**MotifVar: A resource and strategy for amplifying coding variant signal by using repeat protein domains**

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**Abstract**

Large-scale whole genome and exome sequencing holds great promise for the interpretation of protein structures. However, because protein-coding regions are under high selective constraints, their sequences are extremely conserved and variants occur at low frequencies. To address this problem, we have developed the MotifVar approach, which uses the modular structure of a class of repeat protein domains (RPDs) to amplify the conservation signal. In particular, we are able to aggregate variants at the codon level within the human population and compute population genetic metrics to identify potentially functional positions of a RPD protein motif that show stronger conservation signals. This allows us to compare inter- and intra-species conservation directly over different evolutionary timescales. It also enables us to readily visualize population genetic measures on protein structures. We make available the MotifVar results for RPDs as an online resource and illustrate its applicability through a case study on the tetratricopeptide repeat.

**Introduction**

The combined efforts from large-scale human sequencing projects and clinical sequencing have given rise to an exponentially increasing number of human sequences in recent years.1–3 With substantial drop in the sequencing cost and improvement in sequencing technologies and data processing capabilities, we now have the ability to generate a huge catalog of variants that exist in the human population in a fairly rapid and high-throughput fashion. One of the challenges is to provide functional annotations for these variants efficiently and accurately.

Much of the variant annotation work has been performed in the protein-coding regions. A non-synonymous mutation is considered functionally disruptive if it occurs in a region of high conservation, which are considered to be important evolutionarily.4 Evolutionary conservation can be observed at different levels. Inter-species comparison can pick out fixed differences between the dominant homologous sequences of the chosen species across their phylogeny over a long evolutionary time.5–7 At a more recent timescale, intra-species conservation (across a population) has been observed over specific sites in a few large-scale sequencing studies, by aggregating variants over a region or site within the human population.8–10 However, all protein-coding regions are, in general, under high selection pressure. As such, almost all positions in high-impact protein domains tend to be extremely conserved, making it tricky to pinpoint specific positions. Variants also occur sparsely across the coding region and at very low frequencies within a population. Consequently, it is difficult to increase the number of variants for population analyses without increasing the pool of sequenced individuals. To this end, we devise an “intra-genome conservation” approach that is able to “amplify” the variant signal in protein-coding regions within a population.

We focus on a functional category of protein domains that explicitly mediates protein-protein interactions (PPI), known as repeat protein domains (RPDs).11,12 RPDs have been found to be present in almost one in every three human protein.13 As a result, many classes of RPDs have also been studied extensively.14–16 Each RPD is made up of modular repeat motifs of the same class. For example, tetratricopeptide repeat (TPR) domains are made up of only TPR motifs and Ankyrin repeat (ANK) domains of ANK repeat motifs. This modularity gives rise to a strategy that was first introduced in the field of protein engineering to generate protein design templates to create synthetic proteins with desired specificities and affinities.17–19 We adapted the strategy to build a multiple sequence alignment (MSA) profile, which we term a ‘motif-MSA’ profile, for each class of RPD. Using the TPR as an example of a class of PPI RPD, we demonstrate that the motif-MSA strategy can “amplify” variant signal by aggregating the variants from all homologous motifs for each class of RPD within the human genome. Interestingly, we note that such analyses of intra-genome conservation can only be performed using a dataset as large as those from ExAC. Our MotifVar database (<http://motifvar.gersteinlab.org>) contains our results as a resource for annotating variants in 17 PPI RPDs.

**Results**

**MotifVar *database***

Figure 1 shows our strategy that is used to build up the resources in our publicly available MotifVar database (<http://motifvar.gersteinlab.org>) that relates protein residue to genomic information in 17 RPDs. Our strategy first produces a motif sequence alignment profile for a class of repeat domain. Using the TPR repeat domain as an example, we obtain every TPR repeat motif of a given amino acid length in the human proteome (typically the length with the most number of available motifs); in this case, the length is 34 amino acids (see ‘Methods’ for details; Supplementary Figure 1). We then perform an MSA of all the TPR motifs (we term ‘motif-MSA’) to obtain a residue frequency table, which shows the percentage occurrence of each amino acid at each position in the motif. This table can then be translated into a sequence logo for better visualization. For each repeat motif, we then locate its genomic positions in the human genome. Subsequently, we map genomic variants from the ExAC catalog onto the genomic coordinates of the repeat motifs. This allows us to obtain aggregate statistics of variants at each residue positions for each class of repeat domain, namely ratio of the number of non-synonymous SNVs to synonymous ratio (NS/S), enrichment of rare variants (R/C) and the distributions and medians of the derived allele frequencies (DAF) and SIFT scores at each residue position. In our MotifVar database, we provide the residue frequency tables, the SIFT score distributions, median SIFT scores, log(NS/S), log(R/C) and ∆DAF values for each position along the motif for the users.

