To split or not to split: CASP15 targets and their processing into tertiary structure evaluation units

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Abstract

Processing of CASP15 targets into evaluation units (EUs) and assigning them to evolutionary-based prediction classes is presented in this study. The targets were first split into structural domains based on compactness and similarity to other proteins. Models were then evaluated against these domains and their combinations. The domains were joined into larger EUs if predictors' performance on the combined units was similar to that on individual domains. Alternatively, if most predictors performed better on the individual domains, then they were retained as EUs. As a result, 112 evaluation units were created from 77 tertiary structure prediction targets. The EUs were assigned to four prediction classes roughly corresponding to target difficulty categories in previous CASPs: TBM (template-based modeling, easy or hard), FM (free modeling), and the TBM/FM overlap category. More than a third of CASP15 EUs were attributed to the historically most challenging FM class, where homology or structural analogy to proteins of known fold cannot be detected.

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Running Title: CASP15 Domains

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Abstract

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Keywords: CASP15; protein structure; protein structure prediction; protein domains; evaluation units.

Acknowledgements

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1 | INTRODUCTION

CASP has been monitoring progress in the protein tertiary structure prediction for over 25 years[1-4]. Every other year since 1994, CASP organizers contact a wide network of structural biologists in quest of targets for the upcoming protein structure modeling experiment. The latest CASP15 call yielded 93 single-sequence entries representing monomeric proteins or subunits of protein multimeric complexes (https://predictioncenter.org/casp15/targetlist.cgi?view=regular). Eighty-one entries on this list were suggested for tertiary structure prediction, while the remaining twelve were auxiliary structures for other prediction categories (see Table 1). Four targets out of the 81 were canceled due to the lack of structure at the time of evaluation, leaving 77 for the assessment. Below we discuss procedures for splitting these targets into evaluation units (EUs) and assigning them to evolutionary-based prediction classes.

Defining and classifying evaluation units in CASP has been a very important and time-consuming task requiring multiple numerical tests and extensive human inspection. In five out of six recent CASPs these tasks were directed by Lisa Kinch, whose involvement with the structural classification ECOD database[5] and extensive knowledge of protein structure was an invaluable asset[6-10]. In CASP15 we decided to develop a procedure that would mimic the procedure of previous CASPs while requiring only minimal human intervention.

2 | METHODS

2.1 | Defining evaluation units

Domains are basic folding units of proteins. They can be mobile, and their relative position can deviate depending on environmental conditions (e.g., the presence of a ligand), protein's functional state (e.g., open or closed) or structure determination factors (e.g., crystal packing). Thus, evaluating models versus a specific domain conformation in a multi-domain structure can be too restrictive and penalizing. To address this issue, CASP adopted a domain-based approach to evaluating models, where multidomain targets are first split into smaller evaluation units. Even though the recent progress in modeling and the availability of non-rigid body structure comparison methods make splitting of targets into EUs less critical, we kept this practice to allow fair comparison of results across CASPs. A detailed procedure is described below.

Step 1: identifying varying regions.

Multi-chain and multi-model targets were checked for structural consistency by superimposing their chains /models using LGA[11].

If the distance between the corresponding residues in different chains /models exceeded 3.5 Å, then the residue was marked as varying. Local regions of three or more consecutive varying residues were removed from the target. If varying regions were extensive, but superimposed well when treated separately, then they were organized into separate domains.

Step 2: parsing into domains based on structural compactness.

To define domains from structure, we consulted three automatic domain parsing programs, which identify geometrically compact substructures in a protein based on the analysis of inter-residue contacts and evolutionary preserved substructures - DomainParser[12], DDomain[13] and SWORD[14]. The programs were installed at the Prediction Center and run as:

domainparser <TARGET.pdb>

ddomain <TARGET.pdb>

SWORD -i <TARGET.pdb>

We also consulted a newly developed SWORD2 web server[15] for analyzing two particularly large targets with elaborate domain architecture - T1165 and T1169. The SWORD /SWORD2 programs were used in CASP for the first time and proved to be especially helpful (in fact, the only option) for large structures with more than 1200 residues.

If the structure parsers agreed on domain boundaries, the consensus definition was adopted. If the programs disagreed, domain boundaries were defined upon visual inspection, evolutionary analysis (see*Step 3*), and /or functional information received from experimentalists.

Alternative domain definitions were considered for cases where certain regions of proteins were involved in domain swaps.

Step 3: fine-tuning domain boundaries based on similarity to other proteins.

Whole targets and suggested domains from *Step 2* were run through PSI-BLAST [16] and HHblits [17]/HHsearch[18] programs to establish sequence-based similarity to proteins of known fold:

psiblast -query <TARGET.seq> -db <pdbaa_PSIBLAST> -num_iterations 3 -evalue 10.0;

hhblits -i <TARGET.seq> -d <UniRef30_hhb_db> -oa3m <TARGET.a3m> -n 2 -cov 60 -id 90;

hhsearch -i <TARGET.a3m> -d <PDB70_hhs_db> -o <TARGET.hhr>.

The targets were also structurally compared to proteins in the PDB with*Foldseek* [19]:

foldseek easy-search <TARGET.pdb> <./db/FSmmcif> –tmscore-threshold 0.25 –max-seqs 500 -e 0.1 -s 9.5 –alignment-type 1;

and then top 100 *Foldseek* hits were re-checked with *LGA* :

lga <hit_FS.TARGET.pdb> -4 -ie -o2 -sia -d:4 -gdc_sc -swap.

The templates discovered with PSI-BLAST and HHblits/HHsearch will almost certainly be homologous to the target. However, since *Foldseek* is sequence-independent and *LGA* was run in sequence-independent mode, the second step will potentially discover structurally analogous templates as well as homologues too remote for detection by sequence-based searches.

Template-target alignments from these searches were used to adjust domain boundaries. For example, if the domain parsing programs in*Step 2* suggest termination of a domain at residue N , but templates covered the target until residue $N+i$, then the termination point was moved to $N+i$ if this did not contradict the alignment data for the neighboring domain.

Step 4: joining domains into larger evaluation units based on the performance.

Once domains were defined, models were trimmed accordingly and evaluated against the domains and their pair-wise combinations. GDT_TS scores from LGA's sequence-dependent superpositions served as the numerical basis for deciding whether domains should be kept separate or combined into larger Evaluation Units (EUs) for the final evaluation.

A rationale and numeric procedure for combining domains /splitting targets into evaluation units were suggested by Nick Grishin and coworkers in CASP9 [6]. They argued that targets should be split into domains only if this can help reveal interesting predicted features in models. Rephrasing this postulate for the bottom-to-top approach (split first, then consider re-joining), domains should be merged if their separate evaluation does not provide additional benefits for the assessment. A good indicator of this scenario is the similarity of model accuracy scores on the combined and individual domains. To facilitate the decision-making, Kinch et al[6] plotted GDT_TS scores for combined domains versus the weighted sum of scores for individual domains.

Such a graph became later known as the Grishin plot and was adopted for defining EUs in subsequent CASPs[6-10,20]. If the points in such a graph line up close to the diagonal line, then joining a pair of domains into a larger evaluation unit is advised.

In CASP15, domains were joined if the slope of the zero-intercept best fit line in a Grishin plot was <1.2. Three or more domains were joined into one EU when the plots for all pairwise domain combinations supported the merger.

The process was repeated iteratively until no further combining of EUs was needed.

