Intrinsic Disorder in the Human Tear Proteome

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Purpose. We aimed to characterize the proteome of human tears and assess for the presence of intrinsically disordered proteins (IDPs). IDPs, despite lacking a rigid three-dimensional structure, maintain biological functionality and could shed light on the molecular interactions within tears.

Methods. We analyzed a dataset of 1475 proteins identified in the tear film of three healthy subjects. We employed several computational tools, including the Compositional Profiler, Rapid Intrinsic Disorder Analysis Online, Search Tool for the Retrieval of Interacting Genes, and Database of Disordered Protein Predictors to evaluate the intrinsic disorder, protein interactions, and functional characterization of the disordered regions within this proteome.

Results. Our analysis showed a notable inclination toward intrinsic disorder. Two out of 10 order-promoting residues and five out of 10 disorder-promoting residues were found enriched. Using the Predictor of Natural Disordered Regions (PONDR) VSL2 output, 95% of these proteins were classified as highly or moderately disordered. We revealed an extensive protein–protein interaction network with significant interaction enrichment. The most disordered proteins exhibited higher disorder binding sites and diverse post-translational modifications compared to the most ordered ones.

Conclusions. To the best of our knowledge, our study is the first comprehensive analysis of intrinsic disorder in the human tear film proteome, and it revealed an abundance of IDPs and their role in protein function and interaction networks. These findings suggest that variations in the intrinsic disorder of a tear film could be impacted by systemic and ocular conditions, offering promising avenues for disease biomarker identification and drug target development. Further research is needed to understand the implications of these findings in human health and disease.

Keywords: tears, proteomics, intrinsic disorder

Tears, also known as tear film or fluid, are the most anterior component of the ocular globe and cover the surface of epithelial cells. The tear film is composed of a complex mixture of proteins, peptides, electrolytes, lipids, and small molecule metabolites and is essential for maintaining the health of the ocular surface.[1,2] Tears are produced by a combination of glands and cells in the eye, including the main and accessory lacrimal glands, ocular surface epithelial cells, Meibomian glands, goblet cells, and an ultrafiltrate of blood.[1] In addition, the tear film acts as a first line of defense against pathogens, flushes out contaminants, moisturizes the ocular surface, lubricates the lid–cornea interface during blinking and sleeping, nourishes avascular corneal epithelial cells, and improves optical properties by modifying the refractive index of the cornea.[3]

Recent breakthroughs in proteomics, metabolomics, and lipidomics have significantly expanded our understanding of the molecular composition of the tear film.[4–6] These innovations have opened up new avenues for investigating the tear film, revealing previously unappreciated components such as novel proteins, peptides, and lipid species that warrant further investigation. In addition, tear film analysis is a non-invasive and readily available sample for biomarker discovery and diagnostics, with the potential for early disease detection and personalized treatment plans.[1,7,8]

To the best of our knowledge, the presence of intrinsically disordered proteins (IDPs) in the human tear proteome remains unexplored. IDPs (i.e., entire proteins) and intrinsically disordered protein regions (IDPRs, or protein segments) are polypeptides characterized by the absence of three-dimensional (3D) and well-defined structural elements. They are known to be among the most dynamic and functional proteins and protein regions, as evidenced by the recently published literature on this topic.[9–11] Moreover, mounting evidence suggests that IDPs

and IDPRs may represent promising targets for novel drug development and biomarker discovery, given their role in various pathologies, including cancers and neurodegenerative diseases.[12–14] Therefore, it is crucial to consider the presence of IDPs and IDPRs when studying proteins in any cellular compartment or physiological system. Characterizing these entities allows for a deeper understanding of the molecular dynamics that underlie the function of cells and other physiological systems.

In our previous research, we characterized the intrinsic disorder of the aqueous humor proteome, revealing its abundance and potential functional and pathological implications for the eye.[15] Building on this work, our current study focuses on the human tear proteome. Specifically, we hypothesize that the human tear proteome is also rich in IDPs and IDPRs. Our analysis centers on 1543 proteins previously identified in the human tear film proteome by Zhou et al.,[16] the most accurate and extensive set of proteins identified in the human tear proteome reported to date. We aim to utilize the power of advanced computational techniques and neural network–based models to quantify and classify the level of intrinsic disorder of the human tear proteome and explore its network of protein-protein interactions. The presence of IDPs and IDPRs in this microenvironment could yield valuable insights into the molecular functions and interactions and provide a framework for developing biomarkers and novel targets for ocular diseases in the future.

Methods

Proteome Sequence Retrieval

In this study, we used the data from an in-depth analysis of the human tear proteome by Zhou et al.,[16] who identified 1543 proteins in the tears of healthy subjects (three females and one male; average age, 36 ± 14 years) using a high-speed TripleTOF 5600 system (SCIEX, Framingham, MA, USA) with a less than 1% false discovery rate. However, the proteins reported were based on the older International Protein Index identification system, and thus this list of proteins had to be mapped to the more widely accepted identification used in the Universal Protein (UniProt) database.[17] Of the 1543 identified proteins, 74% (1154) were automatically matched to UniProt IDs using SWISS-PROT. The remaining 389 proteins not matched automatically by SWISS-PROT were each matched manually using their corresponding protein and gene names within the UniProt database. Subsequently, 1475 of these identified protein sequences were employed in further analysis, and their protein sequences were then obtained from the UniProt database in FASTA format.

Proteome Composition Analysis

The human tear proteome was analyzed using the Composition Profiler (available at http://www.cprofiler.org/),[18] which allows the measurement and visualization of the enrichment or depletion of common amino acids in the human tear proteome. To generate an amino acid composition profile of the proteins present within the tear proteome, the Composition Profiler was utilized, with the human tear proteome in FASTA format serving as the query set and the Protein Data Bank (PDB) Select 25 as the background set. Additionally, composition profiles for experimentally validated disordered proteins from the DisProt database and the distribution of amino acids in nature from the SWISS-PROT database were generated for comparison. The normalized enrichment or depletion is evaluated as $(C_x - C_{order})/C_{order}$, where C_x is the content of a given residue in its query protein, and C_{order} is the content of the same residue in the PDB Select 25.

Intrinsic Disorder Quantification

Prediction of Disorder Using Commonly Used Predictors. In this stage of our analysis, we aimed to quantify the level of intrinsic disorder in the proteins of interest. To accomplish this, we employed the Predictor of Natural Disordered Regions (PONDR),[19] specifically the PONDR VSL2 model, to quantify intrinsic disorder on a per-residue basis. Additional predictors of intrinsic disorder were employed to further validate our findings and to gain deeper insight into the disorder status of the human tear proteome. Scores and percentages were collected for three additional PONDR predictors: PONDR VL3, PONDR VLXT, and PONDR FIT.[20,21] Additionally, the Prediction of Intrinsically Unstructured Proteins (IUPred2A) platform (available at https://iupred2a.elte.hu/) was used to generate predictions for short and long disordered regions.[20] These per-residue intrinsic disorder predictors were accessed through the novel Rapid Intrinsic Disorder Analysis Online (RIDAO) tool (available at https://www.ridao.app).[21]

Mean Disorder Profile Analysis. A mean disorder profile (MDP) score and percentage were calculated by averaging the predictions of the PONDR and IUPred2A platforms. In these analyses, proteins were classified based on the percentage of predicted disordered residues (PPDR), with two arbitrary cutoffs being used to classify proteins as highly ordered (PPDR < 10%), moderately disordered (10% ≤ PPDR < 30%), and highly disordered (PPDR ≥ 30%).[22] It is also worth noting that the average disorder score (ADS) of a given protein is not directly related to its PPDR value. Therefore, we also evaluated the disorder status of proteins using this criterion. Here, a protein, region, or residue is ordered if it has an ADS < 0.15, moderately disordered or flexible if 0.15 ≤ ADS < 0.5, and disordered if the ADS ≥ 0.5.

