing structural roles, such as those from the collagen. Finally, a minority of genes contain many ASRUs (up to 35), each one made of a few s-repeats (**Fig. 2A**). In such cases, the sequence similarity is at the level of entire domains and the different ASRUs correspond to different parts of these domains (**Fig. 2B**, at the bottom). ATPases, platelet glycoprotein 4, sucrase-isomaltase, and ABC transporters partake in this archetypal situation.

The s-repeats span a broad range of lengths, from 5 to over 5 000 amino acid residues, with a median of 36 residues. Despite this variability, most of the ASRUs display a strong regularity in their s-repeat lengths (**Fig. S4**). We often observed that some s-repeats are exact multiples of others, with multiple similarity hits (**Fig. S4**). Regarding the AS events, mutually exclusive and alternative events are enriched within ASRUs compared to the proportions computed over the entire proteome (**Fig. S5A**). By contrast, insertions are under-represented, reflecting the high evolutionary conservation of the s-repeats (**Fig. S5B**) and in agreement with a recent study (Osmanli et al., 2022). In the following, we adopted a human-centred perspective by focusing on the s-exons and s-repeats present in humans and by taking the human exonic sequences as references.

## Evolutionary signals suggest functional diversification of the repeats

We asked whether the s-repeats belonging to the same ASRU are subject to the same evolutionary constraints. We systematically assessed and compared the conservation profiles of the s-exons ASPRING used to build the ASRUs (see *Materials and Methods*). We found that the vast majority of the positions in the s-exon pair alignments are highly conserved (**Fig. 2C**). More precisely, both s-exons in the pair feature a conserved consensus amino acid (aa) at 85% of the aligned positions, on average. The mutually exclusive pairs display the strongest signal (**Fig. 2C**, MEX), with 90% of their aligned positions conserved in both s-exons. This observation agrees with previous studies emphasising the ancient evolutionary origin and functional importance of such pairs (Martinez Gomez et al., 2021; Abascal et al., 2015). Less than 10% of the positions are variable in both s-exons (**Fig. 2C**).

The s-repeats belonging to the same ASRU are mostly indistinguishable, often because they share the same highly conserved aas (79% of the conserved aligned positions) and in a few cases because they display highly variable and unrelated sets of aas (87% of the remaining positions). Nevertheless, some clear-cut patterns suggest functional diversification of the s-repeats. We illustrate these patterns with the two s-repeats detected in the complement C3d receptor 2 (**Fig. 2D**). At positions marked with a pink star, the same strong selection pressure applies to both s-repeats but for different aas. They correspond to type II specificity-determining site (SDS) in (Chakraborty and Chakrabarti, 2015). For instance, at position 82, the two s-repeats have highly conserved aas exhibiting distinct physicochemical properties, namely Proline and Valine. The same type of pattern occurs at position 9, with the minor difference that Isoleucine replaces Valine in one species, these aas being very similar. This type II SDS contrasts with the preceding positions 2, 4, 5, 7, and 8, where the s-repeats share the same highly conserved aas. Positions 83, 86, and 98 also qualify as type II SDSs, albeit with less striking physicochemical differences or weaker conservation. Positions highlighted with purple triangles indicate a redistribution of the selection pressure over the s-repeat sequences (**Fig. 2D**). They correspond to type I SDS, conserved in one s-repeat and variable in another (Chakraborty and Chakrabarti, 2015).

Beyond these strong conservation signals, several variable positions display non-random covariation patterns suggesting that the same selection pressure has applied to the s-repeats despite phylogenetic divergence (**Fig. 2D**, purple diamonds). For instance, at positions 20 and 87, the two s-repeats are always in match, although the sequences have diverged between rodents and primates. Namely, the Leucine observed in primates at position 20 corresponds to a Serine in rodents. Position 39 features a persistent charge pattern, despite sequence divergence. More precisely, in primates, the first s-repeat displays the negatively charged Glutamate while the second one has the positively charged Arginine. Reversely in rodents, the second s-repeat carries the negative charge and the first s-repeat has the positive one (Lysine).

Overall, we identified 858 ASRUs coming from 628 genes with SDS or covariation sites. They amount to almost two thirds of all ASRUs detected by ASPRING, and most of the time, they contain more than just one type of sites (**Fig. S6A**). The s-exon pairs that do not contain any SDS or covariation sites tend to have more highly conserved positions with a shared amino acid (perfect match) and also less conserved weak matches (**Fig. S6B**).