2.2 | Classifying evaluation units into evolutionary prediction classes

Historically, the outcome of a protein structure modeling exercise was largely predetermined by the evolutionary relationship between the target and experimentally determined structures. Proteins with apparent homology to available structures were typically easier to model, while non-homology targets were at the harder side of the prediction difficulty spectrum. Since targets of different difficulty required different modeling approaches, yielded different degrees of model accuracy, and thus required different evaluation approaches, CASP had previously assessed modeling results separately for different target difficulties. The names of the difficulty categories changed with time, but the major factor defining the difficulty remained the same: availability of structural templates. The classical difficulty schema was shaken in CASP14, where the DeepMind group showed that highly accurate models can be built with AlphaFold 2 (AF2) for practically all targets, independently of the template availability. This suggested that the classical division into largely homology-based difficulty categories may not be needed any more. Acting upon these developments, CASP organizers recommended assessment of tertiary structure prediction in CASP15 in one batch. This analysis is presented elsewhere in this issue[21]. Nevertheless, similarly to splitting targets into EUs (above), the assignment of EUs to evolutionary prediction classes is still needed for comparing CASP15 results with the earlier ones.

In previous CASPs, EUs were classified into difficulty categories based on the availability of similar structures in the PDB, as detected by sequence- and structure-based searches (reflecting estimated difficulty) and predictors' performance (reflecting actual difficulty)[9,10]. Since performance has become more uniform across the whole range of targets, it is no longer useful for their discrimination. To adapt to the situation, we explored automated approaches to target classification, aiming to recapitulate the outcomes of previous CASPs as far as possible, but working solely with the results of automated PDB searches. Each EU was assigned a sequence-based and structure-based similarity score. The sequence-based score was defined as the HHscore [10], which is the product of the HHsearch probability and the alignment coverage of the query for the top-ranked template identified by HHsearch. The structure-based score was the LGA_S score of the highest-ranked structural match according to the procedure described in *section 2.1, Step 3* . These scores were used to automatically assign EUs to prediction classes (see *Results, section 3.2*).

3 | RESULTS

3.1 | To split or not to split

3.1.1 | Summary

From among 77 CASP15 tertiary structure prediction targets, 43 were one-domain targets, 21 had two domains, and the rest - three domains or more (Table 1). For 52 targets no domain rearrangement was necessary, and the targets were evaluated as whole-length structures (41) or unchanged constituent domains (11). For the remaining 25 targets, in 20 cases we merged at least some domains according to Grishin plots, in two cases we merged domains according to other considerations, and in three cases we split targets in more EUs than suggested by the domain parsing programs. The domain splitting and re-joining procedure (*Methods*) yielded 112 evaluation units, 109 of which were included into the final tertiary structure evaluation[21], while three – T1114s1-D2, T1157s1-D2 and -D3 – were cancelled due to the low resolution of the cryo-EM maps in their local areas.

Out of 34 multi-domain targets, 14 were evaluated as one EU and 20 were split into multiple EUs (Table 1).

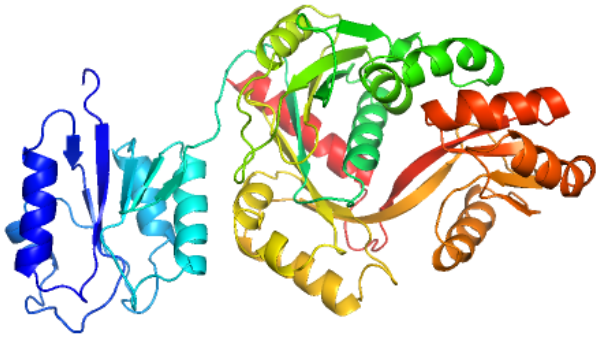
Below we discuss different scenarios of forming evaluation units and present case studies for some targets.

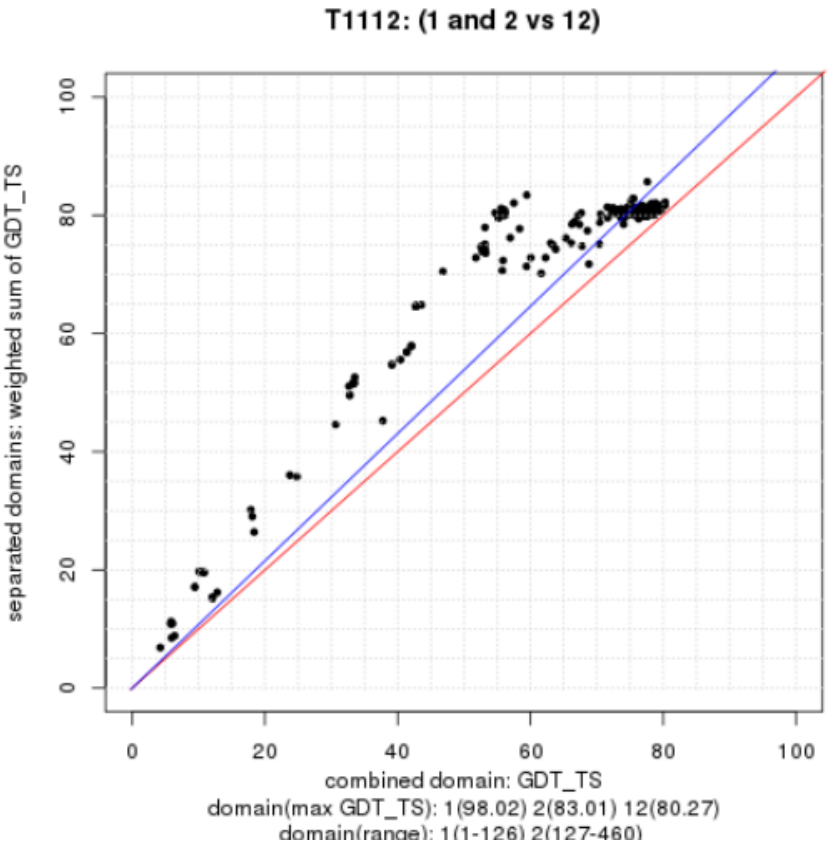
3.1.2 | Multidomain-targets not requiring splitting (14)

Fourteen multi-domain targets (as defined by the automatic parsers - section *2.1, Step 2*) were proposed for the evaluation without splitting into substructures.

In two cases, T1131 and T1133, we disagreed with the automatic domain parsing results and considered the targets as one-domain structures. Target T1131 is a small protein where a long central helix holds two parts of the structure together and is needed for the structural integrity of the protein; while target T1133 (PDB: 8DYS) is a nine-bladed beta-propeller that is fully and reliably covered by templates (e.g., 3WJ9_B) and well-predicted as the whole.

For eleven targets a decision to join domains into single EUs was reached based on the analysis of Grishin plots. Two examples of such targets are shown in Figure 1. Even though the targets are clearly two-domain entities, their whole structures were predicted by most groups as accurately as the constituent domains and thus did not require splitting.





