Protein intrinsically disordered regions have a non-random, modular architecture

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Short title: Protein disordered regions are non-randomly organized

ABSTRACT

Protein sequences can be broadly categorized into two classes: those which adopt stable secondary structure and fold into a domain (i.e., globular proteins), and those that do not. This latter class of sequences are conformationally heterogeneous and are described as being intrinsically disordered. Bioinformatics enable sub-classification of globular sequences by domain type, an approach that has revolutionized how we understand and predict protein functionality. Conversely, it is unknown if sequences of disordered protein regions are subject to broadly generalizable organizational principles that would enable their sub-classification. Here we report the development of a statistical approach that quantifies linear variance in amino acid composition across a sequence. With multiple examples we provide evidence that intrinsically disordered regions, but not globular domains, are organized into statistically non-random modules of specific compositional bias. Modularity is observed for both low and high complexity sequences and, in certain cases, we find that modules are organized in repetitive patterns. These data demonstrate that disordered sequences are non-randomly organized and motivate future experiments to comprehensively classify module types and to determine if modules, like the globular protein's domain, represent functionally separable units.

INTRODUCTION

The basic functional unit of a globular protein is the domain, a polypeptide region that folds into a stable three-dimensional structure (1). Many eukaryotic proteins possess a modular, multi-domain architecture resulting from the genetic duplication and shuffling (2) of the approximately 6,000 protein domain superfamilies (3). In this way evolution has produced a vast repertoire of architecturally distinct and functionally diverse multi-domain proteins. The domain architecture of a protein has traditionally been the realm of structural biology but modern bioinformatic algorithms now enable rapid identification of a protein's separable domains (4). Understanding the modular architecture of a multi-domain protein is key to understanding its function.

Approximately 40% of the eukaryotic proteome does not fold into globular domains (5). These protein sequences, which do not possess stable secondary structure, exist in an ensemble of dynamic configurations and are described as being intrinsically disordered (6). Despite their lack of structure, protein intrinsically disordered regions (IDRs) are known to play essential roles in many physiological and pathophysiological pathways, including transcription (7,8), DNA replication (9), as the etiological agents in certain neurodegenerative proteinopathies (10), and certain IDRs drive protein phase separation which helps organize the cell (11). Regions of protein disorder can be discriminated from globular domains by sequence composition alone (12,13) and there exist many bioinformatic algorithms to predict the disorder propensity of a polypeptide (14).

By definition, IDRs lack a defined spatial architecture and, with the exception of short linear motifs (SLiMs) (15), are thus relatively unrestrained in primary sequence. One exception to this are seemingly non-random regions of disordered sequences that are locally enriched in only a small subset of amino acids, so-called Low Complexity Regions (LCRs). Sequence gazing readily identifies LCRs by their conspicuous local sequence bias but these can also be quantitatively and unbiasedly discriminated on the basis of informational entropy (16). Some IDRs also possess multiple LCR types with distinct functionalities (17,18) and bioinformatic approaches to demarcate unique LCR sequences within a contiguous region of disorder have recently been reported (18,19). However, LCRs are but a fraction of all disordered sequences and it is unknown if sequence-spanning organizational principles, such as local compositional bias, are operative in IDRs generally.

Here we develop a statistically robust computational algorithm to determine if disordered sequences possess local compositional bias. Relying on the Chi-square (χ^2) test of homogeneity, our method quantifies variability in the fractional composition of amino acids between two

sequences. Applied intramolecularly in a moving-window, matrix-based approach, this method can identify sequence-spanning compositional heterogeneity. With multiple examples we show that IDRs of both low and high sequence complexity (but not folded domains) possess local compositional bias that bestows disordered sequences with a non-random, modular architecture. Analogous to the domain architecture of globular proteins, we propose that modules represent functionally separable units of disordered sequences and provide a means for their classification. These data demonstrate that high-level organizational principles are at work in disordered sequences and motivate future functional studies to understand the role of modules in biology.

RESULTS & DISCUSSION

Development of a bioinformatic algorithm to identify local compositional bias in IDRs.

Protein disordered regions lack stable secondary structure and are thus relatively unrestrained in primary sequence. Consistently, IDRs often have weak linear sequence conservation (20,21) and no visually discernible sequence patterns, suggesting they lack a defined organization. We hypothesized that local compositional bias may bestow disordered sequences with a sequence-spanning level of organization undetectable by current methods. We therefore developed a bioinformatic algorithm to determine in an unbiased and quantitative fashion if amino acids are non-randomly distributed across the length of a protein sequence. Our approach implements the Chi-square (χ^2) test statistic to compare the fractional content of amino acids between two sequences as a measure of compositional dispersion (see **METHODS AND ALGORITHMS**). Applied in this way, chi-score analysis quantifies how different the amino acid proportions (i.e., composition) are between two sequences.

The chi-score metric can be applied intramolecularly to identify compositionally biased regions (**Fig 1A**). In the first step of our algorithm, the sequence is broken into all possible sub-sequences for a specified window size and all pairwise chi-scores are calculated (**Fig 1A-a**). Each chi-score

is then converted to a Pearson's correlation coefficient which better resolves compositionally biased regions and sequence-spanning patterns (**Fig 1A-b**). Subsequently, for each residue position we calculate the mean correlation coefficient contained within a 10-residue long line perpendicular to the main diagonal and plot these "insulation scores" against residue position (**Fig 1A-c**). Finally, local minima from the insulation score plot are calculated and recorded as potential boundaries between compositionally distinct regions (**Fig 1A-d**). These steps are completed for nine sets of pairwise chi-scores – each using a different odd integer window size between 5 and 21 to define the original sub-sequences – which results in nine sets of boundaries (**Fig 1A-d**).