Charge–Hydropathy and Cumulative Distribution Function Plot Analysis. Additionally, to evaluate intrinsic disorder on a whole protein basis, we employed two binary predictors of disorder: the charge–hydropathy (CH) plot and the cumulative distribution function (CDF; available at http://www.pondr.com/). These binary predictors are combined to classify a protein as either completely ordered or disordered and generate a CH–CDF plot.[23,24] The data used to generate these plots were sourced from the RIDAO platform.[21]

Protein–Protein Interaction Network

To evaluate the degree of interactivity between the human tear proteins, we employed the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; available at https://string-db.org/).[25] STRING generates a protein–protein interaction (PPI) network based on predicted and experimentally validated information on the interaction partners for a protein of interest or a set of proteins. The analysis was carried out for 1475 human tear film proteins, which were the primary input for the STRING analysis.

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Analysis of the Most Disordered Proteins

Quantification of Disorder. To gain a deeper understanding of the disorder status of the proteins in our set, we identified the five most disordered and five most ordered proteins based on their PONDR VSL2 scores. We used PONDR VSL2, a predictor chosen for its exceptional performance at the recent Critical Assessment of protein Intrinsic Disorder prediction (CAID) experiment,[26] where it was ranked third among 43 methods evaluated on a dataset of 646 proteins from DisProt.[27]

Structural Assessment. To gain further insight into the structures of the most disordered human tear proteins, we employed the capabilities of AlphaFold. This cutting-edge deep learning algorithm incorporates physical and biological information about protein structures to produce highly accurate predictions of protein structures.[28,29] Specifically, we utilized AlphaFold to create a gallery of structures for the identified disordered tear fluid proteins.

Disorder Profiles. Additional intrinsic disorder predictors were used to validate these results and gain a more comprehensive understanding of the disorder of these selected proteins. These were used to generate per-residue intrinsic disorder profiles using the six intrinsic disorder predictors PONDR VLXT, PONDR VL3, PONDR VSL2, PONDR FIT, IUPred Short, and IUPred Long.

Functional Disorder Profiles. Selected most ordered and most disordered proteins were further characterized by the Database of Disordered Protein Predictions (D^2P^2; available at https://d2p2.pro/). This platform integrates outputs of nine disorder predictors, allowing for a consolidated visualization of the disorder prediction tools. It provides protein structural classifications and predicts superfamily affiliations, sequence annotations, and disorder-based binding sites. Additionally, it predicts disorder-based binding regions, which may convert from a disordered to an ordered state when interacting with specific partners (i.e., Molecular Recognition Features [MoRFs]), with specific regions folding into ordered structures when engaged.[30] The D^2P^2 platform also predicts possible posttranslational modifications (PTMs).[31]

Results

Proteome Composition Analysis

The composition of the 1475 human tear proteins was analyzed and compared through visualization (Fig. 1) with the experimentally validated protein composition profile from the DisProt database and the natural distribution of amino acids from the SWISS-PROT database. The amino acids were ranked according to their propensity to promote either order or disorder, with positive values indicating enrichment and negative values indicating depletion. Out of the 10 order-promoting residues (C, W, I, Y, F, L, H, V, N, and M), only two (C and L) showed enrichment. Conversely, five out of the 10 disorder-promoting residues (R, T, D, G, A, K, Q, S, E, and P) showed enrichment. The composition profile of human tear proteins mostly displayed similarities with the DisProt protein set, particularly in the enrichment of the most disorder-promoting residues (Q, S, E, and P), which was inconsistent with the SWISS-PROT protein set.

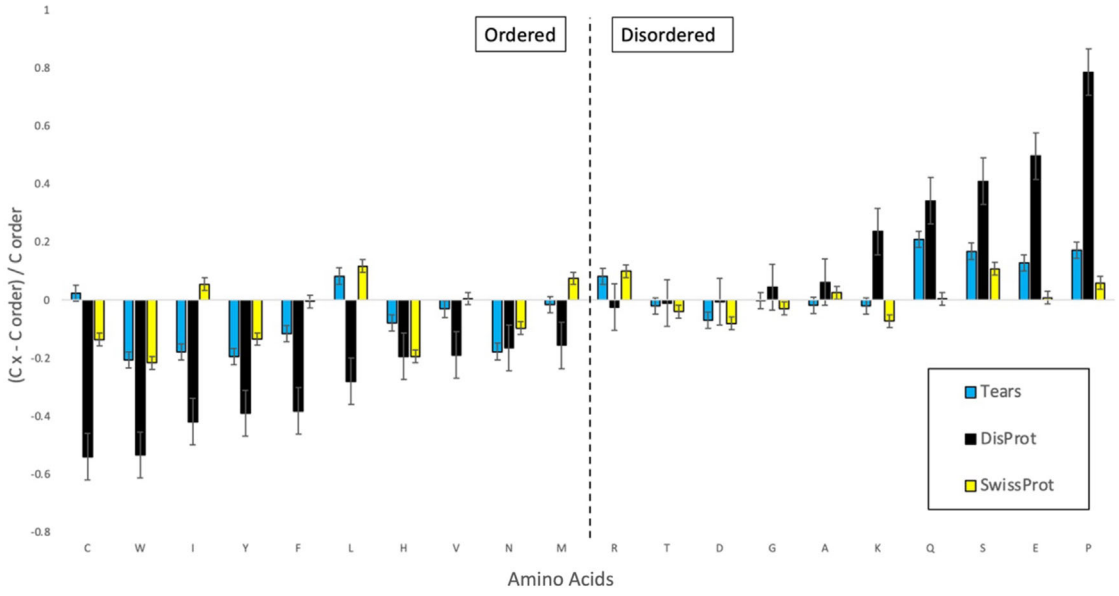

Figure 1. Amino acid composition profile of 1475 human tear proteins (*blue bars*). The fractional difference is calculated as $(C_x - C_{order})/C_{order}$, where C_x is the content of a given amino acid in the query set (1475 amino acid sequences of human tear proteins or proteins from the SWISS-PROT database), and C_{order} is the content of a given amino acid in the background set (Protein Databank Select 25). The amino acid residues are ranked from most order-promoting residue to most disorder-promoting residue. Positive values indicate enrichment, and negative values indicate depletion of a particular amino acid. The composition profile generated for experimentally validated disordered proteins from the DisProt database (*black bars*)[27] and the distribution of amino acids in nature from the SWISS-PROT database (*yellow bars*)[60] are shown for comparison. In both cases, *error bars* correspond to standard deviations over 10,000 bootstrap iterations. The composition profile analysis showed that two of 10 order-promoting residues (C and L) and five of 10 disorder-promoting amino acid residues (R, Q, S, E, and P) were enriched ($P < 0.05$).