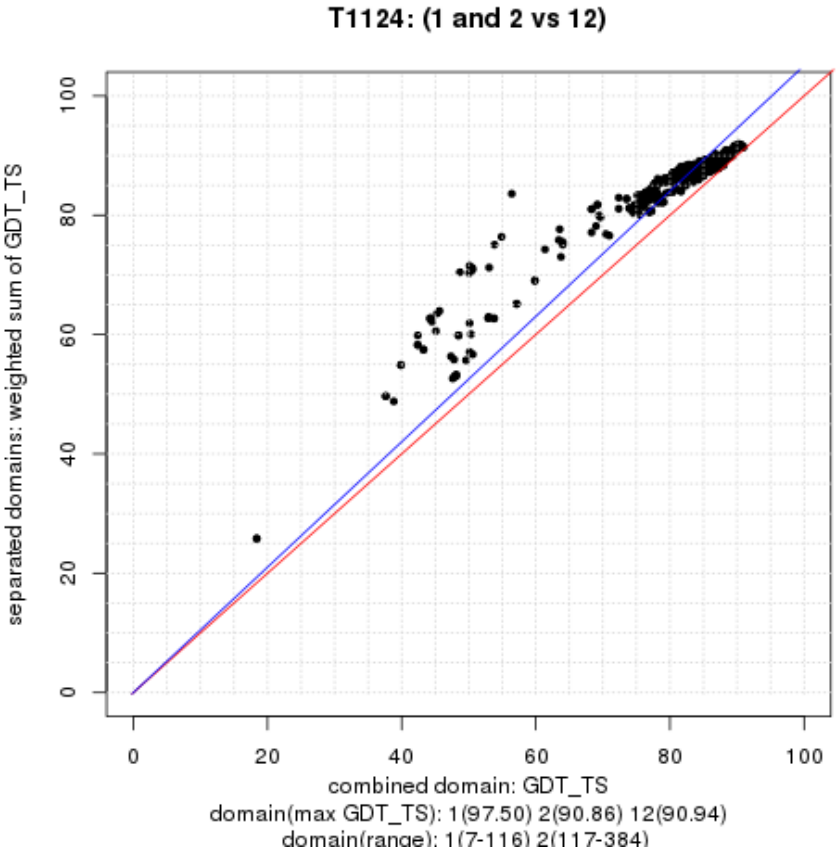


Figure 1. PyMOL[22] target renderings (left) and Grishin plots (right) for two two-domain targets: ***(A)*** *target T1112, a protein involved in the synthesis of an osmolyte involved in thermoadaptation, and* ***(B)*** *target T1124, a methyltransferase MfnG (PDB: 7UX8). Grishin plots are built on the GDT_TS scores for all collected models. The plots suggest evaluating domains together as the angle between the data trend line and the diagonal is small (i.e., the evaluation scores for the combined domains (X-axis) and individual domains (Y-axis) are similar for most groups).*

The last target in this category, T1180, is an exception to the splitting rule (*section 2.1*). Even though the Grishin plot advised splitting, we did not proceed with that as the target is a fusion enzyme of two known domains, where the only prediction interest was to model inter-domain orientation.

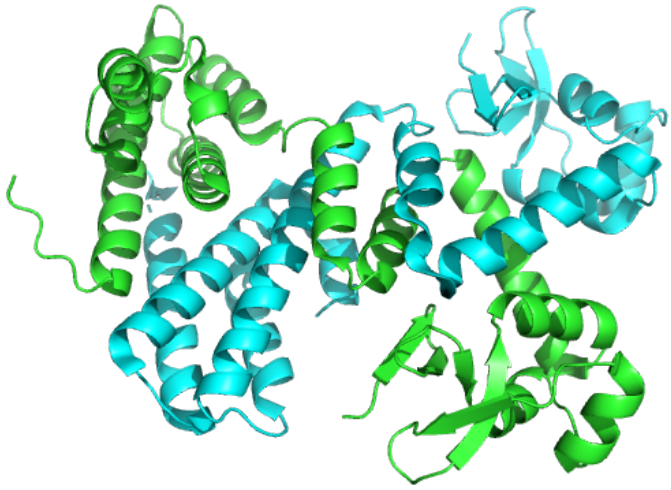
3.1.3 | Multidomain-targets requiring splitting (20)

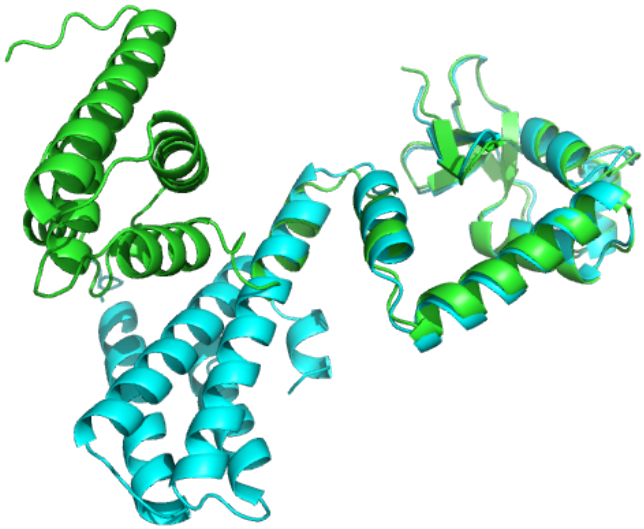
For half of the 20 targets that required splitting, the number of EUs was determined by the number of structural domains (no merging was necessary), and for the other half, some domains but not all were joined.

In several cases splitting was required because different chains exhibited different folding patterns.

For example, target T1120, a DNA-binding protein DdrC, is composed of an N-terminal winged helix-turn-helix motif and a C-terminal four-helix bundle, that folds as an asymmetric domain-swapped dimer (Figure 2A)[23]. Superposition of the two chains revealed the distortion of the long central helix hA in chain A (cyan) into two smaller, non-colinear helices (hB_1 and hB_2) in chain B (green) and cause a shift in the relative position of the C-terminal domain with regards to the anchor N-terminal domain (Figure 2B). This

prompted splitting of the target into two EUs at the break point (residue 125). Such a split is strongly supported by the Grishin plot (Figure 2C).