The boundaries, which were originally grouped by window size, are then clustered by residue proximity (**Fig 1Ad**) and the optimal boundary positions are determined by selecting the placements within each group that maximize the mean chi-score between the surrounding regions (**Fig 1A-e**). The statistical strength of each boundary is then determined by calculating a z-score. To do this, the two juxtaposed regions separated by each boundary are scrambled 500 times and the position resulting in the maximum chi-score identified. This results in a set of 500 chi-scores, one for each scramble, from which the mean (and standard deviation) is determined and used to calculate the z-score for the corresponding boundary (**Fig 1A-f**). Finally, low scoring boundaries are iteratively removed and those that remain are re-optimized and scored until only high-confidence boundaries remain.

To demonstrate the utility of this algorithm in identifying compositional bias, we first tested its effectiveness at differentiating human languages (**Fig 1B**). We translated a quote into English (Eng), Japanese (Jpn), Yoruba (Yrb), Spanish (Spn), and Polish (Pol), appended the translations one after another, and then analyzed the resulting string of text by the Chi-score method. This approach proved highly effective at discriminating between languages based on their differential character usage (all texts were Romanized and composed of the same 26 letter alphabet).

Regions off the diagonal with relatively high correlation reveal languages with more similar alphabet usage, such as English and Spanish or Yoruba and Japanese. Conversely, regions off the diagonal with relatively low correlation reveal languages with differential alphabet usage, such as Polish and Yoruba. We next applied the method to a protein sequence to determine whether it can parse a sequence by "molecular language" (**Fig 1C**). Fused in Sarcoma (FUS) is known to possess multiple compositionally biased regions with unique functionality (22,23) and our method accurately identifies these, revealing three major language types: G/S-Y-G/S repeats, RGG repeats, and a more complex sequence type which corresponds to the folded domains of the protein (RRM and Zn finger domains). Analysis of off-diagonal correlated regions reveals homology amongst the three RGG-enriched regions and the two regions which have a globular structure. These data establish the utility of Chi-score analysis in parsing sequences by local compositional bias.

IDRs have a non-random, modular organization.

Having established the algorithm on a sequence with conspicuous local sequence bias (**Fig 1C**), we next applied it to a disordered region that lacks recognizable sequence patterning. The Origin Recognition Complex (ORC, composed of Orc1-6) is an essential DNA replication initiation factor that contains an IDR in the Orc1 subunit that is necessary for recruitment to chromatin (9). In *C. elegans*, the Orc1 IDR is predicted to be 245 amino acids long and, except for two short, low complexity regions, has no apparent sequence organization (**Fig 2A**). However, chi-score analysis of local compositional bias revealed a strikingly non-random, sequence-spanning level of organization with regions of distinct compositional bias juxtaposed in a repetitive pattern (**Fig 2B**). Given the modular appearance, we will refer to these compositionally biased regions as modules. The Orc1 IDR has three module types which can be broadly categorized as either basic, neutral, or acidic. In this sequence the basic modules (residues 1-51, 106-167, 186-232) and

acidic modules (residues 52-68, 80-105, 168-185, 233-245) alternately repeat throughout the sequence, with the neutral module type appearing only once (residues 69-79).

The discovery of a statistically non-random, sequence-spanning level of organization in the Orc1 IDR was unexpected. Importantly, amino acid content alone does not produce this type of organization. To demonstrate this, we reran the analysis on a random scramble of the Orc1 IDR (**Fig 2C**). In this example, modules are not only visually absent but there were additionally no statistically significant boundaries output by our algorithm. Likewise, compositionally biased modules are absent from Orc1's AAA+ domain (**S2 Fig**) which, compared to the IDR, has a far more uniform sequence landscape. This is consistent with prior work showing that the lengthwise distribution of amino acids in globular domains does not differ substantially from randomized sequences containing equivalent proportions of amino acids (24–26). These data suggest that local compositional bias is an organizational principle unique to disordered sequences.

Many disordered sequences have local compositional bias.

The strikingly modular architecture of FUS (**Fig 1C**) – a low complexity IDR – as well as Orc1 (**Fig 2B**) – a high complexity sequence – prompted us to investigate whether this type of organization is widely operative in disordered sequences. We therefore extended our studies to assess modularity of several other protein disordered regions with known biological and pathological significance, including human TDP-43 (**Fig 3A**), Spt6 (**Fig 3B**), Nucleolin (NCL, **Fig 3C**), KMT2B (**Fig 3D**), and *Caulobacter crescentus* Ribonuclease (RNase) E (**Fig 3E**). These analyses, which we briefly describe below, show that local compositional bias is pervasive amongst IDRs and bestows disordered sequences with a modular architecture.

We analyzed an internal IDR of the protein TDP-43 (residues 259-414, **Fig 3A**), an RNA processing factor that forms pathological aggregates in neurodegenerative disease. Our analysis

identified several modules, one of which corresponds precisely to a region harboring an abundance of pathological missense mutations and which functions independently as TDP-43's amyloidogenic core (residues 320-PAMMAAAQAALQSSWGMMGMLAS-342) (27–30). These data suggest that modules, like a folded domain, can behave as functionally separable units. On the other hand, our analysis of the N-terminal IDR of Spt6 (residues 1-310, **Fig 3B**) suggests that modules can also function cooperatively. Specifically, we identified a repetitive arrangement of basic and acidic modules which recent data suggest mediate selective partitioning into MED1 condensates to control transcriptional activation (8). We anticipate that understanding module types and patterning will help elucidate the rules underlying selective partitioning in biomolecular condensates. Finally, we identified numerous modules of variable composition and size within full-length Nucleolin (NCL, **Fig 3C**), the N-terminal IDR of KMT2B (**Fig 3D**), and the C-terminal IDR of RNase E (**Fig 3E**). Altogether, these data suggest that local compositional bias represents an organizational principle that is widely operative in disordered sequences and inspires fundamental questions about the role of IDR modularity in biology and disease.