Table 1. Average Disorder of the Human Tear Proteome as Predicted by Various Intrinsic Disorder Predictors*

	Disorder Predictor						
	VLXT	VSL2B	VL3	PFIT	IUPred Short	IUPred Long	MDP
ADS	0.35041	0.44781	0.36447	0.32947	0.27698	0.30347	0.32393
PPDR	31.53%	36.84%	27.56%	23.86%	16.02%	16.53%	20.37%

*Includes Predictor of Natural Disordered Regions (PONDR) VLXT, VSL2B, VL3, PFIT, Intrinsically Unstructured Regions of Proteins Short (IUPred Short), IUPred Long, and mean disorder profile (MDP). Each of the terms VLXT, VSL2B, VL3, PFIT, IUPred Short, and IUPred Long represents a unique computational tool used to predict protein disorder. The average disorder score (ADS) represents the disorder prediction score of each protein, and the percent of predicted disordered residues (PPDR) refers to residues with disorder scores above 0.5. The MDP is derived from the average disorder prediction of these six individual predictors.

Intrinsic Disorder Quantification

Prediction of Disorder Using Commonly Used Predictors. After establishing the amino acid composition propensity of the human tear proteome for disorder, we quantified the intrinsic disorder in the 1475 protein query sample with six per-amino acid residue predictors, which included PONDR VLXT, PONDR VSL2B, PONDR VL3, PONDR FIT, IUPred Short, and IUPred Long (Table 1). For each predictor, we quantified the overall disorder of proteins based on two measures, the ADS and the PPDR (percentage of residues with disorder scores above 0.5). The ADSs for each predictor were as follows: PONDR VLXT, 0.35041; PONDR VSL2B, 0.44781; PONDR VL3, 0.36447; PONDR FIT, 0.32947; IUPred Short, 0.27698; and IUPred Long, 0.30347. The PPDRs for each predictor were as follows: PONDR VLXT, 31.53%; PONDR VSL2B, 36.84%; PONDR VL3, 27.56%; PONDR FIT, 23.86%; IUPred Short, 16.02%; and IUPred Long, 16.53%. The MDPs (average disorder predictions of the six individual predictors) for the 1475 proteins in the human tear film were 0.3239 (ADS-based MDP) and 20.37% (PPDR-based MDP).

On a per-amino acid residue basis, the PONDR VSL2 output confirms the existence of numerous intrinsically disordered or partially intrinsically disordered proteins in the human tears (Fig. 2). Specifically, of the 1475 proteins found in the human tear proteome, 401 proteins (27.2%) are predicted to be highly disordered, 1011 proteins (68.5%) demonstrate moderate disorder or conformational flexibility (679 + 332), and only 63 proteins (4.3%) are predicted to be highly ordered.

MDP Analysis. The proteins were further analyzed with the aid of several other PONDR predictors, including PONDR VL3, PONDR VLXT, and PONDR FIT. The resulting PPDR (VSL2B Percent) and ADS (VSL2 Score) values for all 1475 proteins present in human tears are provided in Supplementary Table S1. The PONDR-centric analysis consistently showed agreement among the intrinsic disorder predispositions determined by the various tools, where a protein predicted to be highly disordered by one PONDR predictor was likely to be predicted as highly disordered by the others. An additional predictor of intrinsic disorder, the web server for IUPred, was utilized to validate the PONDR findings. The resulting per-residue disorder profiles generated by IUPred were used to calculate the corresponding PPDR and ADS values, as summarized in Supplementary Table S1. This analysis demonstrated reasonable agreement between the outputs of various PONDRs and the results generated by IUPred run in both long and short modes. As a final approach for the quantitative evaluation of disorder, mean disorder profiles (MDPs) were generated for all

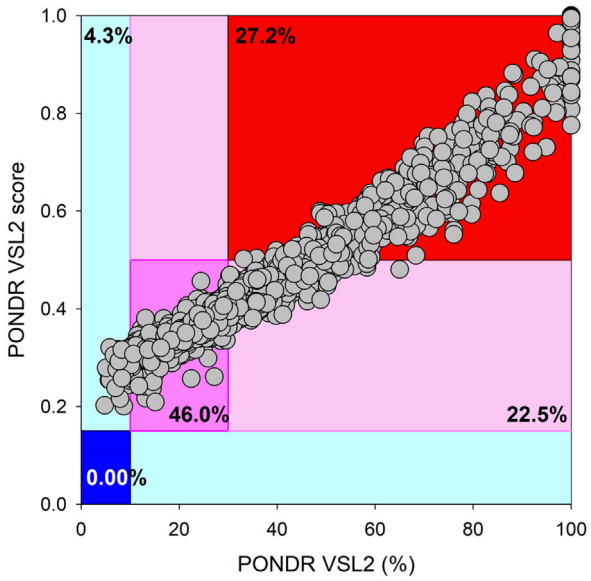

Figure 2. PONDR VSL2 output for 1475 human tear proteins. The PONDR VSL2 score is the ADS for a protein. PONDR VSL2 (%) is a PPDR (residues with disorder scores above 0.5). *Color blocks* indicate regions in which proteins are mostly ordered (*blue* and *light blue*), moderately disordered (*pink* and *light pink*), or mostly disordered (*red*). If the two parameters agree, the corresponding part of the background is dark (*blue* or *pink*), whereas *light blue* and *light pink* reflect areas in which only one of these criteria applies. The boundaries of the colored regions represent arbitrary and accepted cutoffs for ADS (*y*-axis) and the PPDR (*x*-axis). Of the 1475 proteins found in the human tear proteome, 401 proteins (27.2%) are predicted to be highly disordered, 1011 proteins (68.5%) demonstrate moderate disorder or conformational flexibility, and only 63 proteins (4.3%) are predicted to be highly ordered.

1475 proteins present in the human tear proteome, and their corresponding PPDR and ADS values were calculated (refer to Supplementary Table S1). This additional quantification of intrinsic disorder strongly supported the original findings that the human tear proteome contains proteins with varying levels of disorder.