***Comparing species- and motif-MSA***

An MSA is more typically performed using homologous sequences from multiple species (we term ‘species-MSA’). Here, we perform species-MSA for the first three TPR motif sequences in the TPR-containing protein TTC21B, using orthologous sequences from 66 species (see ‘Methods’ for details) (Figure 2a). TTC21B contains about 16-19 TPR motifs, with almost all of them having a length of 34 amino acids and is a cilia-specific protein that is necessary for retrograde intra-flagellar transport.20 Expectantly, most positions are comparably high in sequence conservation (Figure 2a). In contrast, the motif-MSA profile exhibits substantially differential sequence conservation among the motif positions (Figure 2b). We were able to easily identify positions 8, 11, 20, 24 and 27 as more conserved within the TPR repeat motif.

***Motif-MSA amplifies variant signals to compute population genetic metrics***

The conventional species-MSA profile is restricted to the sequence of a single human protein (since the alignment is based on orthologs), hence even with a large catalog of human exonic variants, only a maximum of three human variants can occur for each residue’s codon position (Figure 2c). However, in the TPR motif-MSA, variants are aggregated from all 34-amino-acid TPR motifs within the human genome. This accumulation of variants amplifies the variant signal, thereby facilitating the computation of various population genetic metrics to investigate the selective constraints in the protein domains.

In Figure 3, we use the TPR domains as an example to show the results of four aggregate statistics derived from the accumulation of genomic variants on the motif-MSA, namely the distribution of SIFT scores (Figure 3a), rare-to-common-SNVs ratio (R/C) (Figure 3b), non-synonymous-to-synonymous-SNVs ratio (NS/S) (Figure 3c) and change in delta derived allele frequency (∆DAF). We use the SIFT score of a non-synonymous SNV as an estimate for inter-species conservation, with lower SIFT score denoting a greater likelihood of an SNV being deleterious, most likely due to high residue conservation.4 As a proxy for intra-species conservation within the human population, we compute R/C, with an enrichment of rare variants (or depletion of common variants) signifying high conservation.8–10 Since protein-coding regions are generally under high selective constraints across species, almost all positions of highly functional PPI domains tend to have very low median SIFT scores across the motif. In the TPR motif-MSA, the most highly conserved position 20 exemplified this observation (Figure 3a and 3d). Over a shorter evolutionary timescale, we find high rare variant enrichment across the motif-MSA profiles of all classes of RPDs, regardless of residue or positional conservation within the repeat motifs (Figure 3b).

We further compute the NS/S for each position in the motif-MSA profile (Figure 3c). The use of NS/S has been traditionally useful in the estimation of selection pressures in the protein-coding regions typically at the gene level.21 Here, rather than at the gene level, the accumulation of variants enables NS/S to be calculated at the codon level (Figure 3c). We observe that most of the positions in the TPR motif with very low NS/S coincide very well with positions of high sequence conservation in the motif-MSA profile. In fact, if we arbitrarily take the top five positions with the lowest NS/S, four of them are the positions with the four most conserved position in the TPR motif-MSA, reinforcing the utility of motif-MSA in picking out functionally important residue positions (Figure 3c).

At this juncture, we also note that this result was observable only with the ExAC data (60,706 exomes), but not when solely with the 1000 Genomes Project Phase 1 data (1000GP; 1,092 whole genomes)8 nor its combination with the Exome Sequencing Project (ESP; 6,500 exomes)10, which total more than 7,500 protein-coding exome data (Supplementary Figure 2 and Supplementary Table 1). The fact that only the largest dataset with more than 60K exomes and 7M SNVs yields interpretable results underscores the importance of still having more genome sequences and rare variants.