CONCLUSION

We find that the sequences of both low and high complexity IDRs are non-randomly organized into regions with local compositional bias. This concept, like the structural hierarchy (1° – 4° structure), bears the hallmarks of a fundamental organizational principle: it is generalizable to any sequence, it appears to be broadly operative, and it enables the unbiased sub-division of a sequence into component parts. These features have enabled the structural hierarchy to provide a comprehensive classification of folded sequence space, a long-standing objective in the field of protein disorder. Collectively, these findings warrant a conceptual shift away from IDRs as indivisible functional units to viewing them as modular sequences with region-specific physical, chemical, and functional properties (**Fig 3F**). To this extent, individual IDR modules and their combinations may represent the functional analog of the globular protein's domain, whose many

types and arrangements produce the diversity of cellular functions that are needed for life. However, correlating module function with a specific tier(s) in the structural hierarchy will require an understanding of how modularity dictates conformational ensembles.

This work calls for the comprehensive classification of module types and their combinations to determine whether there exist distinct classes or a continuum of compositional varieties. Such work will benefit from other sequence characterization parameters, such as charge distribution (31) and binary sequence patterning (32). While the evolutionary preservation of modules provides strong *a priori* evidence that modules are important for biology, future studies are needed to systematically relate module type with functionality. Beyond this, many other important questions remain, including the use of genetic mechanisms to shuffle modules and produce novel functionalities, the biophysical properties of isolated modules and emergent properties of multi-module sequences, and whether a modular view of IDRs clarifies the mechanism of disease-associated mutations and rationalizes specificity in IDR-enriched biomolecular condensates. In the immediate, we hope the concept of IDR modularity as a generalizable organizational principle provides a rational approach for dissecting the functional mechanisms of this enigmatic class of sequences (**Fig 3F**). To facilitate these types of studies, the code required to run these analyses is freely available and easily implemented (see **METHODS AND ALGORITHMS**).

METHODS AND ALGORITHMS

4.1 Applying the Chi-square test to compare sequence composition.

The Chi-Square Test of Homogeneity is used to determine whether two distributions are from, or were sampled from, the same population. Traditionally, the Chi-square test statistic is calculated and used to either reject or accept the null hypothesis. Here, the test statistic is instead used as a metric scoring the compositional difference between two sequences; a high-test statistic indicates a high degree of compositional distinction.

The Chi-square test can be applied directly when comparing sequences of equal length, such as in the intramolecular, matrix-based approach we elaborate on here. It can also be applied intermolecularly to compare the composition of sequences of different lengths. However, for this latter application the sequence lengths must first be normalized. Since length normalization does not impact comparison of equal length sequences, we have implemented length normalization in our algorithm to make the chi-score adaptable to both types of comparison. Thus, the instances of each residue are normalized to a sequence length of 1000. Subsequently, the number of each residue is taken as the observed values (O) for the Chi-square formula. For each observed value, a corresponding expected value (E) is calculated with the following equation.

$$E_{r,n} = \frac{1}{2}\left(O_{r,1} + O_{r,2}\right)$$

In this formula, *n* refers to the sequence (either 1 or 2) and *r* refers to the residue (one of twenty amino acids). The total number of residue *r* in the two sequences is multiplied by the ratio of the length of sequence *n* to the total length of both sequences. Because the sequences are normalized to 1000 residues each, this value is always equal to 1/2. The test statistic can then be calculated with each observed/expected pair using the following equation.

$$\chi^2{}_{r,n} = \frac{\left(O_{r,n} - E_{r,n}\right)^2}{\left(E_{r,n}\right)^2}$$

Finally, the 40 unique scores – one for each amino acid and sequence pair – are summed, and the test statistic is normalized between zero and 1 (by dividing by the maximum possible sum, 2000). Sequences that have no residues in common will always receive a normalized score of

one, and sequences with identical amino acid compositions will receive a normalized score of zero.

In addition to the Chi-square method, Euclidean distance has also been applied to quantitate the similarity in amino acid composition between disordered sequences (33,34). This method takes the fractional content of amino acids as Cartesian coordinates in a 20-dimensional Euclidean space and the “distance” between any two sequences is quantified. Our Chi-square method builds on Euclidean distance in several ways. First, chi-score values can be readily decomposed to see the contribution of each amino acid to the overall score, thereby determining the residue type(s) that most distinguishes one sequence from another. Second, Euclidean distance is influenced by sequence complexity while Chi-square is not. Finally, the Chi-square test possesses inherent statistical power which we apply to determine whether sub-sequences are compositionally distinct compared to random scrambles of the same sequence.

4.2 Applying the Chi-score metric to protein sequences to identify regions of local compositional bias.

The Chi-square method can be applied intramolecularly to identify regions of distinct amino acid compositional bias. During the first step of this analysis, the sequence is broken up into all possible subsequences of nine different window sizes (all odd integers between 5 and 21) and for each window size the chi-score is calculated for all subsequence pairs. The pairwise scores are then converted to Pearson’s Correlation Coefficients with the following equation:

$$r = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{[n\Sigma x^2 - (\Sigma x)^2][n\Sigma y^2 - (\Sigma y)^2]}}$$

After plotting the coefficients as a two-dimensional matrix with the subsequences along each axis, we then calculate insulation scores (as used in Hi-C data processing) for each residue position. These scores are calculated by taking the average value in a ten-residue long line that extends perpendicular to the main diagonal. Insulation score values are high when this line is within a region of distinct amino acid composition and low when the line is near the boundaries between them. Therefore, we then take the residue positions of the local minima of these insulation scores as potential boundaries between compositionally distinct modules.