The MDP-based PPDR analysis revealed that 368 proteins (24.86%) and 380 proteins (25.68%) were expected to be highly or moderately disordered, with 727 proteins (49.12%) classified as ordered. Additionally, in good agreement with the data depicted in Figure 2, the MDP-based ADS analysis indicated that 47 proteins (3.18%), 1225 proteins (82.77%), and 203 proteins (13.72%) were expected to be highly ordered, moderately disordered/flexible, and disordered, respectively (Fig. 3). Hence, the results of this study,

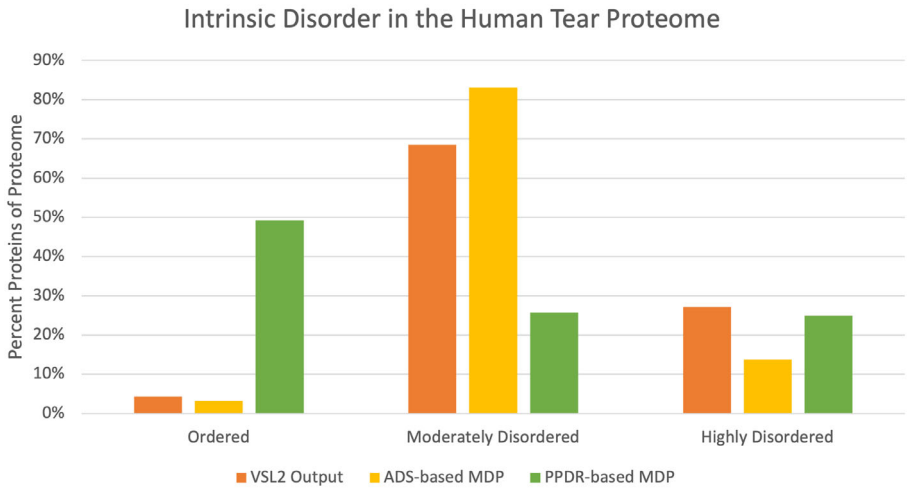

Figure 3. Classification of disordered proteins in the human tear proteome predicted by PONDR VSL2 output, ADS-based MDP, and PPDR-based MDP. ADS-based MDP is calculated by averaging the disorder score from six commonly used predictors, and PPDR-based MDP is calculated by averaging the disorder percent per residue from six commonly used predictors.

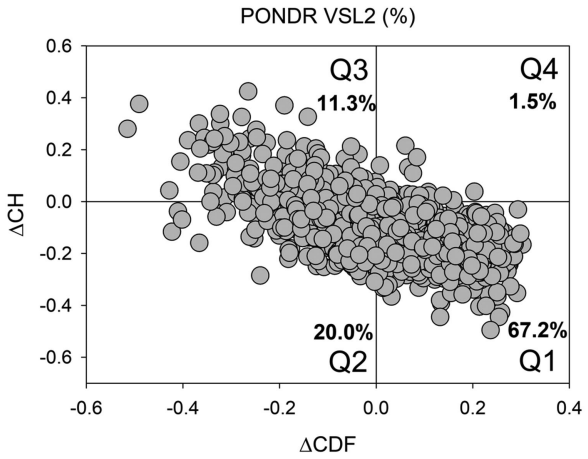

Figure 4. CH–CDF plot for 1475 human tear proteins (*gray dots*). The *y*-coordinate is calculated as the distance of the corresponding protein from the boundary in the CH plot. The *x*-coordinate is calculated as the average distance of the corresponding protein's *x*-curve from the CDF boundary. The quadrant where the protein is located determines its classification. Q1, protein predicted to be disordered by CH plot and ordered by CDF; Q2, protein predicted to be ordered by CH plot and CDF; Q3, protein predicted to be ordered by CH plot and disordered by CDF plot; Q4, protein predicted to be disordered by CH plot and CDF.

regardless of the tool used for analysis, indicated that a substantial proportion of the human tear proteome is expected to be disordered.

CH–CDF Plot Analysis. The CH–CDF plot, a combined binary predictor of intrinsic disorder, was used to verify the widespread presence of intrinsic disorder in the 1475 human tear proteins (Fig. 4). This plot enabled the categorization of each protein based on its plotted quadrant. In quadrant Q1, 991 proteins were found and were therefore predicted to be ordered by both predictors. In quadrant Q2, 295 proteins were identified as either molten globular proteins (compact but without clear 3D structures) or hybrid proteins consisting of balanced levels of ordered and disordered residues; these proteins were predicted to be ordered/compact by the CH plot and disordered by the CDF. In quadrant Q3, 167 proteins were highly disordered and were predicted to be disordered by both the CH plot and CDF plot. Finally, 22 proteins in quadrant Q4 were predicted to be disordered by the CH plot and ordered by the CDF plot. These findings indicate that 484 proteins from the human tear proteome were expected to have substantial levels of disorder. These results further reinforce the existence of intrinsic disorder in human tears.

Protein–Protein Interaction Network

The interaction network for human tear proteins was created using STRING. Most of these proteins are found to be involved in forming a densely interconnected network. The input of 1475 UniProt IDs was mapped by STRING to 1354 unique proteins, with the available interactivity information, whereas no STRING entries were found for the remaining 121 proteins. Of the 1354 predicted nodes, 21 (1.55%) were loners, whereas the remaining proteins were engaged in 32,373 interactions, thereby organizing a network with an average node degree of 47.8 (i.e., on average, each protein interacts with 47.8 partners). The network is characterized by an average local clustering coefficient of 0.395. The average local clustering coefficient quantifies the proximity of neighbors to form a complete clique. If a local clustering coefficient equals 1, then every neighbor connected to a given node N_i is also connected to every other node in the neighborhood. If the local clustering coefficient equals 0, then no node connected to a given node N_i connects to any other node. This PPI network generated for human tear proteins using STRING showed significant enrichment in interactions compared to the expected number of 21,577 interactions in a randomly selected set of human proteins of similar size. Therefore, the PPI network among the proteins in the human tear proteome is characterized by a PPI enrichment $P < 10^{-16}$. This finding indicates that the query proteins in the analyzed network have more interactions with one another than would be expected by chance. As a result, this enrichment suggests that the proteins are biologically connected as a group to some extent.

Table 2. Five Most and Five Least Disordered Proteins in the Human Tear Proteome Identified by PONDR VSL2, Their UniProt IDs, and Molecular Functions Listed on UniProt

	Protein Name	Abbreviation	Molecular Function	UniProt ID	PONDR VSL2 Score
Most disordered	Parathymosin	PTMS	Histone binding	P20962	0.9982467
	Translation machinery-associated protein 7	TMA7	—	Q9Y2S6	0.9958404
	Non-histone chromosomal protein HMG-14	HMGN1	DNA binding and chromatin binding	P05114	0.9944657
	Myristoylated alanine-rich C-kinase substrate	MARCKS	Cytoskeletal protein binding, calmodulin binding, identical protein binding, and protein kinase C binding	P29966	0.9944657
	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	NUCKS1	DNA binding, RNA binding, transcription regulator activity, chromatin binding, DNA-binding transcription factor binding	Q9H1E3	0.9878788
Least disordered	Transmembrane ascorbate-dependent reductase CYB561	CY561	Oxidoreductase activity, metal ion binding	P49447	0.21685
	Cytochrome c oxidase subunit 2	COX2	Transporter and oxidoreductase activity, copper ion binding	P00403	0.21603
	Proteolipid protein 2	PLP2	Transporter activity, chemokine binding	Q04941	0.20903
	Prolactin-inducible protein	PIP	Cytoskeletal protein binding, hydrolase, catalytic activity, identical protein binding, immunoglobulin G binding	P12273	0.20253
	Solute carrier family 22 member 10	S22AA	Transporter activity	Q63ZE4	0.20073

Analysis of the Most Disordered Proteins

Quantification of Disorder. We comprehensively examined the human tear protein set, focusing on its most and least disordered members using the PONDR VSL2 predictor. The five proteins that exhibited the highest level of disorder, as determined by PONDR VSL2, were small proline-rich protein 2A (SPR2A; PONDR score = 0.9992), cornifin-A (SPR1A; PONDR score = 0.99962), cornifin-B (SPR1B; PONDR score = 0.99906), parathymosin (PTMS; PONDR score = 0.99824), and translation machinery-associated protein 7 (TMA7; PONDR score =