∆DAF results show the degree of population differentiation (between pairs of populations) at each residue position.8,22 We observe that highly conserved positions have lower ∆DAF medians and narrower distributions. More interestingly, we can identify some residue positions that are differentiated between certain populations (Figure 3d/e).

***Combining protein and genomic information to identify important residues***

Finally, using the motif-MSA, we are able to integrate both protein (from MSA) and genomic information (SNVs) to better pinpoint positions that might be more functionally important. By combining positions with the highest five sequence conservation in the TPR motif-MSA and the lowest five median SIFT scores and NS/S ratio, we are able to identify eight positions (out of 34 positions on the TPR motif), with four positions that fulfil at least two of the three selective constraint conditions (Figure 3d). The differences in R/C between positions within the TPR motif-MSA are too subtle to be used.

***Mapping genomic information onto protein structures***

In order to visualize the eight residue positions in a spatial context, we further integrated genomic information with protein structures. We use the X-ray crystal structure of a three-motif TPR domain (TPR1) from the human protein Hsp-organizing protein (HOP) bound to its cognate ligand, a short peptide sequence consisting of seven amino acids, PTIEEVD (PDB ID: 1ELW).23 We map the eight positions derived from Figure 3d onto the protein structure (Supplementary Figure 3). Except for position 17 in each of the three TPR motifs in TPR1, we found that all the other seven residue positions with high selective constraints (from either low median SIFT scores, low log (NS/S) or high motif sequence conservation) are buried residues in the PPI domain (Supplementary Figure 3a), in line with a previous study.24

***Relating residues positions to clinically-relevant and disease-related mutation data***

Using two databases, ClinVar25 and the proprietary Human Gene Mutation Database (HGMD)26, we found that the highly constrained positions have some of the most occurrences of clinically-relevant or disease-related mutations along the TPR motif-MSA profile, including the highest two at positions 6 and 7 (Supplementary Figure 3b). In fact, mechanistic studies of a number of these mutations show that the occurrence of certain NS mutations on these positions give rise to diseases precisely as a result of ablation of protein-protein interactions.27,28

**Discussion**

For decades, the focus in research on PPI has typically been the investigation of protein interfaces that directly take part in the protein interaction. Most studies involved the use of 3D protein structures, for instance, to identify protein-protein interfaces,29,30 investigate interfacial properties31,32 or to predict interacting ‘hotspots’33–35. While extremely useful in protein engineering and drug design, it is also very limited by the number of available protein structures. On the other hand, the amount of human sequencing data has been growing dramatically over the past decade, in particular, the number of protein-coding exome sequences.36 This huge trove of sequence information should be leveraged upon for variant annotation in protein-coding regions, especially in complementing protein data with the copious amount of human genomic data. Our introduction of the motif-MSA facilitates genomic analyses with protein information (and vice versa) in several ways.

Firstly, motif-MSA removes the limitation imposed by species-MSA. Thus far, the utility of protein sequences has been largely focused on the more traditional perspective of sequence conservation across multiple species based on homology.5,6,37 By using information from the same motif class, we can systematically aggregate variants from similar protein regions within the genome of a single species in a reasonable manner. This aggregation is key to achieving the variant statistics required to perform analyses that are meaningful, especially in light of the observation that even a combined set of 1000GP and ESP6500 variant data, derived from almost 7600 exomes, was not sufficient to yield immediately-interpretable results (Supplementary Figure 2 and Supplementary Table 1). At this point, it is also important to note that intra-genome conservation, while allowing amplification, conflates genomic variant information not only from long and short evolutionary time scales, but also from the evolution of the same class of repeat motifs within the genome. Thus, the interpretation of selective constraints in metrics such as log(NS/S) is a confluence of evolutionary timescales and mutation processes.