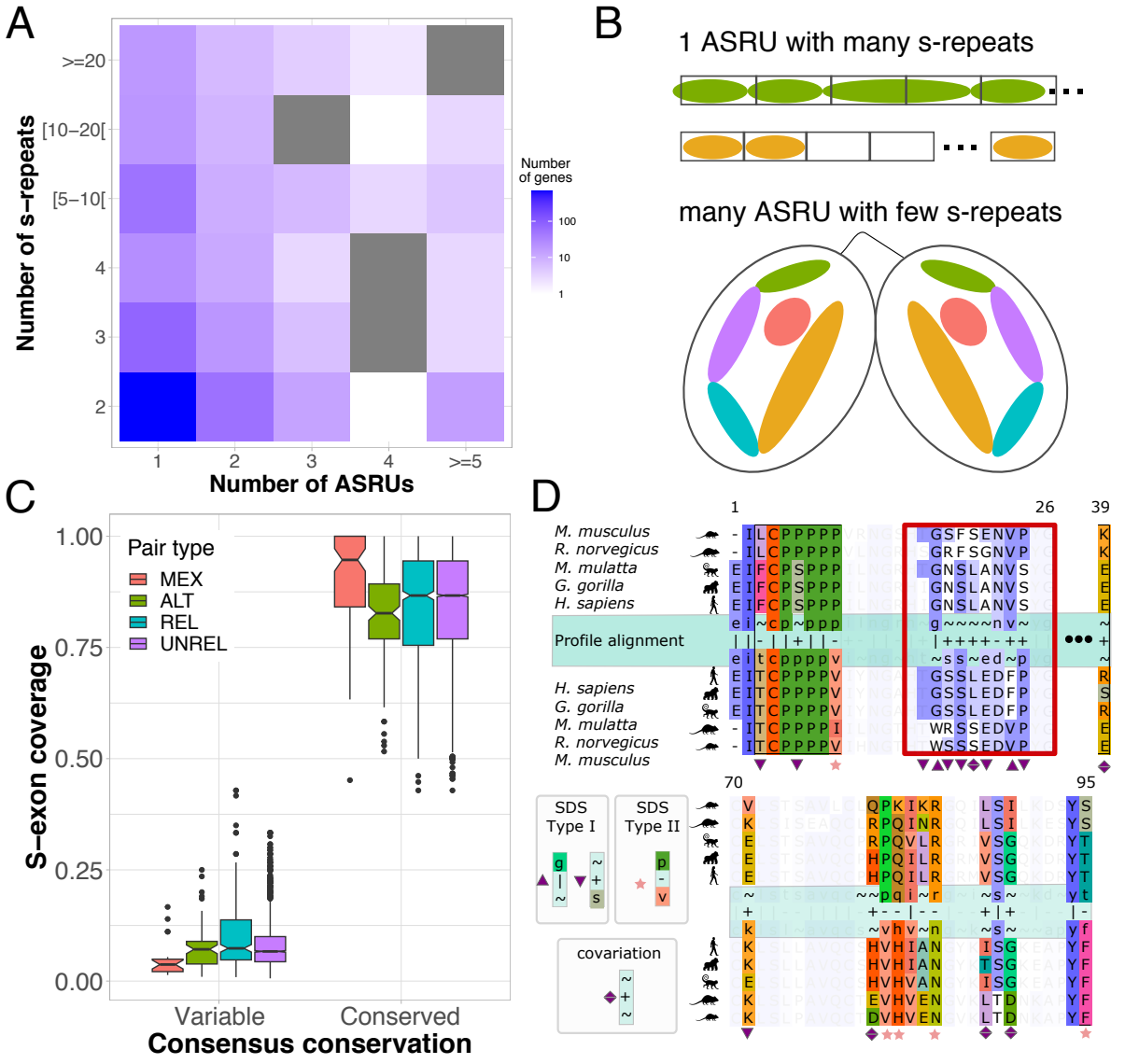


Figure 2: **Overview of the landscape of alternatively used repeats in the human proteome.** **A**. Heatmap showing the distribution of ASRUs and their s-repeat composition. Each cell $(x, y)$ gives the number of genes with $x$ ASRUs and whose largest ASRU contains $y$ s-repeats. The color scale is in logarithm and grey cells are not populated. **B**. Three Archetypal situations. On top, 1 ASRU made of many tandem repeats detected in a row (in green) or repeats located at more distant locations in the protein (in orange). The s-repeat boundaries approximately match those of the known repeats (black rectangles). At the bottom, many ASRUs (each colored differently) made of a couple of s-repeats coming from two domains (delineated in black). **C**. Distributions of the proportion of aligned positions between two s-exons that are variable or conserved. Variable (respectively, conserved) positions have their major amino acids conserved in less (resp. more) than 40% of the species within each s-exon. **D**. Detection of sequence signatures (gene CR2). The alignment between the profile HMM for s-exons 1_2 and 1_3 is shown in the middle (green band), together with the MSA for each of the s-exons. For each pair of s-repeats, we analyse the profile-profile alignment to detect positions that harbour different alignment signature. Each possible signature is based on the degree of conservation within the s-repeat (~: not conserved), and on the score of the aligned position between the s-repeats (matching positions: | or +). Three types of signatures are detailed in the figure: SDS Type I, SDS Type II, and covariation.

## 3D structural properties and interactions of the repeats

About two thirds of the s-repeats identified by ASPRING are predicted to fold into a stable 3D structure (**Fig. 3A**). The distribution of intrinsic disorder content in the s-repeats is highly bimodal (**Fig. 3A**). The vast majority of them (85%) are predicted as either fully disordered (>80% of their residues) or fully ordered (<20%). This result indicates that the s-repeat boundaries tend to agree with structural elements boundaries. The quality of the MSAs are similar for the two classes (**Fig. 3A**, inset). For a third of the s-repeats, we could cross the disorder prediction with experimental structural information from the PDB about their interactions with protein partners (**Fig. 3B**). Among them, 351 are in direct physical contact with another protein in a physiologically relevant macromolecular 3D complex.