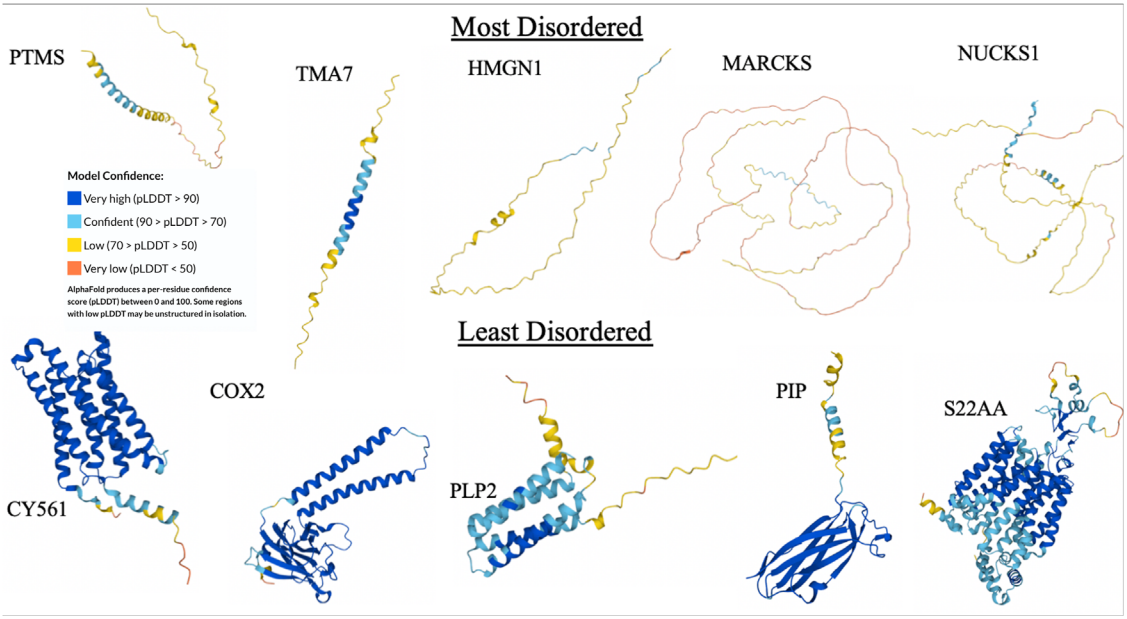

Figure 5. AlphaFold2 generated structures for the five most and five least disordered human tear proteins. The most disordered proteins are characterized by regions with low per-residue confidence scores, suggesting that they are unstructured. These regions are depicted in *yellow* and *orange*.

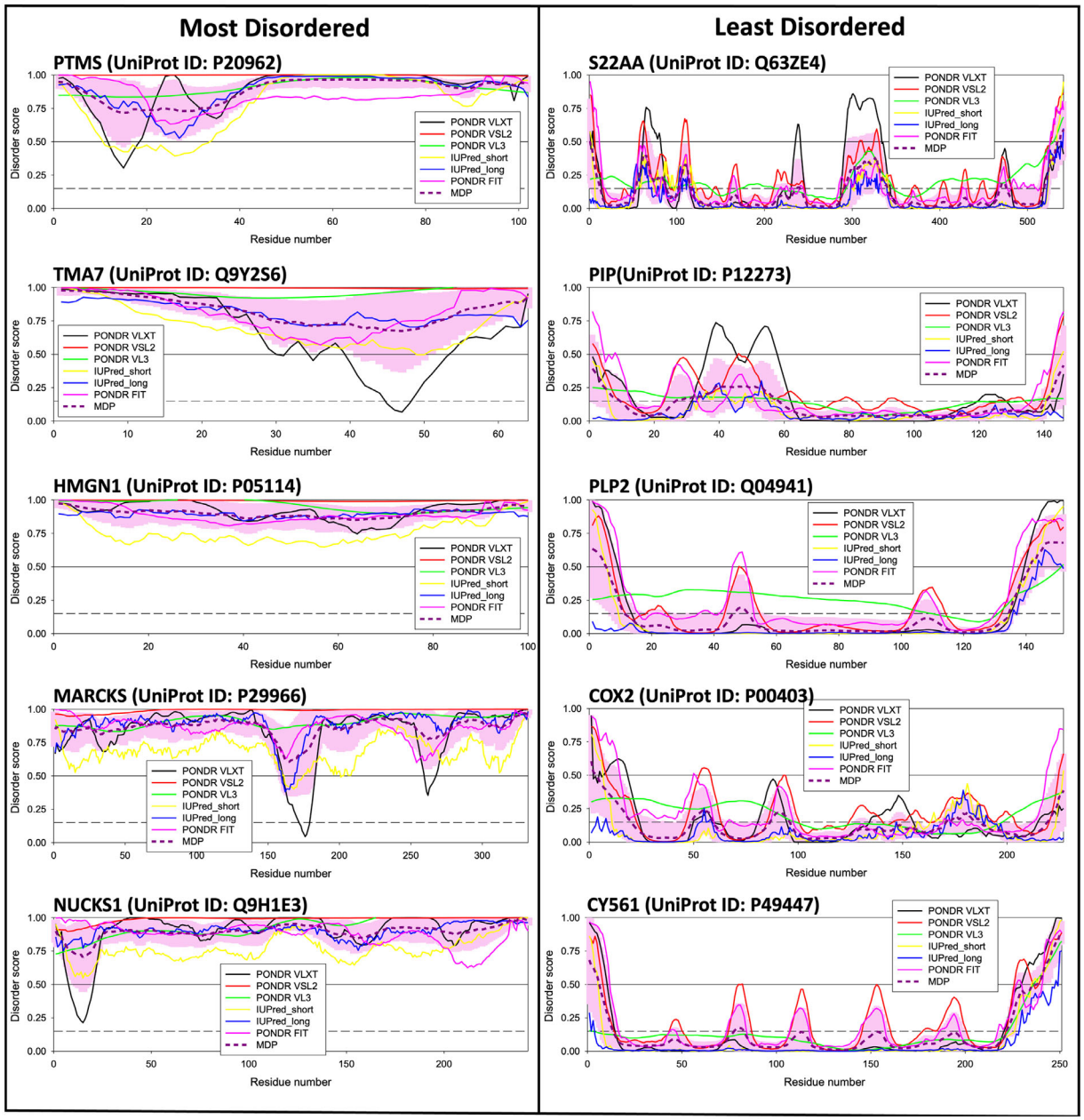

Figure 6. Per-residue disorder profiles generated for the five most disordered and five least disordered human tear proteins. Profiles were generated by RIDAO. The output aggregates the results from several well-known disorder predictors: PONDR VLXT,[61] PONDR VL3,[62] PONDR VLS2B,[19] PONDR FIT,[20] IUPred Short, and IUPred Long.[63,64] The outputs of evaluating the per-residue disorder propensity by these tools are represented as real numbers between 0 (ideal prediction of order) and 1 (ideal prediction of disorder). A threshold of ≥0.5 is used to identify disordered residues and regions in query proteins.