Secondly, the ability to gain statistical power from variant aggregation makes motif-MSA an extremely powerful platform in investigating selective constraints using genomic information. Potentially, motif-MSA is amenable to the entire repertoire of genomic metrics. We used four metrics to demonstrate how motif positions and residues that show evidence for clinical and disease relevance can be identified, and would have been missed otherwise.9

Thirdly, motif-MSA is also able to reflect protein structural properties and their roles in PPI. Conventional species-MSA aligns sequence orthologs that are similar in function and structure. Hence, highly conserved residues or positions are a mix of structural and functional residues. On the other hand, because the protein motifs are classified by their structural folds, sequence features in a motif-MSA are important structural features that determine the folds of the PPI domains. These features are observed as buried residues within the interior of PPI domains (Supplementary Figure 3). In addition, it has been suggested that because motifs in motif-MSA are from a myriad of proteins with diverse binding partners, positions that are low in sequence conservation, or ‘hypervariable’, are found in the binding pockets of the corresponding domains.24,38 Similarly, we noticed hypervariable positions, such as position 2 in TPR motifs, harbor a good number of disease-related variants.

Lastly, the motif-MSA strategy presents an opportunity to extend its application beyond protein motifs to whole domains (domain-MSA), which has been shown to be very informative in uncovering domain-specific protein features that are not observed in a motif-MSA.39 However, the construction of a domain-MSA profile is largely constrained by the number of domains with a certain number motifs or size of motif. For example, the number of domains declines drastically as one uses domains with 5 TPR motifs (instead of three), and/or motifs with 33 amino acids (Supplementary Figure 1). Nonetheless, a domain-MSA, while limited by numbers, can be extremely useful in uncovering domain-specific features important for PPI.

The motif-MSA approach provides a powerful and versatile platform to facilitate the combination of protein and genome information for use in the annotation of protein structures. It enables the leveraging of the vast amount of human sequencing data currently available. This will become increasingly more important and urgent in the future as human genome sequencing becomes more commonplace and personal genome interpretation takes center stage.

**Methods**

**MotifVar *database***

Our publicly available MotifVar database (<http://motifvar.gersteinlab.org>) provides data files for for 17 RPDs, including TPRs. Each class of RPD is a tarball, which contains residue frequency tables (to rebuild the sequence logo), the SIFT score distributions, median SIFT scores, log(NS/S), log(R/C) and values for each position along each RPD motif to allow versatile thresholding by the users. The resource and scripts used in the pipeline are freely downloadable at the database.

***Multiple sequence alignment (MSA)***

All protein, motif and domain information are extracted from Ensembl database version 73 and SMART database, under the ‘genomic’ mode, for species, *Homo sapiens* (downloaded Oct 25, 2013).40 The 17 PPI repeat domains are manually selected based on their availability in the SMART database.

We will use the TPR domains as an example to illustrate the process of motif- and species-MSA in our study.

To obtain a motif-MSA sequence profile, (1) we first extract all TPR domains in the human proteome and break them up into its constituent motifs. (2) Here, the motif-MSA is performed based on the most representative size of the motif. Hence, in order to select the motif size, a histogram of all sizes of TPR motifs is constructed (Supplementary Figure 1) and the most common motif size is selected for motif-MSA alignment; in TPR motifs, the most common motif size is 34 amino acids. There are a total of 114 human proteins (from unique genes) with 571 unique 34-amino-acid TPR motif sequences; we only keep one motif when there are multiple with 100% sequence identity. (3) MSA is then performed on of these 571 TPR motifs with 34 amino acids, with no gaps allowed, i.e. we line up all sequences by position end to end. This ‘ungapped’ alignment allows the derivation of a 20-by-*n* frequency table for 20 residues and *n* positions on the motif profile, and subsequently, visualization, using a sequence logo constructed by WebLogo 3.2.41

The TPR species-MSA is obtained by aligning the homologous protein sequences of TTC21B from 43 species in an ‘ungapped’ fashion. Using the MEGA5 software42, we extracted the TPR domain from the 45-sequence alignment, based on the human TTC21B information in SMART database. There are 16 TPR motifs in TTC21B found in the SMART database. We remove two orthologs due to the existence of gaps in at least one of the 16 TPRs. Finally, we construct the sequence logo of all 16 TPRs using WebLogo 3.2.41 We show the alignment of only the first three TPR motifs of TTC21B in Figure 2.