We illustrate these results with two proteins, namely the cleavage and polyadenylation specificity factor subunit 4 (CPSF4) and the integrin $\beta 1$ (ITB1), both containing one ASRU comprised of two s-repeats with interacting residues and Type II SDS (**Fig. 3C-D**). The two s-repeats of CPSF4 are highly conserved from human to zebrafish. They are consecutive in the protein and have a REL relationship, where the larger s-repeat is always expressed and the smaller one is deleted in some transcripts. The 3D structures of CPSF4 bound to the influenza NS1A protein (PDB code: 2RHK) and of the pre-mRNA 3'-end processing complex (PDB code: 6URO) contain the full-length longer s-repeat and half of the shorter one. Both s-repeats are involved in physical contacts and some of these contacts are established by the SDS. The two s-repeats of ITB1 also show high evolutionary conservation, from human to frog. They are predicted as fully disordered. They are mutually exclusive and thus located at the same place in the protein, namely at its C-terminus. They have been partially resolved experimentally with Talin-2 and ICAP1, respectively. They establish extensive contacts with their partners in the complex structures, suggesting disorder-to-order transition upon binding to their partners.

Out of the ∼90 000 possible binary interactions between the ASRU-containing proteins that have a 3D structure in the PDB, 248 are supported by a physiologically relevant experimental complex structure (**Fig. S7**). A couple of proteins, namely Polyubiquitin-C and Calmodulin, have more than 3 partners in the set. To extend the scope of the analysis, we considered the 60 000 AlphaFold-predicted binary complex models (Burke et al., 2023) available for the Human Reference Interactome (HuRI) (Luck et al., 2020). These interactions have been experimentally identified in Human. The ASRU-containing proteins detected by ASPRING are involved in a bit less than 10% of these complexes. Each 3D model is associated with a predicted DockQ score, a continuous measure of protein-protein complex model quality (Basu and Wallner, 2016). We retained only the models with a predicted DockQ score (pDockQ) higher than 0.23. At this threshold, 70% of the complexes are expected to be correctly modelled (Burke et al., 2023). We identified 55 models representing an interaction between two ASRU-containing proteins (heterodimer) or between two copies of the same ASRU-containing protein (homodimer, **Fig. 4**). In essentially all these complexes, some s-repeats are structured and take part in the binding interface. Nevertheless, in most of the heterodimers, *e.g.* between keratin 78 and keratin 16, the structured interfacial s-repeats come from only one partner, the s-repeats of the other partner being modelled with low confidence.