0.99584). However, further analysis showed that some of these proteins had a high content of cysteine residues, which can mislead other predictors, such as those from the IUPred series. Therefore, we selected the five proteins identified as highly disordered by all of the predictors used in the study. These five proteins are parathymosin (UniProt ID P20962; PONDR score = 0.99825), TMA7 (PONDR score = 0.99584), non-histone chromosomal protein HMG-14 (HMGN1; PONDR score = 0.9944657), myristoylated alanine-rich C-kinase substrate (MARCKS; PONDR score = 0.9944657), and nuclear ubiquitous casein and cyclin-dependent kinase substrate 1 (NUCKS1; PONDR score = 0.9878788) (Table 2). For comparison, the five most ordered proteins as determined by PONDR VSL2 were transmembrane ascorbate-dependent reductase CYB561 (CY561; PONDR score = 0.21685), cytochrome c oxidase subunit 2 (COX2; PONDR score = 0.21603), proteolipid protein 2 (PLP2; PONDR score = 0.20903), prolactin-inducible protein (PIP; PONDR score = 0.20253), and solute carrier family 22 member 10 (S22AA; PONDR score = 0.20073) (Table 2). The amino acid sequences of these 10 proteins are shown in the Supplementary Materials (Supplementary Fig. S1).

Structural Assessment. AlphaFold was used to generate 3D structures of the five most and least disordered

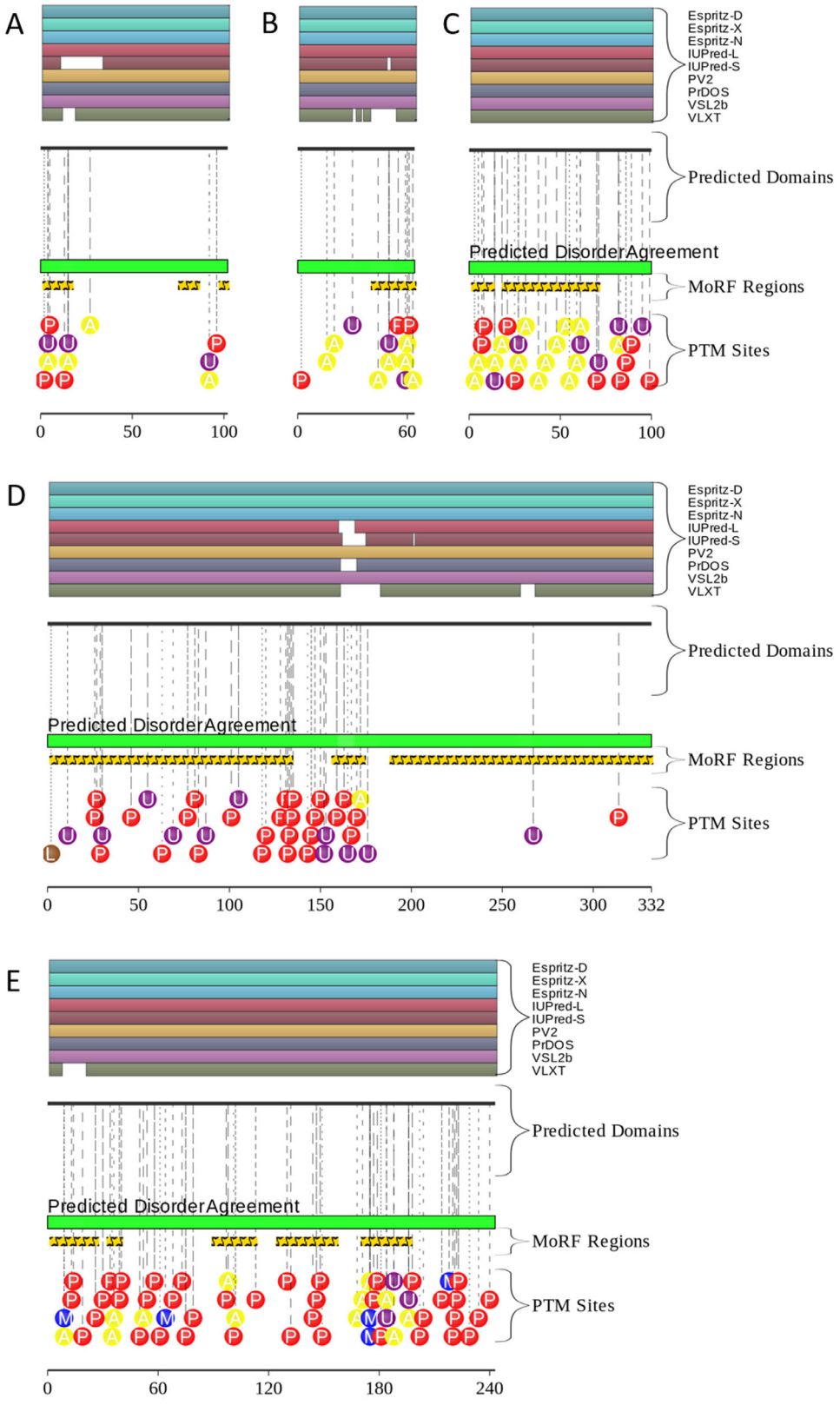

Figure 7. The D^2P^2 platform output assesses the functional disorder profile for five highly disordered proteins from human tear proteome: (**A**) PTMS, (**B**) TMA6, (**C**) HGMN1, (**D**) MARKCS, and (**E**) NUCKS1. The *top right* of the figure shows outputs from various per-residue disorder predictors (i.e., Espritz-D, Espritz-X, Espritz-N, IUPred-L, IUPred-S, PV2, PrDOS, VSL2b, VLXT); the *second bracket* shows predicted protein domains; the *third level* represents predicted disorder agreement; the *third bracket* shows where molecular recognition features are located (MoRFs, disordered regions that become ordered when binding); and the *fourth bracket* shows PTM sites.