Because this is done with nine different window sizes, we now have multiple sets of potential boundaries grouped by the window size used to calculate them. Modules of different length and/or complexity will be best identified with different window sizes, so the precise positions for each boundary will vary between the nine groups. We then regroup these boundaries spatially so that they are clustered with other potential positions for the same boundary. The optimal position for each boundary is then determined by selecting those that maximize the mean chi-score between the resulting modules.

The boundaries can now be statistically verified by calculating a z-score for each. First, the two modules separated by a boundary are juxtaposed and randomly scrambled 500 times. Then, the greatest chi-score between two modules that can be achieved with a comparable boundary is determined for each scramble. Finally, these scores are used to convert the raw chi-score for that boundary into a z-score, which tells us how likely it is that the predicted boundary separates truly distinct modules or simply identifies local biases occurring by chance. Boundaries with low z-scores are iteratively removed until only those with significant scores remain. After a boundary is removed, the positions are reoptimized and the z-scores are recalculated for those that remain; the placements and z-scores are also stored so that they can be recalled as desired.

4.3 Python implementation and accessibility

The code for these analyses was designed with Python Version 3.10.8 and executed in Jupyter Notebook. The complete analysis, which can readily analyze individual amino acid sequences, along with the code required to reproduce all matrices shown in **Fig 1-3**, have been made freely available for use and are easily implemented. For the notebook files, as well as instructions for how to run the analysis, please see: https://github.com/MWPlabUTSW/Chi-Score-Analysis.git.

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FIGURES, FIGURE TITLES AND LEGENDS

FIGURE 1

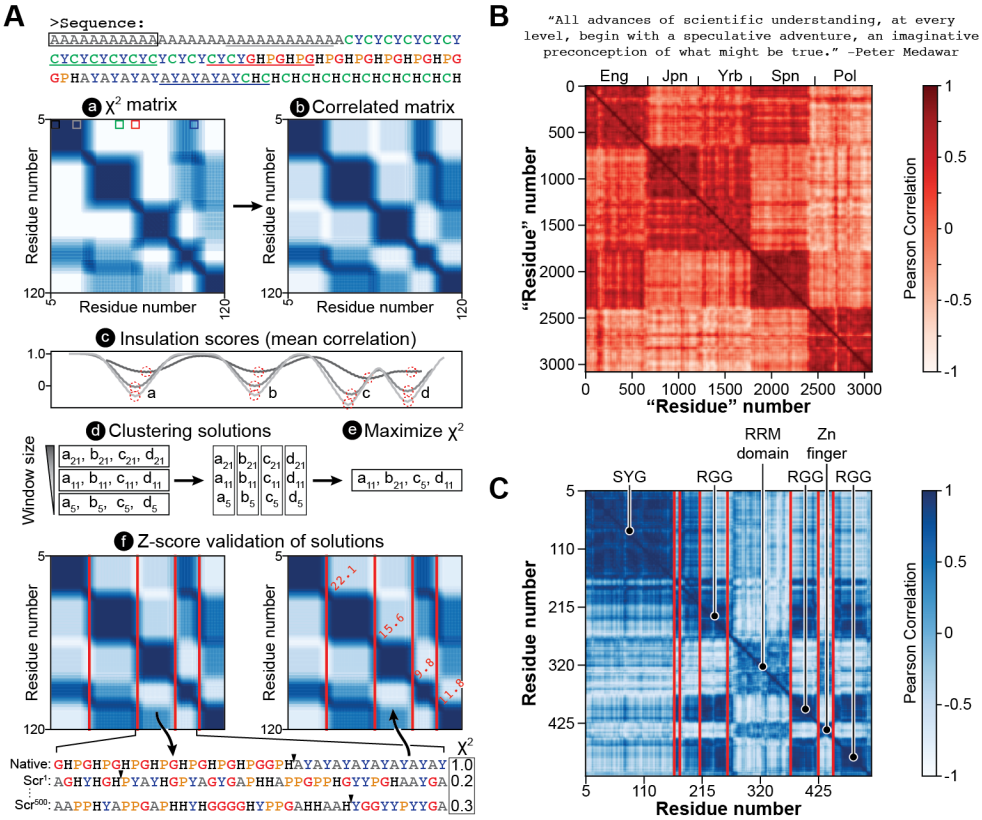


Figure 1: A bioinformatic algorithm to measure compositional dispersion across a sequence. A) For a given sequence, a) pairwise chi-scores are calculated for all window-defined sub-sequences and plotted as a matrix. Window sizes include every odd size from 5-21. Then, for each window size, b) chi-scores are converted to Pearson's correlation coefficients, c) insulation scores are calculated to define local minima which represent compositional boundaries, and d) the minima identified for all window sizes are grouped according to proximity. From these groups, e) each boundary placement is optimized to maximize chi-score and f) z-scores are calculated for each boundary element and are used to iteratively remove non-significant boundaries. B) The chi-score method can distinguish between human languages on the basis of alphabet usage. A quote from Peter Medawar's Romane Lecture was translated into five languages and each translation was strung end-to-end for analysis. "Eng" = English, "Jpn" = Japanese, "Yrb" = Yoruba, "Spn" =

Spanish, and “Pol” = Polish. C) The chi-score method can distinguish between regions of sequence bias in the human protein FUS. Boundaries are shown for 95% confidence level.