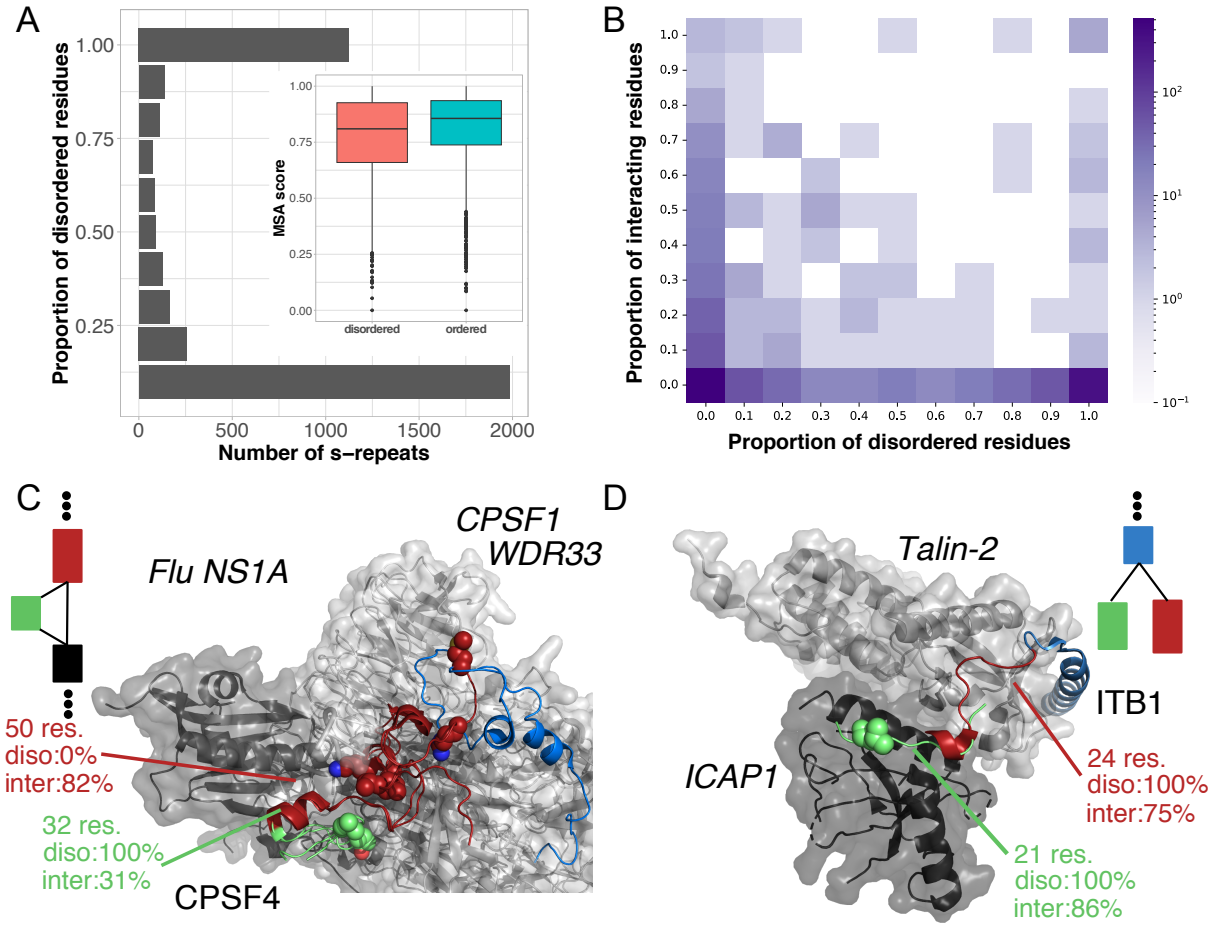


Figure 3: **3D structures and interactions of the s-repeats.A.** Distribution of the content of intrinsic disorder predicted in the s-repeats (see *Supplementary methods*). The plot in inset compares the distributions of MSA scores for the disordered (salmon) versus ordered (turquoise) s-repeats. **B.** Heatmap of the proportion of s-repeat residues involved in the physical interaction with a partner versus the proportion of residues predicted as disordered. We considered only the s-repeats belonging to a protein with experimental structural information (see *Supplementary methods*). **C-D.** Examples of s-repeats in interaction with protein partners. In both cases, the ASRU-containing protein is displayed as cartoons and the protein partners are displayed as transparent surfaces, with their names highlighted in italics. The s-repeats are highlighted in firebrick and lime. Type II SDS are indicated by spheres. The proportion of residues predicted as disordered and of residues in interaction are given for the two s-repeats, along with their lengths. A portion of the ESG showing the AS event linking the two s-repeats is also depicted. **C.** PDB codes 2RHK and 6URO for the complexes of CPSF4 bound to Flu NS1A and CPSF1-WDR33, respectively. **D.** PDB codes 3G9W and 4DX9 for the complexes of ITB1 bound to Talin-2 and ICAP1, respectively.