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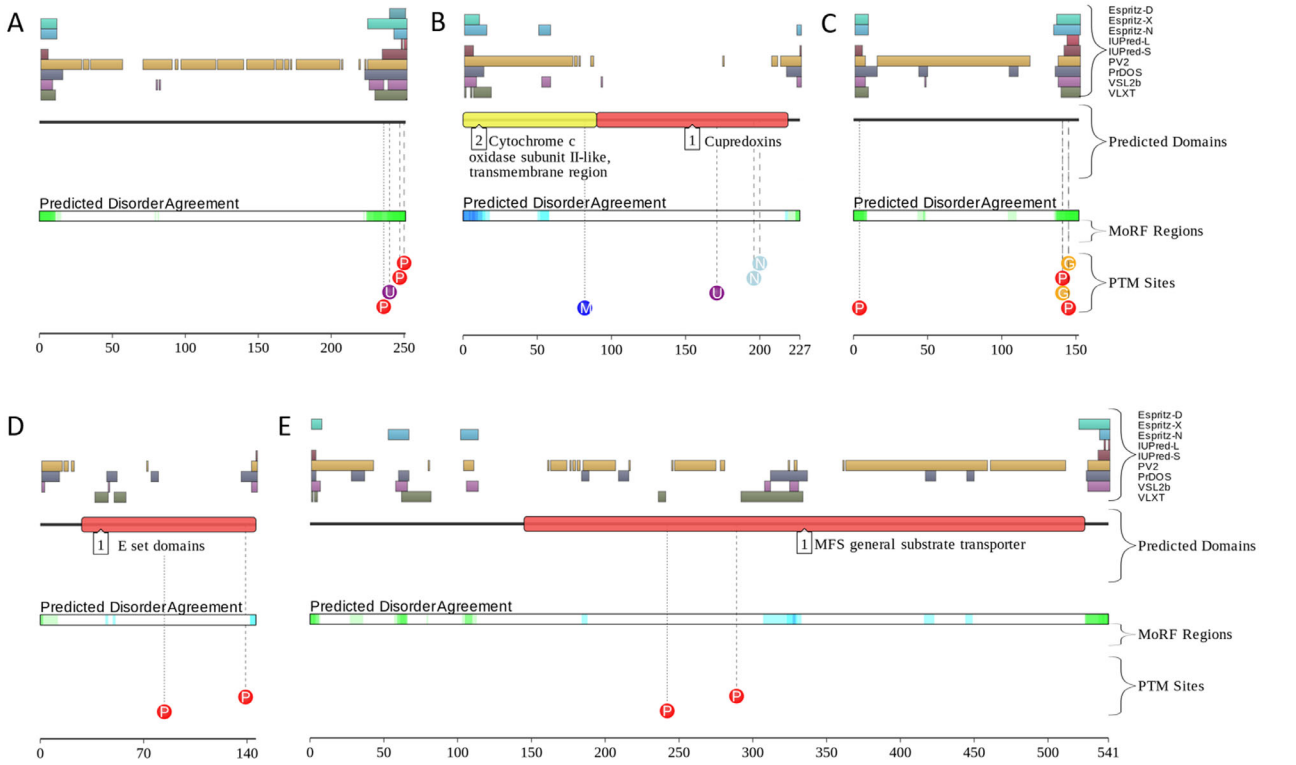

Figure 8. The D^2P^2 platform output assesses the functional disorder profile for five least disordered proteins from human tear proteome: (**A**) CY561; (**B**) COX2; (**C**) PLP2, (**D**) PIP, and (**E**) S22AA.

proteins in the human tear proteome. Figure 5 provides a visual representation of the respective structures, demonstrating the high degree of disorder in five proteins that were classified as intrinsically disordered based on the results of our bioinformatics analyses. This observation is further supported by the comparative analysis with the least disordered proteins, providing a clear visualization of the findings. The results of this study align with the evaluation of the global disorder predisposition in proteins (Table 1).

Disorder Profiles. Per-residue disorder profiles generated for the five most and five least disordered proteins by several commonly used predictors are assembled in Figure 6. The stark difference between highly disordered and highly ordered proteins can be visualized in the figure. Additionally, the per-residue disorder profiles can help us visualize that, although a protein may be disordered overall, certain regions exhibit varying disorder levels, which can be compared visually with the generated AlphaFold structures. Finally, a remarkable agreement between the outputs of six tools used to evaluate disorder predisposition is observed for all 10 proteins.

Functional Disorder Profiles. To elucidate the potential biological functionality of intrinsic disorder, next we utilized the D^2P^2 platform. The corresponding outputs for the five most disordered and five most ordered proteins are shown in Figures 7 and 8, respectively. As disordered proteins are typically characterized by low sequence conservation,[19,32–34] it was not surprising to find that none of the five most disordered proteins contained evolutionary conserved functional domains (Fig. 7), whereas four of the least disordered proteins exhibited such domains (Fig. 8).

Remarkably, all of the highly disordered proteins contained disordered binding sites (MoRFs) capable of binding-induced folding. As shown in Figure 7, PTMS has three MoRFs, TMA7 has a single MoRF, HMGN1 has two MoRFs, MARCKS carries three MoRFs covering a significant portion of its sequence, and NUCKS1 interacts via five MoRFs. Contrarily, none of the least disordered proteins was predicted to possess MoRFs (Fig. 8).

A comparison of the functional disorder profiles of the most and least disordered proteins revealed a noticeable difference in their levels and diversity of PTMs. The highly disordered proteins PTMS, TMA7, HMGN1, MARCKS, and NUCKS1 were heavily decorated by diverse PTMs (Fig. 7), whereas the less disordered proteins CY561, COX2, PLP2, PIP, and S22AA had fewer PTMs, which were often located within their respective IDPRs (Fig. 8).

Discussion

Our work represents the first study to evaluate the human tear proteome for intrinsic disorder, building on previous studies that primarily focused on protein identification. We leveraged the most comprehensive human tear proteome available, which is over three times larger than those in previous studies.[35] This was made possible by advancements in liquid chromatography and mass spectrometry, which are powerful techniques for tear proteomics with high sensitivity, accuracy, and reproducibility.[16,36] Our study demonstrates that intrinsic disorder is abundant in the 1475 human tear film proteins we analyzed. The amino acid composition of the human tear proteome is enriched in many disorder-promoting residues. The amino acid composition of the human tear film demonstrates a propensity for intrinsic

disorder, as two of 10 order-promoting residues and five of 10 disorder-promoting amino acid residues were enriched. Cysteine (C) was an exception, which was highly enriched in human tear proteins but highly depleted in the DisProt protein set. This discrepancy may be due to the extracellular nature of tear fluid, where C is frequently utilized to stabilize proteins by forming disulfide bonds.

Per amino acid residue disorder predictors and binary disorder predictors provide additional evidence that intrinsic disorder is likely abundant in the human tear film proteome. The MDPs (average disorder prediction of the six individual predictors) for the 1475 proteins in the human tear film were 0.3239 (ADS-based) and 20.37% (PPDR-based). The PONDR VSL2 score versus PONDR VSL2 percent classification identified 401 proteins (27.2%) as highly disordered. The CH–CDF plotted two binary disorder predictors against each other and predicted that 991 proteins (67.2%) were ordered. The remaining 32.8% of the proteins were predicted to be disordered by one or both binary predictors.

Our analysis of the PPI network in the human tear film proteome further emphasizes the complex interplay of proteins in biological systems. The network density and complexity shown by our analysis confirm a distinct enrichment of protein–protein interactions, which is significantly greater than the expected number for a randomly selected set of human proteins of similar size ($P < 10^{-16}$). This enrichment indicates that the proteins in the human tear film proteome are not merely randomly interacting entities but are biologically connected, functioning as an interdependent group. These insights further underline the pivotal role of intrinsic disorder in protein interactivity, strengthening the idea that disorder may be an essential factor driving protein functionality and interaction within the tear film proteome. Hundreds of proteins are predicted to contain high levels of disorder and may be integral in forming such a dynamic interaction network. Finally, we show a wide range of intrinsic disorder among the proteins of the human tear film (PONDR VSL2 range, 0.20073–0.99825), which hints at the interplay between ordered and disordered proteins. The more disordered proteins are likely more promiscuous binders than the ordered proteins, given their ability to exist in many conformation states. Moreover, even the proteins typically categorized as highly ordered reveal regions of intrinsic disorder (refer to Figs. 5 and 6). This observation underscores the nuanced nature of protein order and disorder in the human tear film, indicating that it is not an all-or-none phenomenon but rather a spectrum of varying degrees of disorder.