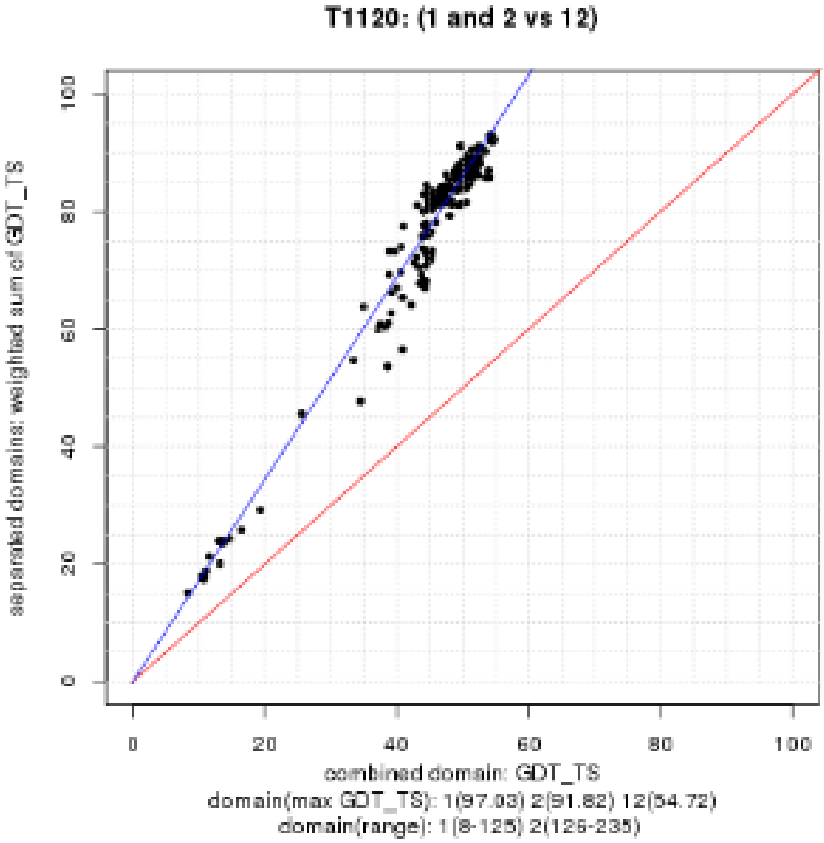
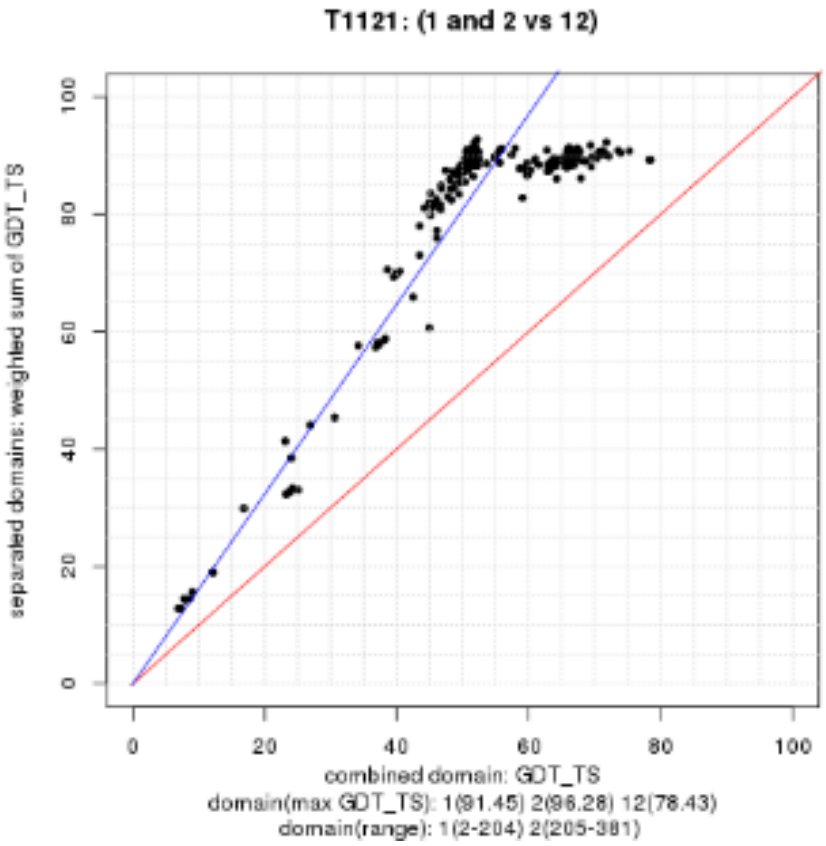


Figure 2. Target T1120, a DNA-binding protein DdrC (PDB: 7QVB). ***(A)*** *a homodimer with two chains colored as cyan and green;* ***(B)*** *superposition of two chains showing the break point in the helix hA at residue LEU 125;* ***(C)*** *a Grishin plot showing the need for splitting (large angle between the data trend line and the diagonal). The plot was built on the GDT_ TS results for all participating groups on the constituent domains D1: 8-125 and D2: 126-235 and the whole target in the chain A configuration.*

Another example of such obligated splitting is target T1121, the Wadjet nuclease subunit JetD[24]. It is a homo-dimeric protein (Figure 3A) containing two domains that are flexibly linked and whose relative orientation differs in the two chains (Figure 3B). Because of that, the target was split into two EUs at the hinge point (residue 204). As in the previous case, such a split is strongly supported by the performance data (Figure 3C).



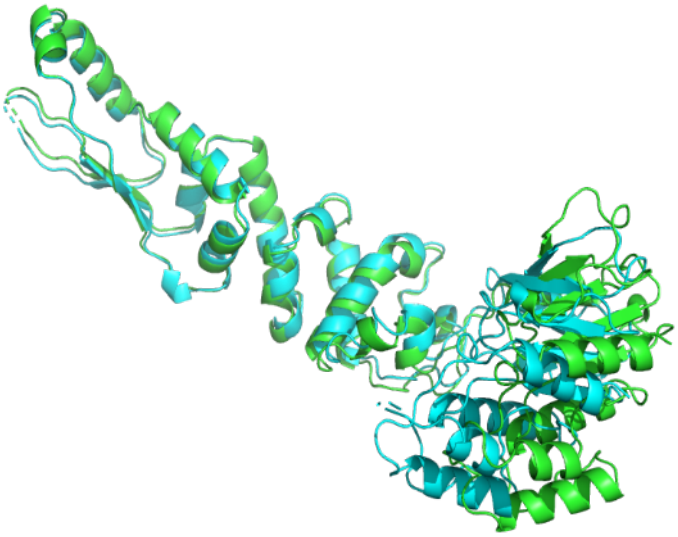
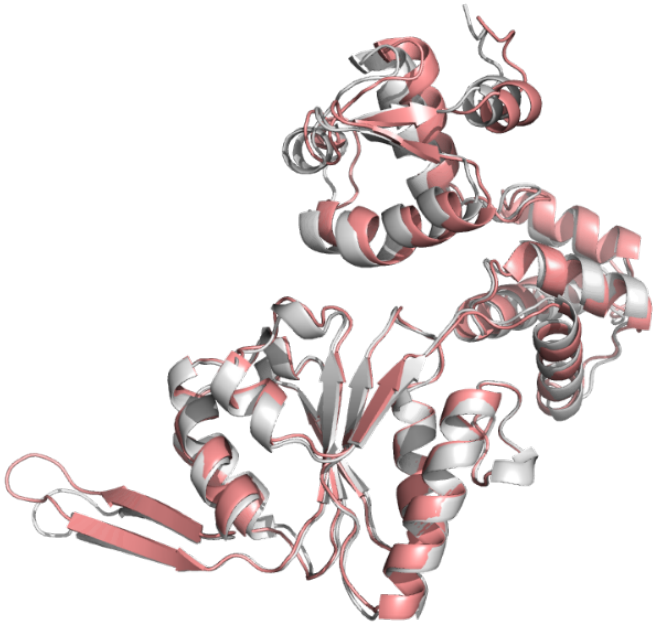
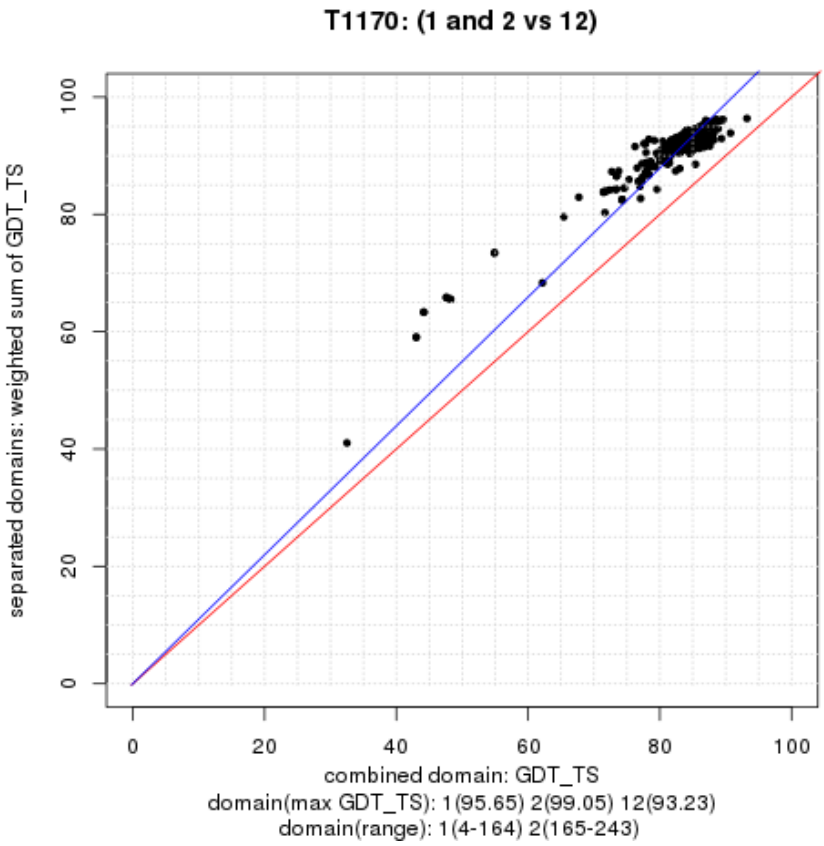
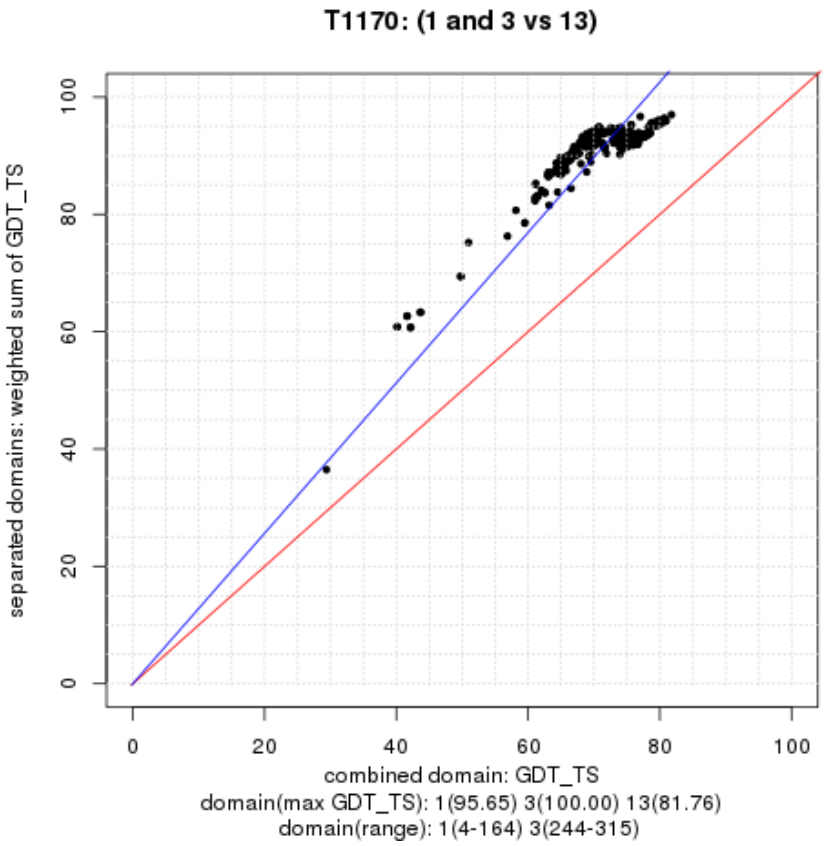