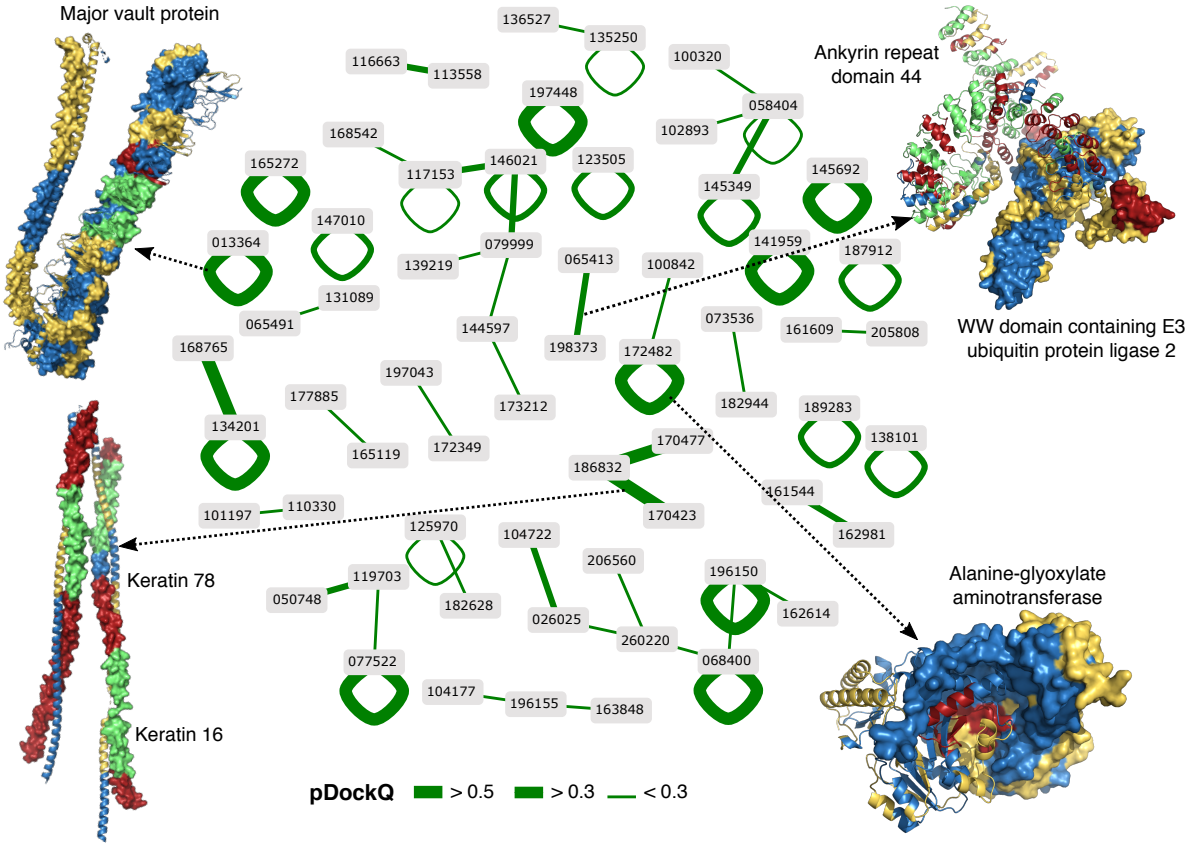


Figure 4: **AlphaFold-predicted 3D interaction network among the ASRU-containing proteins.** Each node represents an ASRU-containing gene and is labelled with its Ensembl id (prefix: ENSG00000). The thickness of the edges indicates the predicted model quality (pDockQ score). On the illustrative 3D structures, one partner is shown as cartoons and the other one as surface. The low-confidence regions (pLDDT<0.5) are not shown. The s-repeats detected by ASPRING are alternatively colored in green and firebrick while the remaining s-exons are in blue and yellow. The structural models were taken from (Burke et al., 2023) and the interactions were experimentally verified in (Luck et al., 2020). the network was rendered with LEVELNET (Behbahani et al., 2023).