***Sequence logo visualization***

All sequence logos are created by WebLogo 3.241, using the following parameters:

-A protein -U bits --composition "{'L':9.975,'A':7.013,'S':8.326,'V':5.961,'G':6.577,'K':5.723,'T':5.346,'I':4.332','E':7.096,'P':6.316,'R':5.650,'D':4.728,'F':3.658,'Q':4.758,'N':3.586,'Y':2.653,'C':2.307,'H':2.639,'M':2.131,'W':1.216}" -n 34 -c chemistry --stack-width 25 --errorbar no

For the ‘composition’ parameter (used for the relative entropy calculation), we provided manually the background distribution of the amino acids in the entire SMART database (‘genomic’ mode), in order to be in line with our input data from the SMART database; the values above are in percentages. We separately computed these values from the SMART database. Unless the sequence logos are in monochrome (as in Figure 2), they are colored by amino acid chemistry, where polar residues (G, S, T, Y, C) are colored green, neutral residues (Q, N) purple, basic residues (K, R, H) blue, acidic residues (D, E) red, and hydrophobic residues (A, V, L ,I ,P ,W, F, M) black.

***Variant information from exomes***

For all the analyses in this study, we use the SNVs and their minor allele frequencies from 60,706 exomes found in the ExAC database3 (Version 0.3, downloaded February 1 2015), after removing the variants from the sex chromosomes and singletons (those variants that only occur in one chromosome in the entire ExAC dataset). This ends up with 7,202,445 autosomal SNVs. We obtained SIFT scores, and non-synonymous nature of the SNVs on the proteins using the VEP tool (Version 73) from Ensembl release 73.43

Similarly, we have also used a combined number of 1,328,447 unique, non-singleton, and autosomal SNVs from the 1000 Genomes Project Phase 1 (1,092 whole genomes)8 and Exome Sequencing Project data (6,500 exomes)10, to produce Supplementary Figure 2 and Supplementary Table 1.

All coordinates are based on the human reference genome assembly version of hg19.

***Relating genomic and protein information***

Custom scripts are written to relate genomic to protein information. The key portion is in identifying codon coordinates. We first obtain all genomic coordinates and strand information of protein-coding exons and residue coordinates of SMART protein domains from Ensembl 73 and GENCODE 18 on the reference genome, hg19. The exon information will give us the exact genomic coordinates of the codons for each protein-coding gene, using the locations of the exon-intron junctions. This allows mapping of genomic variants to specific codons, enabling positional accumulation of variant information across a motif-MSA profile. These scripts are part of the pipeline available for download in the MotifVar database.

***Protein structure visualization***

The X-ray crystal structures from Protein Data Bank (PDB) are created using Pymol 1.3.44

***Clinically-relevant and disease-related variants***

Clinically-relevant and disease-related variants in GRCh37 were downloaded from ClinVar25 on July 8, 2015 and the proprietary HGMD Professional Database downloaded on July 27, 2015.26

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**Figure Legends**

**Figure 1. Our motif-MSA approach.** (1) We first query a database and obtain all the proteins with the desired domains or motifs. We use the TPR motifs as an example in this figure. These motifs have to be the same length. Here, we select TPR motifs that are 34 amino acids since they are the most frequently-occurring size. (2) Subsequently, we perform an ‘ungapped’ multiple sequence alignment (MSA) of the TPR motifs by lining them up end to end, to obtain a sequence conservation profile. This motif-based MSA typically exhibits differential sequence conservation among the positions across the length of the motif. (3) The third step involves collecting genomic single nucleotide variants (SNVs) for each amino acid position of the motif-based alignment profile. For TPR domains, we obtain the specific genomic coordinates of each codon, and then we locate all variants that fall into each codon. (4) For each motif-MSA, we then host the results on our MotifVar database, including residue frequency tables, log(NS/S), log(R/C) and SIFT score distributions.

**Figure 2. Motif-MSA can uncover important domain positions missed by species-MSA and it also serves as a “variant information amplifier”.** This figure uses TPR as an example. (a) We perform a species-MSA using orthologous TTC21B from 66 species (species-MSA). Here, we show the alignment profiles for the first three TPR motifs (red, blue and green sequence logos), out of the possible 16. We observe that almost all the positions are highly conserved. (b) In contrast to conventional species-MSA, there is a differential sequence conservation profile across the TPR motif-MSA (black sequence logo), which facilitates the identification of more conserved motif positions that are potentially important (positions are highlighted in yellow). (c) In order to integrate the vast amount of sequencing data, we can directly map genomic variants (black diamonds) onto the coordinates of TPR motifs in protein-coding genes. We can use species-MSA to align orthologous sequences across multiple species, as in (a). However, because we are focusing on proteins and sequencing data in humans, the number of variants at each amino acid position or codon in a species-MSA profile will never exceed a maximum of three. On the contrary, a motif-MSA profile is able to aggregate variants across all motifs within the human genome, thereby amplifying variant information sufficiently for further downstream analyses.