Figure 3. Target T1121, a DNA-cleavage protein JetD (PDB: 7TIL). ***(A)*** *a homodimer with two chains colored as cyan and green;* ***(B)*** *superposition of its two chains showing flexibility of the C-term domain (Pfam DUF2220, right) with respect to the N-term arm-like domain (DUF3322, left);* ***(C)*** *a Grishin plot showing the need for splitting. The plot was built on the GDT_ TS results for all groups on the constituent domains D1: 2-204 and D2: 205-381 and the whole target in the chain A configuration.*

The last example in this category is target T1170, a Holliday junction hexamer where some chains deform to accommodate DNA[25] in such a way that the overall structures of domains remain largely unchanged, yet their relative position varies (Figure 4AB). Non-crystallographic symmetry of the structure requires separate treatment of parts that have different relative orientation. The target was originally split into three domains (1: 4-164; 2: 165-243; 3: 244-315) and analyzed if any of those need to be merged for the final evaluation. The Grishin plots (Fig 4C) advised that domains 1 and 2 should be merged, while 3 should remain a separate evaluation entity. Thus, for the final evaluation, this target was represented by two EUs: D1: 4-243 and D2: 244-315 (encircled).







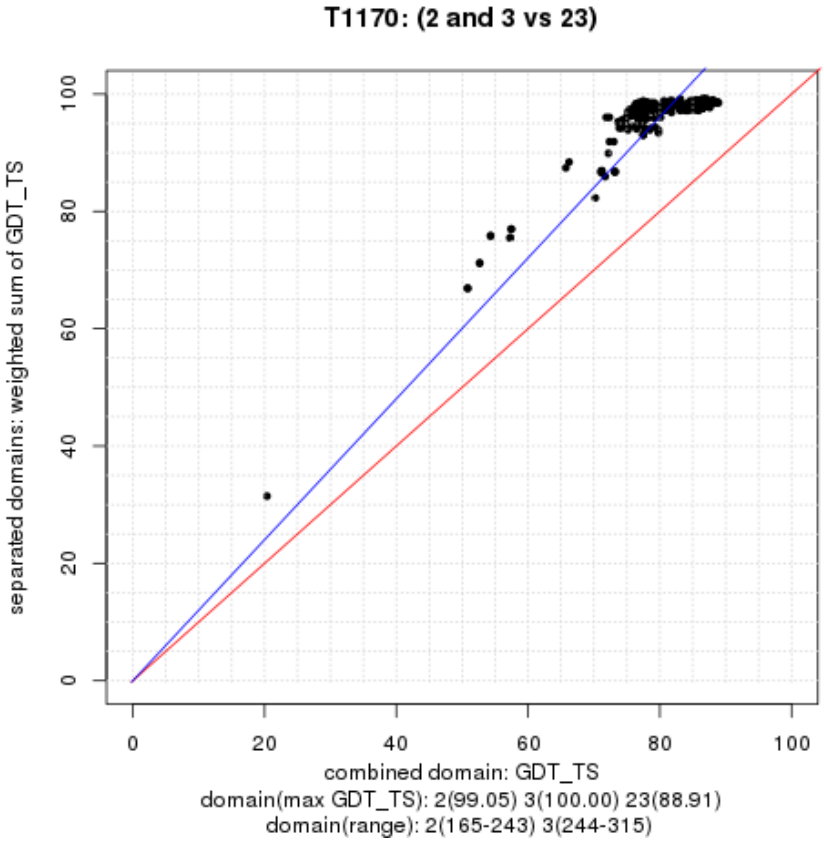


Figure 4. Target T1170, a Holliday junction hexamer (PDB: 7PBR). ***(A)*** *superposition of two deformed chains versus* ***(B)*** *four undeformed chains in the same frame of reference. The domain that moves the most with respect to other two is encircled.* ***(C)*** *Grishin plots for the original target split into three domains show the similarity of results on domains 1, 2 and their combination 12 (left panel, points close to the diagonal), and the dissimilarity of results on the combined substructures 13 and 23 and their constituent domains (middle and right).*

In all other targets, except for T1120, T1121 and T1170 discussed above, chains were largely similar, and the decision on domain splitting was dictated purely by Grishin plots. Below we discuss three cases of some of the most difficult domain rearrangements.