## Comparison with known structure-based tandem repeats

The detection of ASRUs by ASPRING is conditioned by the presence of sufficiently similar sequences linked by some conserved AS event. We tested whether ASPRING could detect structure-based repeats annotated in RepeatsDB (Paladin et al., 2021). We selected a set of 10 proteins for which the mapping between structural repeats and exons has been extensively described in (Paladin et al., 2020). By lowering down the sequence identify cutoff at 30%, ASPRING could detect ASRUs in three ankyrin repeat (ANK) and leucine rich-repeat (LRR) containing proteins (**Fig. 5**). The ASRU detected in human ankyrin-1 comprises 19 s-repeats, all highly conserved from human to frog (**Fig. 5A**). Ten of the s-repeats have exactly the length of a classical Ankyrin unit (33 residues, highlighted by one triangle). Two of them have double length (66 residues, highlighted by two triangles) and span two units. These observations are in line with those reported in (Paladin et al., 2020). Deviations from these classical patterns are visible at the extremities, and also in the middle of the protein, where two s-repeats, each made of two s-exons, namely *9_13-9_14* and *9_16-9_17*, are alternatively included or excluded in some transcripts from rat and frog (**Fig. 5A**, ESG on top). The relatively small (16 residue-long) s-exon *9_15* (in blue) serves as an anchor for these two AS events. In murine tankyrase-1, the detected ASRU contains 16 s-repeats, all highly conserved from human to frog and comprising each a single s-exon (**Fig. 5B**). In the experimentally resolved part of the protein (rectangle) we observed that the s-exon boundaries match well the structure-based repeats annotated in RepeatsDB, in agreement with (Paladin et al., 2020). The AlphaFold model allows substantially expanding the structural coverage of the protein, uncovering previously unresolved repeats. The AS event linking the s-repeats substitutes the 744 C-terminal residues of the protein, encompassing 6 s-repeats, by a 34-long unrelated segment. The human leucine rich-repeat (LRR) containing G-protein coupled receptor 5 features ten single-s-exon s-repeats, conserved from human to frog (**Fig. 5C**). They encompass up to 3 structural repeats annotated in RepeatsDB, most of them (8/10) matching well a single structural repeat. As noted in (Paladin et al., 2020), we observe a very regular pattern, with s-repeat boundaries slightly shifted (by 2-3 amino acids) compared to the structural repeats. Some of the later (5 out of 18 are not included in the ASRU due to their high sequence divergence. The number of s-repeats is modulated by three deletion event, two of them conserved across species and one observed only in frog. In all three proteins, the s-repeats display several type I and type II SDS. ASPRING did not detect any ASRU in the other solenoids and $\beta$-propellers emphasized in (Paladin et al., 2020) due to a too low sequence similarity between the structural repeats.

# Conclusion

This work proposes an automated method for detecting alternatively used protein repeats through the prism of alternative splicing in evolution. In contrast to other works, we do not aim at exhaustively enumerating repeats but rather at guiding the users to focus on protein "units" sharing complex relationships, namely sequence similarity, conservation in evolution, and alternative usage. Our method is highly versatile, allowing the users to adapt their experimental set up depending on the level of similarity and of conservation they expect or target. We applied the method at large scale over hundreds of million years of evolution. We relied solely on gene annotations from the Ensembl database. We expect such annotations to grow rapidly in the coming years, thanks to the advent