FIGURE 2

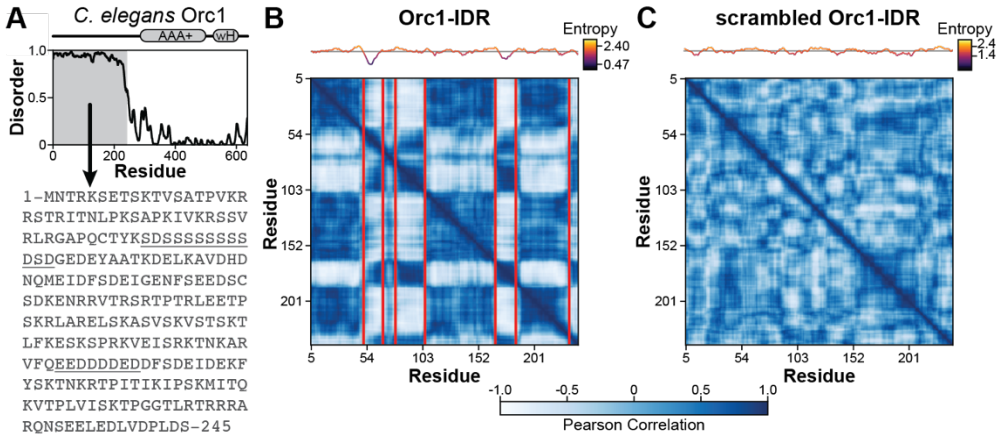


Figure 2: High-complexity disordered regions are non-randomly organized. A) The *C. elegans* Orc1 IDR (Orc1-IDR) has a long N-terminal IDR (residues 1-245) as predicted by Metapredict (35) but no obvious sequence patterning. B) Chi-score analysis reveals a strikingly modular architecture for Orc1-IDR with alternately repeating blocks of like-type sequences. C) Modularity is lost when the sequence of Orc1-IDR is randomly scrambled. Boundaries are shown for 95% confidence level.

FIGURE 3

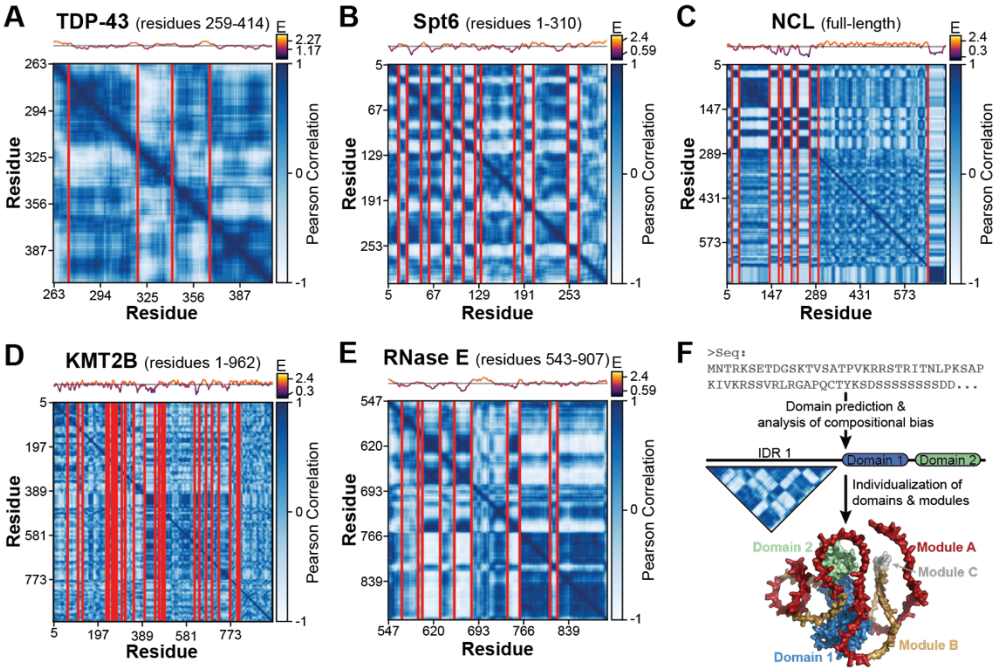
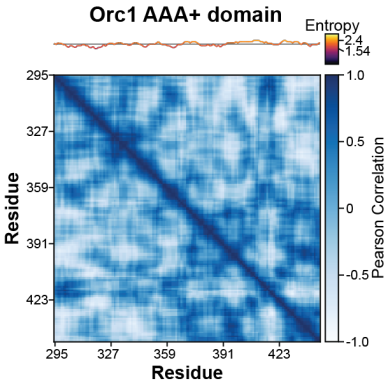


Figure 3: Many intrinsically disordered regions have a modular architecture. Chi-score analysis for A) human TDP-43 IDR (residues 259-414), B) human Spt6 N-IDR (residues 1-310), C) human Nucleolin (NCL), D) human KMT2B N-IDR (residues 1-962), and E) *C. crescentus* RNaseE C-IDR (residues 543-907). Boundaries are shown for 95% confidence level. NCL contains four tandem RNA binding domains which are contained within the largest module in the matrix and appear compositionally uniform. F) IDRs should not be thought of as indivisible units but as modular sequences with region-specific physiochemical features and biological function.

S2 FIG



S2 Fig: High-complexity disordered regions are non-randomly organized. Chi-score analysis of the Orc1 AAA+ domain reveals a compositionally uniform and non-modular sequence landscape. At a 95% confidence level, no statistically significant boundaries are identified.