Target T1158 is a type IV ABC transporter, which is a common fold (see review [26]). In CASP15, this protein family was represented by five targets - T1158 (Figure 5A) and T1158v1-v4, which differ by rigid body movements of the two halves of the transporter with respect to one another, and no significant rearrangements within the subunits (Figure 5B). When submitted to domain parsing programs, T1158 was split in several ways, none of which made functional sense. The suggested split was either too fragmented (6 domains by DDomain, or 5/8/7 by the top three SWORD assignments) or too coarse-grained (2 domains by DomainParser: the C-terminal globular domain (red) in Figure 5A (48-1022) and the rest). We split this target into two EUs (Figure 5C) reflecting the conformational changes that the transporter undergoes performing its biological function of opening and closing gates in bound and unbound states. In other words, evaluation units for T1158 were defined not from a single structure, but from a set of structures from the same superfamily. A Grishin plot for the target (not shown) supports the suggested split.

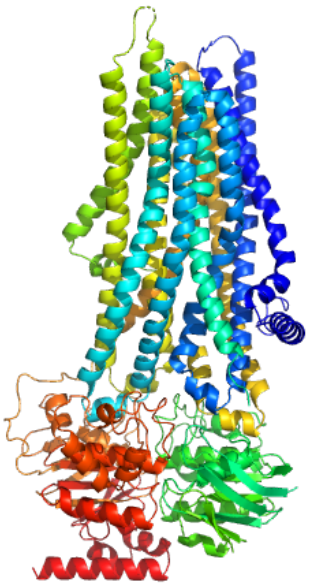
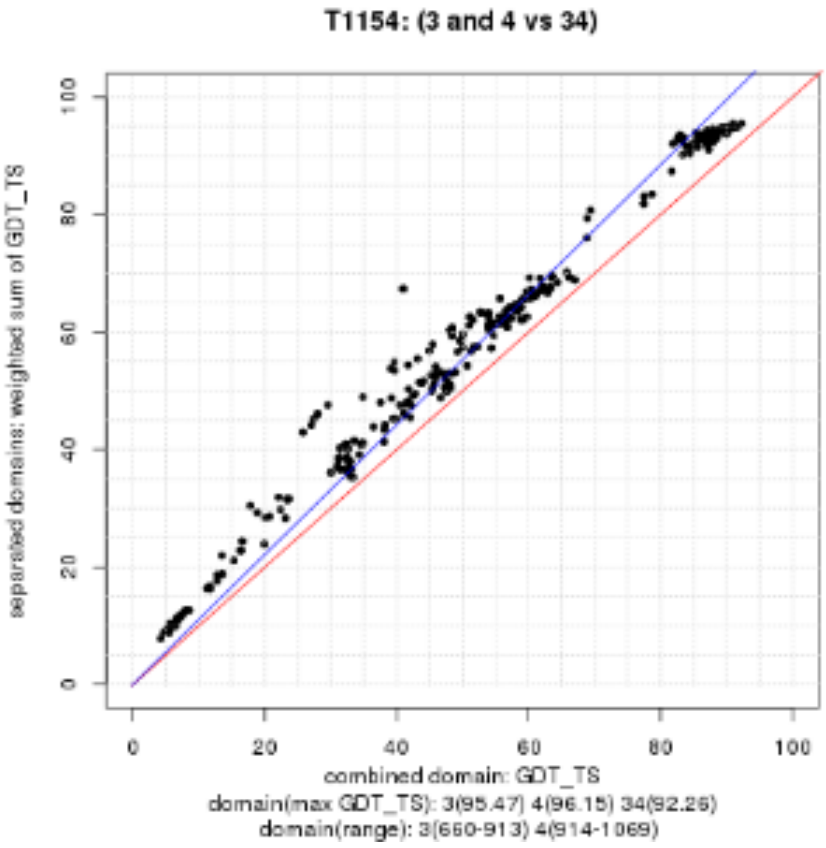
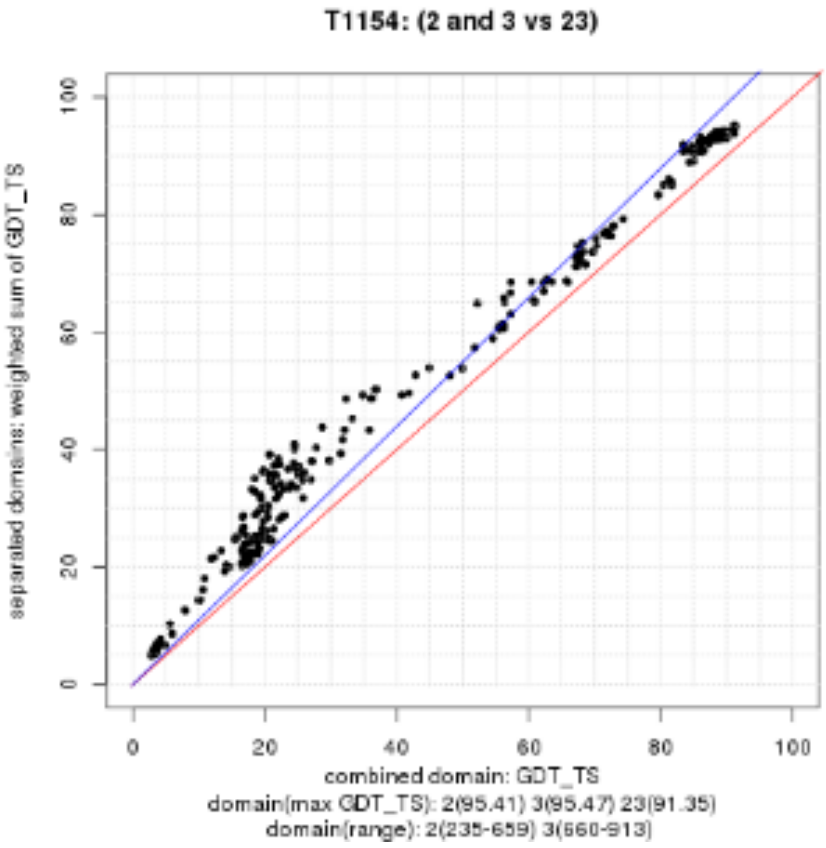
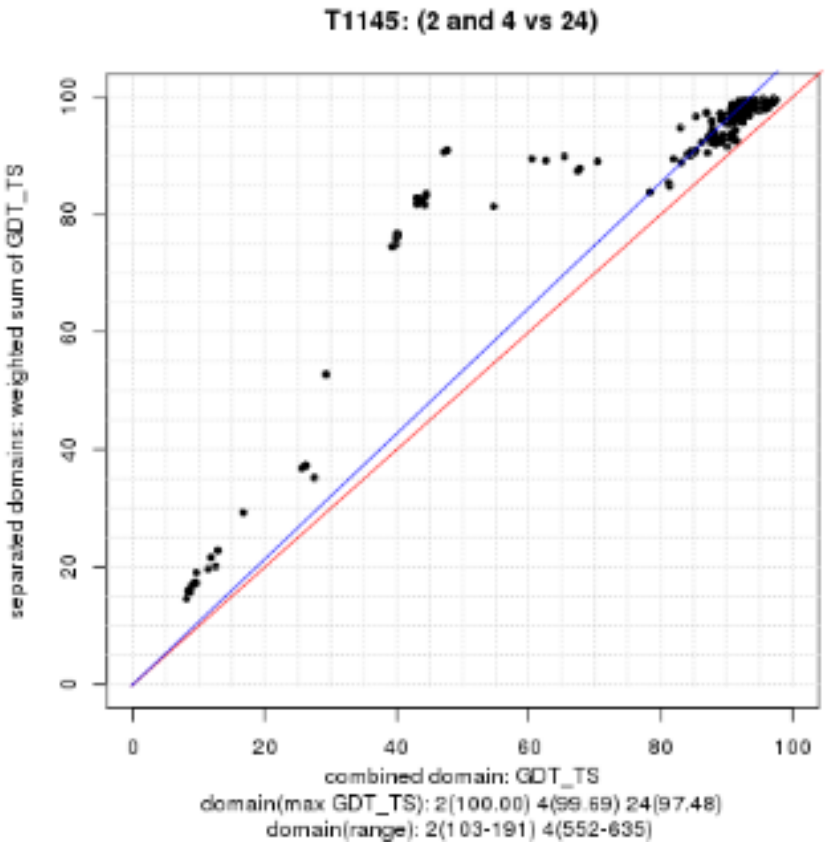