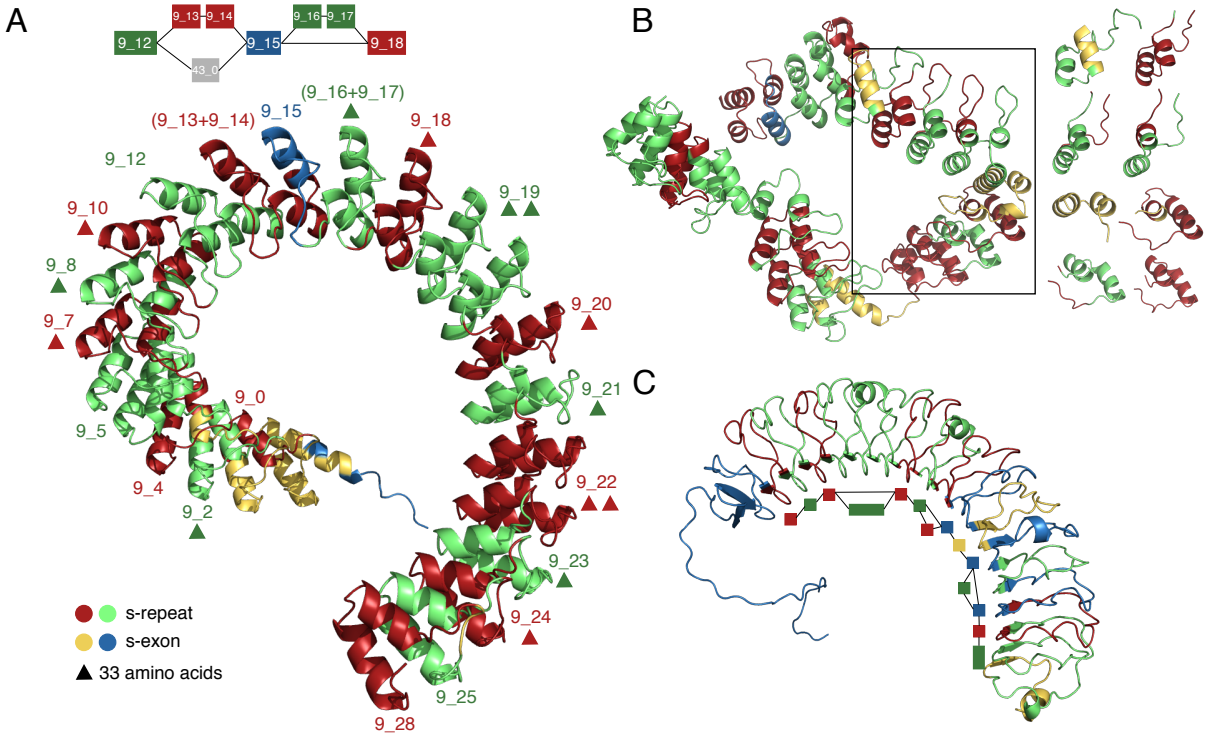


Figure 5: **Detection of alternatively spliced repetitive units in solenoids.** The s-repeats detected by ASPRING are mapped onto AlphaFold 3D models and are highlighted in green and red. The s-exons that are not part of a s-repeat are colored in blue and yellow. For ease of visualisation, we used an alternating coloring scheme. The analysis was performed on groups of orthologous genes across 12 species, from human to nematode. **A**. Human ankyrin 1 (Ensembl v105 gene Id: ENSG00000029534, transcript id: ENST00000347528, residues 1-795). A portion of the ESG is shown on top, with the coloring matching that of the 3D model. **B**. Murine tankyrase-1 (ENSMUSG00000031529:ENSMUST00000033929, residues 174-937). The structural repeats annotated in RepeatsDB, all located in the black rectangle, are displayed separately on the right. **C**. Human leucine rich-repeat (LRR) containing G-protein coupled receptor 5 (ENSG00000139292:ENST00000266674, residues 1-482). The ESG is displayed with a layout following the 3D structure, and using the same coloring.

of long-read sequencing technologies. In this context, methods such as ASPRING will become instrumental to shed light on protein diversity and protein interaction modulation through repeats. Future work will focus on expanding the detection to paralogous families and integrating raw RNA-Seq data. Our joint analysis of the sequence patterns, structural properties and interaction propensities exhibited by the repeats, at the amino acid resolution, provides a first step toward improving our understanding of protein functional diversification and protein interaction specificity. Limitations of the present analysis include the focus on humans, the dependence on experimentally resolved PDB structures covering the repeats, and the fact that it did not account for interactions with nucleic acids. Future works will aim at overcoming these limitations. Another future direction will concern the development of integrated frameworks and learning algorithms to leverage these curated data for targeting protein interactions.

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# Author contributions

Elodie Laine: Conceptualization, Methodology, Software, Validation, Formal analysis, Writing - Original draft preparation, Visualization, Supervision, Funding acquisition. Hugues Richard: Conceptualization, Methodology, Validation, Formal analysis, Data Curation, Writing - Review and editing, Visualization, Supervision, Funding acquisition. Antoine Szatkownik: Methodology, Software, Validation, Formal analysis, Investigation, Writing - Original draft preparation, Visualization. Diego Javier Zea: Software, Writing - Review and editing.

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