**Figure 3. Using genomic variant information in the motif-MSA profile to investigate selective constraints in PPI motifs.** Using SNVs from the ExAC dataset, we use various SNV properties to investigate the extent of selective constraints at each position in the motif-MSA profile. (a) For each non-synonymous SNV, a score can be computed from the SIFT tool to approximate its deleteriousness phylogenetically, based on sequence conservation over multiple species, where a lower SIFT score means more deleterious. Each blue violin plot represents the distribution of SIFT scores at each position in the TPR motif, with the width of the plot approximating frequency density and the black dot denoting the median SIFT score. The distribution provides an estimation of the selective constraints based on intra-species comparison. (b) For each SNV, the minor allele frequency (MAF) in the human population can determine whether an SNV is rare (MAF ≤ 0.005) or otherwise, common. The log ratio of the number of rare versus common variants (log R/C) represents the enrichment of rare variants, which has been used as a metric for estimating selective constraints based on intra-species comparison. All positions have an enrichment of rare variants, with position 25 having no common variants (log ratio with a zero denominator is undefined). (c) We can also calculate the log ratio of non-synonymous (NS) versus synonymous (S) SNVs. A depletion of NS variants with respect to the background of S SNVs suggests a position might be functionally significant. (d) The five positions with the least median SIFT scores are numbered in blue according to their rank (there are four positions tied at rank 2). The five positions with the lowest log (NS/S) are ranked in red. The top five most conserved positions in the TPR motif are highlighted in yellow. There are seven candidate positions which fulfil at least one of the above criteria of the lowest SIFT median scores, log(NS/S) and motif-MSA sequence conservation, with four positions satisfying at least two.

**Supplementary Figure 1.** The most frequent size of the TPR motif is 34 amino acids.

**Supplementary Figure 2.** We compare the utility among three variant sets, namely from 1000 Genomes Project Phase 1 (1000GP; green bars), the combined set of 1000GP and the Exome Sequencing Project (1000GP+ESP6500; blue bars) and the ExAC dataset. We can see that there are subtle differences in log(NS/S) for each position along the TPR motif, when using variant datasets from 1000GP to 1000GP+ESP6500. We were able to make meaningful interpretations only when we use variant data from ExAC (grey bars).

**Supplementary Figure 3.** Mapping genomic information onto protein structures and disease-related mutation data.(a) We choose the TPR domain, TPR1, found on the Hsp-organizing protein (HOP; PDB ID: 1ELW), as a basis of mapping candidate positions. TPR1 contains three 34-amino-acid TPR motifs (e.g. there are three position 20s). We find that all positions with high selective constraints are found buried within the PPI domains (red residues on protein structure), except for position 17 on each of the TPR motifs. The colors are overlaid in order: positions with lowest median SIFT scores (light blue numbers and residues in structure), with lowest log(NS/S) (red numbers and residues in structure), then finally positions with highest sequence conservation in the motif-MSA profile (orange highlights in motif sequence and residues in structure). The ligand-binding convex profile of the TPR1 domain (the cognate ligand is represented by the green stick sticks) is rotated 180o to reveal the concave profile of the same TPR1 domain. (b) We also use two databases, ClinVar and HGMD, to demonstrate which TPR motif positions accumulates more clinically-relevant and disease-related SNVs.

**Supplementary Table 1.** The 1000 Genomes Project (1000GP) provides the least number of autosomal SNVs, followed by an approximate 6-fold increase in number of exomes in the combined set of 1000GP and Exome Sequencing Project (ESP6500); this is a corresponding ~3-fold increase in the number of autosomal SNVs. Our study uses the dataset from ExAC, with 60,706 individuals, an almost 8-fold increase from the combined set of 1000GP+ESP6500; this is a corresponding ~5-fold increase in the number of autosomal SNVs.

**Supplementary Table 2.** The lists of repeat and non-repeat domains that we performed the motif-MSA approach and are included in the MotifVar repository.