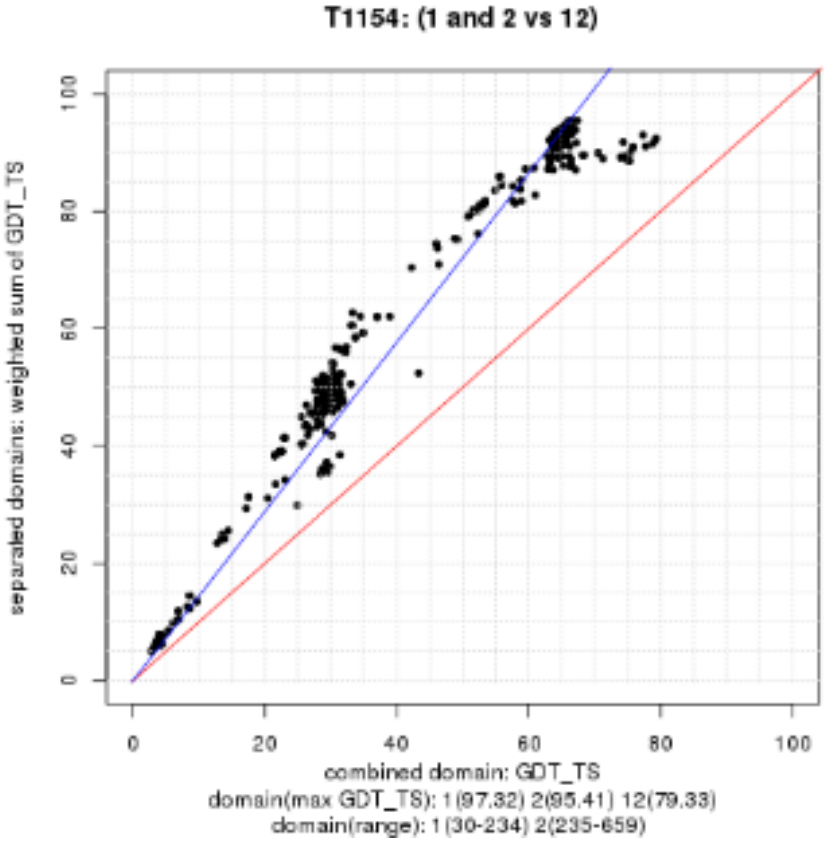
*Figure 5. An ABC transporter **(A)** in apo state, T1158, colored from N-terminal (blue) to C-terminal (red); **(B)** in one of the bound states, T1158v4, colored from N-terminal to C-terminal; and **(C)** as split into two EUs: D1 (blue): 48-234,347-394,409-615,861-974 and D2 (red): 235-346,692-860,975-1296.*

Target T1145 is a starch binding protein Sas6[27] (Figure 6A). DDomain classifies it into four domains (as numbered in the figure), while DomainParser and SWORD suggest a three-domain arrangement with domains 2 and 4 joined. Starting with the most disjoint 4-domain version and based on the Grishin plot analysis (B) we joined domains 2, 3 and 4 into one EU, while leaving domain 1 separate.









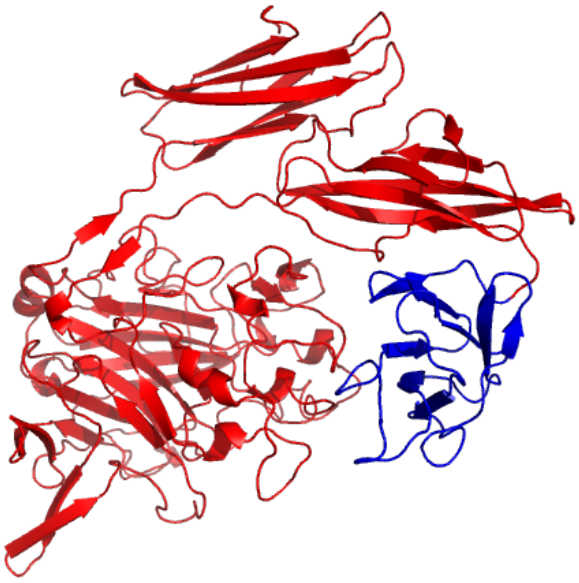
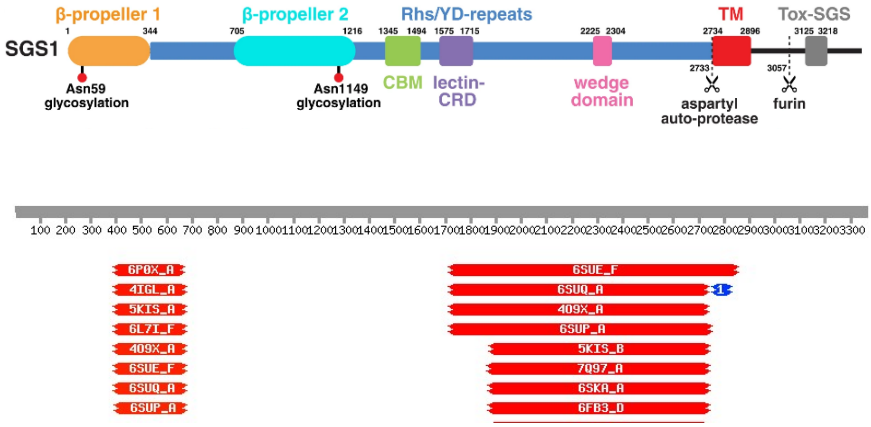


Figure 6. ***(A)*** *Target T1145 as split into two EUs.* ***(B)*** *Grishin plots for four original domains of T1145 as marked in panel A. The upper left panel in section (B) shows that domains 1 and 2 should be split, while domains 2, 3 and 4 (the remaining 3 panels) should be joined.*

The last example is target T1169, a mosquito salivary protein SGS1 involved in mosquito-borne diseases[28]. It is the largest monomeric target in the history of CASP (3364 residues in the sequence; 2735 residues resolved in the structure). It has a cocoon-shaped structure with multiple domains and extensive inter-domain interactions (Figure 7), thus presenting a significant challenge in defining EUs. The top-ranked SWORD/SWORD2 splitting schema suggested 7 domains; the domain definition from the authors (Figure 7B[28]) and the results of HHsearch homology searches (Figure 7C) offered additional help in defining domains. Domains were originally defined so that the following 7 areas were separated: the N-term β-propeller (blue in panel A, orange in panel B), region between the two β-propellers (HHsearch), β-propeller 2, region after the beta-propeller, CBM domain, lectin-CRD domain, the area containing the wedge domain up to the TM domain (HHsearch). The Grishin plot analysis suggested merging of two domains surrounding β-propeller 2, and merging of CBM, lectin-CRD and wedge-containing domains. In the end we split T1169 into four evaluation units, as colored in Figure 7A. A long linker between D1 and D4 and orphan helices in the middle of the cocoon (grey) were not assigned to any of the EUs.