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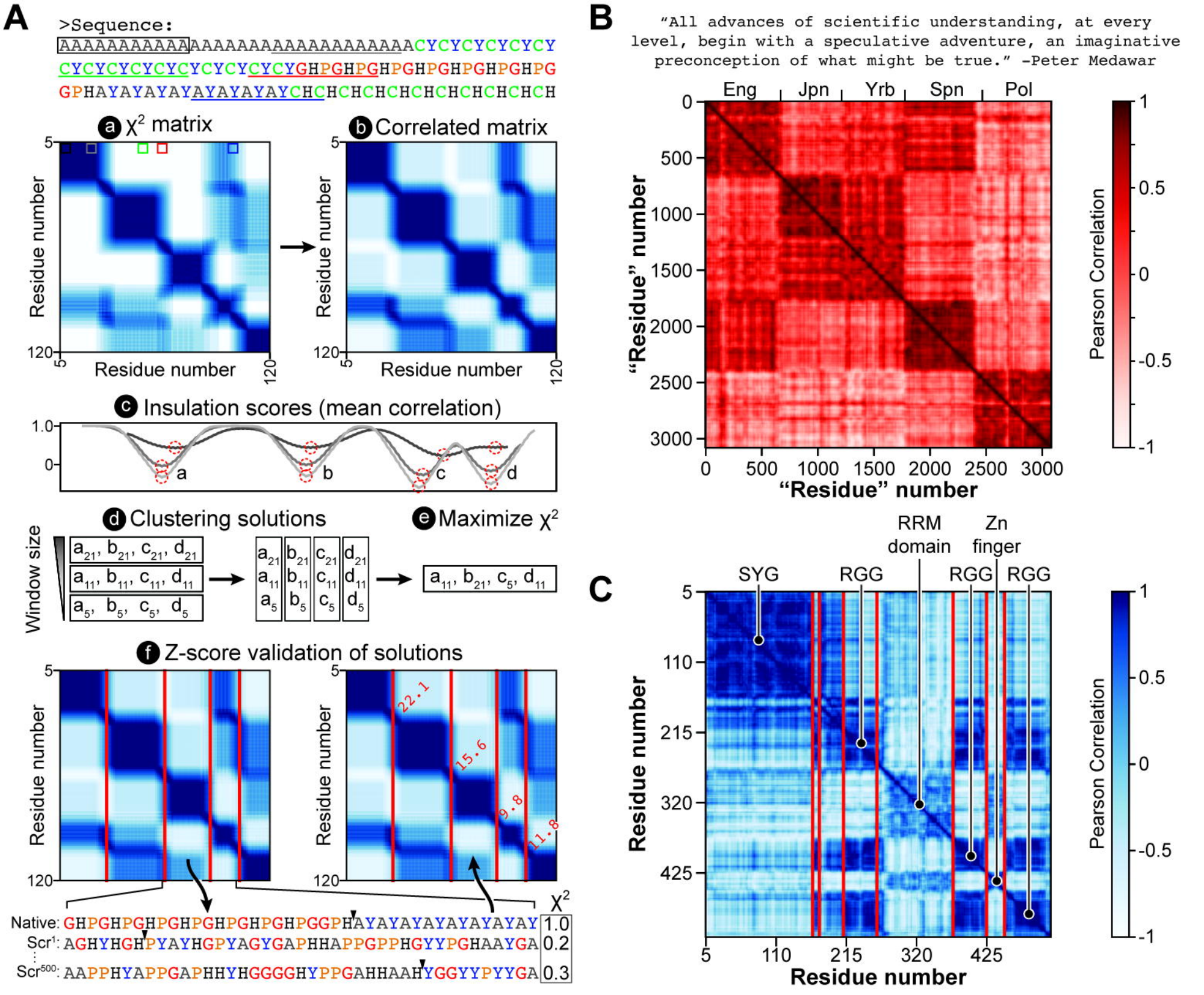
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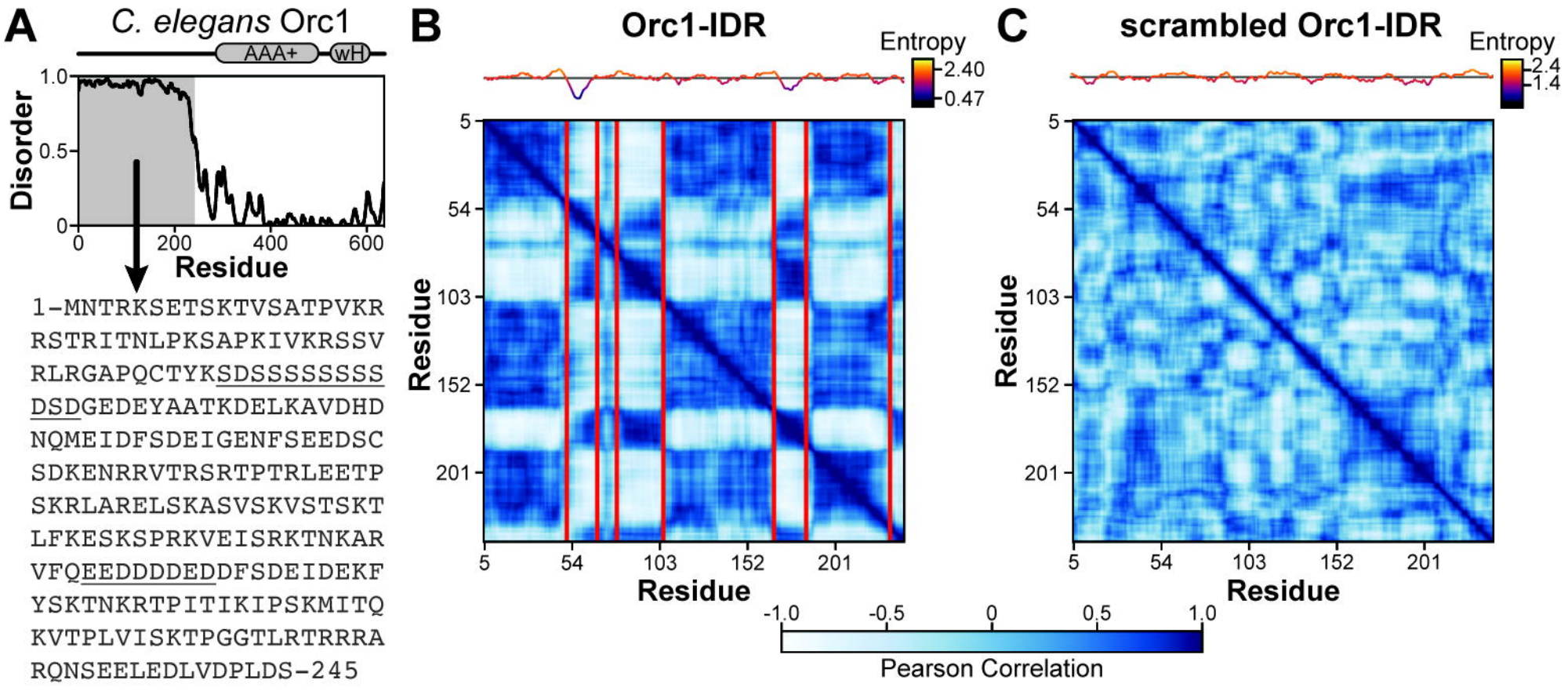
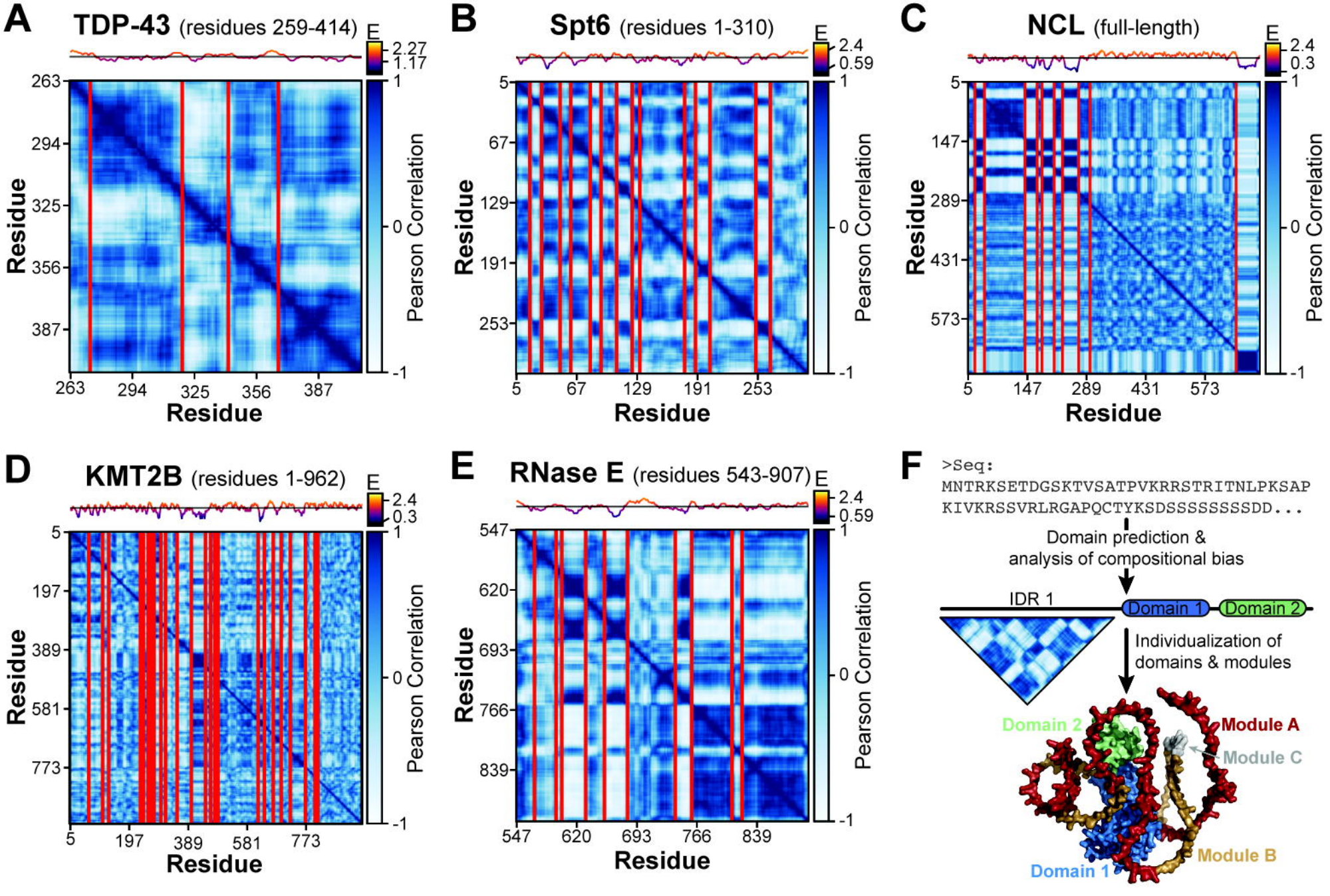
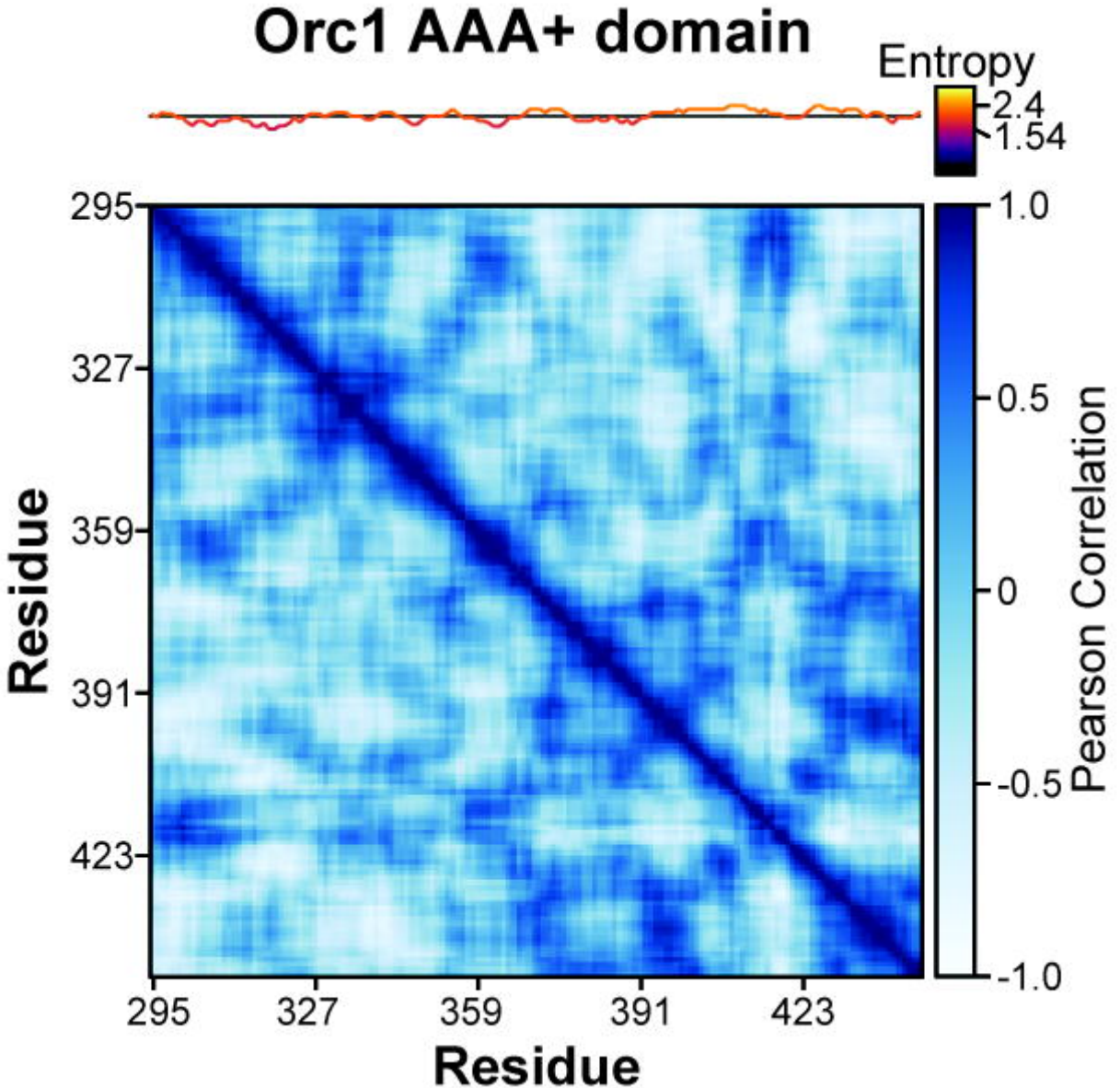
a χ2 matrix
b Correlated matrix
Residue number
c Insulation scores (mean correlation)
a
b
c
d
d Clustering solutions
e Maximize χ2
Window size
a21, b21, c21, d21
a11, b11, c11, d11
a5, b5, c5, d5
a11, b21, c5, d11
f Z-score validation of solutions
22.1
15.6
9.8
11.8
χ2
Native: GHPGHPGHPGHPGHPGHPGHPGHPGGPHAYAYAYAYAYAYAYAY 1.0
Scr1: AGHYHGHPYAYHGPYAGYGAPHHAPPGPPHGYYPGHAAYGA 0.2
Scr500: AAPPHYAPPGAPHHYHGGGGHYPPGAHHAAHYGGYYPYYGA 0.3
B
"All advances of scientific understanding, at every level, begin with a speculative adventure, an imaginative preconception of what might be true." -Peter Medawar
Eng
Jpn
Yrb
Spn
Pol
"Residue" number
Pearson Correlation
C
SYG
RGG
RRM domain
Zn finger
Residue number