Our analysis of the functional disorder profiles of tear film proteins using the D^2P^2 platform, as shown in Figures 7 and 8, underscores the potential functionality of intrinsic disorder in proteins and its implications in their biological roles. Our findings highlight that the five most disordered proteins do not contain evolutionary conserved functional domains, which aligns with previous research suggesting that disordered proteins typically exhibit low sequence conservation.[19,33,34] Conversely, four out of the five least disordered proteins possess such conserved functional domains, further supporting the concept that proteins with a high degree of order maintain functional domains conserved through evolutionary processes.

However, what sets highly disordered proteins apart is their possession of disordered binding sites, or MoRFs. These regions are crucial for enabling binding-induced folding, suggesting that disordered proteins may play essential roles in diverse interactions through these dynamic domains. This is corroborated by the high prevalence of MoRFs in the five most disordered proteins, potentially explaining their binding promiscuity and multifunctionality.

Our comparison of the functional disorder profiles also revealed significant differences in the levels and diversity of PTMs between the most and least disordered proteins. The most disordered proteins are heavily decorated by diverse PTMs, whereas the least disordered proteins feature considerably fewer PTMs, often located within the IDPRs. This observation aligns with the established knowledge that enzymatically catalyzed PTMs are preferentially located within the IDPRs.[37–40] These modifications can drastically influence protein function, providing another dimension to the functional versatility of disordered proteins. Taken together, our results underscore the functional implications of intrinsic disorder within proteins, substantiating the notion that such disorder contributes to the multifaceted functionality of proteins through its influence on binding promiscuity and posttranslational modifications. These highly disordered proteins, given their critical roles and complex functional profiles, may contribute to tear dysfunction and potential ocular surface morbidity. Therefore, future analyses could potentially focus on these protein targets as therapeutic avenues for the treatment of tear dysfunction.

Tears are a potentially rich source of information about the human body, with their complex composition reflecting environmental and systemic factors and ocular diseases. As a readily available and non-invasive sample (e.g., Schirmer's strips, glass capillary uptake),[41] the tear film is an ideal medium for biomarker discovery and diagnostic purposes that can be replenished in the blink of an eye.[8,42] A review article by Azkargorta et al.[7] provides a comprehensive overview that highlights the various advances in the use of proteomics and peptidomics to analyze the human tear fluid, as well as the translation of these techniques into clinical practice. Identifying biomarkers within the proteome of human tears holds the promise of early disease detection and more targeted, personalized treatment plans. Recent studies have shown how protein analysis can be a valuable tool for the objective assessment of disease-related changes and systemic conditions, such as dry eye,[43] blepharitis,[44] Sjögren's syndrome,[45] conjunctivochalasis,[46] keratoconus,[47] and autoimmune thyroid eye disease.[48] Our study shows that the intrinsic disorder phenomenon may play a role in the physiological function of tears and could be leveraged for diagnosing or managing diseases not previously considered. Although there are currently no known intrinsic disorder protein targets in human tear proteins, these proteins should be considered as potential targets for future research, as this could lead to the discovery of novel therapeutics or alter the management of various diseases. However, more research is needed to fully understand the significance of these findings in relation to patient care.

The role that IDPs have in the tears is likely multifactorial. One potential role of these disordered proteins is involvement in the liquid–liquid phase separation (LLPS), which is a thermodynamically driven, reversible phenomenon that separates biomacromolecules into distinct liquid-like condensates with different solute concentrations.[49] LLPS plays a crucial role in the assembly of membrane-less organelles (MLOs), which are dynamic compartments that modulate various cellular processes, such as gene expression, signal transduction, and stress responses.[49–51] IDPs

have been associated with LLPS in both intracellular membrane-less structures and extracellular tissue, as their dynamic structure enables them to engage in LLPS.[52] IDPs and IDPRs are key players in LLPS, as they can undergo multivalent and transient interactions with other biomolecules, leading to the formation of liquid condensates.[49,50,53,54] The extracellular milieu of tears is subject to constant fluctuations in various physicochemical parameters, such as temperature, pH, osmolarity, and oxygen tension.[55] These factors could influence the LLPS of these proteins by modulating their solubility and phase transitions. Changes in the tear environment in conditions such as dry eye disease could trigger the assembly and disassembly of liquid condensates and could have consequences for the orchestrations of extracellular signaling and functions. According to a study conducted by Azharuddin et al.,[56] dry eye disease may be a protein conformational disease associated with abnormal protein aggregation, potentially triggered by oxidative stress and inflammation. Through these mechanisms, it is possible that LLPS of tear proteins could modulate their functions and interactions and their response to environmental stimuli and pathogens. It is feasible that the most disordered proteins in tears may participate in LLPS, offering insight into their molecular behavior and potentially serving as a promising target for biomaterials and drug development.[57]

As with all studies, our study has limitations. We studied 1475 proteins that were collected from three patients.[16] Although the protein evaluations were extensive, the samples may not be representative of every human tear film proteome. Moreover, our reliance on the database reported by Zhou et al.[16] may not encompass the full spectrum of genomic diversity, which could influence tear protein composition and their intrinsic disorder characteristics. In addition, if the patient has an ophthalmic or systematic disease, it will likely alter the differential expression of proteins.[58] Our study analyzed proteins published in 2012, and there have been advancements in mass spectroscopy that enhance the depth, speed, and accuracy of proteomic studies of the human tear film.[59] In addition, our study compared the levels of disorder based on a one-to-one comparison between all proteins. We did not account for the overall expression of each protein. To enhance the generalizability and depth of our findings, we propose a multifaceted approach for future research. It would be beneficial to explore larger and more diverse cohorts, thus widening the scope of proteomic variations accounted for. Concurrently, the integration of databases that encapsulate a broader array of genomic diversity could offer a more comprehensive view of the influence on tear protein composition. Finally, employing robust statistical analysis that takes into account the variability in protein expression could yield more nuanced insights, providing a more holistic understanding of the intricate protein dynamics in human tear film.

Our findings indicate that the most disordered proteins in tears may engage in LLPS, providing crucial insights into their molecular behaviors. This could pave the way for designing biomaterials inspired by the tear protein condensates, potentially leading to a new generation of biocompatible materials with unique properties. In the realm of drug development, such proteins, due to their binding promiscuity and dynamic nature, might be considered potential targets for therapeutics. Advancements in computational modeling and proteomics could facilitate these explorations, but further interdisciplinary research is needed to fully realize these potential applications. Future studies should clarify the mechanisms by which disordered tear proteins contribute to LLPS and the functional implications of this process.

Conclusions

In this study, to the best of our knowledge, we are the first to demonstrate that intrinsic disorder is abundant in the tear film proteome. The results of our computational analysis indicate that intrinsic disorder is prevalent in human tear proteins. We have identified many highly disordered proteins in the human tear film. These insights provide additional insights into this readily accessible proteome. Intrinsic disorder is embedded into the proteome of the human tear film and likely has a key role in physiology. Ocular or systemic conditions may also alter the degree of intrinsic disorder in the tear proteome, which may be a valuable biomarker for diagnosing and managing diseases. The implications of these findings should be researched further to translate them into direct patient care.

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