A
C. elegans Orc1
AAA+
wH
Disorder
1.0
0.5
0
0
200
400
600
Residue
1-MNTRKSETSKTVSATPVKR
RSTRITNLPKSAPKIVKRSSV
RLRGAPQCTYKSDSSSSSSSS
DSDGEDEYAATKDELKAVDHD
NQMEIDFSDEIGENFSEEDSC
SDKENRRVTRSRTPTRLEETP
SKRLARELSKASVSKVSTSKT
LFKESKSPRKVEISRKTNKAR
VFQEEDDDDEDDFSDEIDEKF
YSKTNKRTPITIKIPSKMITQ
KVTPLVISKTPGGTLRTRRRA
RQNSEELEDLVDPLDS-245
B
Orc1-IDR
Entropy
2.40
0.47
Residue
5
54
103
152
201
C
scrambled Orc1-IDR
Entropy
2.4
1.4
-1.0
-0.5
0
0.5
1.0
Pearson Correlation


A
TDP-43 (residues 259-414)
E
2.27
1.17
263
294
325
356
387
Residue
Residue
Pearson Correlation
1
0
-1
B
Spt6 (residues 1-310)
E
2.4
0.59
5
67
129
191
253
Residue
Residue
Pearson Correlation
C
NCL (full-length)
E
2.4
0.3
5
147
289
431
573
Residue
Residue
Pearson Correlation
D
KMT2B (residues 1-962)
E
2.4
0.3
5
197
389
581
773
Residue
Residue
Pearson Correlation
E
RNase E (residues 543-907)
E
2.4
0.59
547
620
693
766
839
Residue
Residue
Pearson Correlation
F
>Seq:
MNTRKSETDGSKTVSATPVKRRSTRITNLPKSAP
KIVKRSSVRLRGAPQCTYKSDSSSSSSSSDD...
Domain prediction &
analysis of compositional bias
IDR 1
Domain 1
Domain 2
Individualization of
domains & modules
Domain 2
Module A
Module C
Module B
Domain 1


Orc1 AAA+ domain
Entropy
2.4
1.54
295
327
359
391
423
Residue
295
327
359
391
423
Residue
1.0
0.5
0
-0.5
-1.0
Pearson Correlation