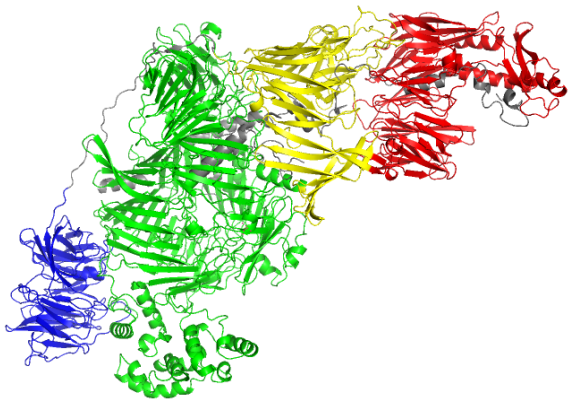


Figure 7. ***(A)*** *Target T1169, a mosquito protein relevant to pathogen transmission (PDB:8FJP) with four evaluation units defined: D1: 1-345; D2: 1302-2735; D3: 378-699,1223-1301; D4: 700-1222.* ***(B)*** *Parsing of SGS1 into domains as suggested by the authors of the structure [28].* ***(C)*** *Top HHsearch hits showing similarity of the query sequence to known folds in two areas: 395-670 (intermediate domain between the two beta-propellers - see panel B) and 1718-2735 (region after the lectin-CRD domain and up to the TM domain).*

3.1.4 | Targets that were split into more EUs than suggested by Grishin plots

Two single-domain targets as suggested by the domain parsers (T1137s2 and T1137s3) were split into two domains for consistency with the other subunits of the same heteromeric complex. Target H1137 (PDB: 8fef) is a hetero 9-mer with six subunits forming an intertwined obligatory complex. The split was made in agreement with the results of template searches and splits of other related subunits.

Another target, T1125, was split into 6 domains instead of 5 suggested by the domain parsers. In this target the C-terminal region penetrates the N-terminal part forming one structural domain, but predictors were unable to model the circular fold of the protein. Thus, for the evaluation, the N-terminal domain (#1) and C-terminal domain (#6) were considered separately.

3.1.5 | Domain swaps

Four targets in CASP15 included domains involved in domain swaps: T1109, T1113, T1120 and T1176. Target T1120 was discussed above (3.1.3). The remaining three targets were un-swapped, and models were evaluated versus both swapped and un-swapped versions of the targets. For T1109 and T1113, models scored higher versus the original (swapped) version, and thus the original targets were used for the final evaluation; for T1176, the evaluation scores were higher for the un-swapped version, and that version was used as the target (T1176-D9: A1-138 + B139-170).

3.2 | Prediction classes

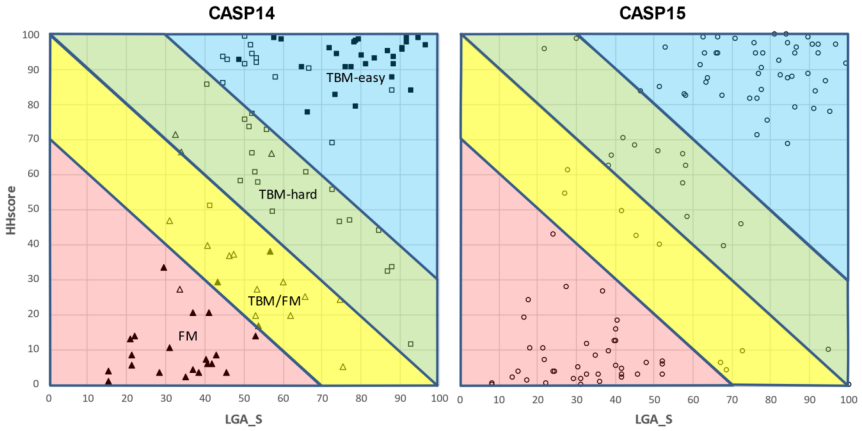


*Figure 8. Scatter plot of evaluation units in CASP14 (**A**, left) and CASP15 (**B**, right) represented by sequence (HHscore, Y-axis) and structure (LGA_S, X-axis) scores of the top template. Evaluation units in the left panel are marked according to the difficulty categories as manually assigned in CASP14: full squares – TBM-easy; hollow squares – TBM-hard; hollow triangles –TBM/FM; full triangles – FM. Targets of the same difficulty cluster together in the suggested (X,Y) axes. An automatic delineation of EUs into four classes (X+Y<70, red; 70-100, yellow; 100-130 green; >130, blue) based on the results of sequence- and structure-based searches of the PDB is suggested to mimic the CASP14 difficulty categories. The schema is applied to define target prediction classes in CASP15 (right panel).*

Two scores, HHscore and LGA_S, for sequence- and structure-based relationships of the target with PDB entries, were defined in Methods. They are plotted against each other for all EUs in CASP14 and CASP15 (Figure 8). The classification of the CASP14 data resulting from the previous procedures [10]– based partly on predictor performance and involving manual intervention – is indicated by symbols in panel A. This reveals that TBM-easy and FM EUs cluster in these coordinates in the upper right and lower left corners respectively, while TBM-hard and TBM/FM EUs predominantly occupy areas immediately above and below the diagonal, respectively. It also can be seen that all triangle markers but two (FM and TBM/FM targets) are below the diagonal and all squares but one (TBM-easy and TBM-hard) are above. Thus, if we consider the diagonal line (HHscore+LGA_S=100) as a boundary between the wider TBM (TBM-easy and TBM-hard together) and FM categories (FM and TBM/FM), then there are only three targets for which the prior CASP14 and current automated classifications schemes disagree.

To further delineate TBM-easy from TBM-hard, and FM from TBM/FM we draw two lines parallel to the diagonal. These lines were drawn symmetrically so that the areas between them and the diagonal include the majority of the TBM-hard (upper) and TBM/FM (lower) EUs yet not encroaching deeply into the TBM-easy and FM territory. Based on the CASP14 data, the split lines were drawn at HHscore+LGA_S=70 and 130 levels. As a side note, we want to mention that we experimented with several other splitting schemas (like rectangular or spherical divisions) and found the linear split to be the simplest and best fitting the CASP14 and CASP13 target classifications. When the suggested schema is applied to the classification of CASP15 EUs (Figure 8B), we see that the points in the graph are nicely separated, with particularly clear clustering in the FM and TBM-easy zones.

Using this classification approach, the CASP15 EUs were automatically assigned to four largely homology-based prediction classes (see Figure 1B and Table 1). Forty-seven EUs were assigned to the TBM-easy class, 15 to TBM-hard, 8 to TBM/FM, and 39 (˜35%) to FM - a class with the weakest or no evolutionary relation to available folds. These data show that the CASP15 target set was one of the most difficult (homology-wise) in the whole history of CASP. For comparison, the FM class constituted only 24% of all targets in CASP14, and 27% in CASP13. Conceivably this rise may already illustrate the impact of AF2 on target selection in structural biology: experimentalists may be switching attention to more structurally novel targets with which AF2 still struggles.

As discussed in more detail elsewhere[2129], it is clear that FM targets comprise the majority of those with which even the top predictive methods struggled, even though some FM targets were well-predicted. Thus, even though it is well known that AF2 (on which most predictive methods were based) generalizes beyond its training set, the absence of similar structural folds in the PDB still leads to a greater risk of predictive failure. Factors further predisposing a target to less accurate prediction appear to include shallow Multiple Sequence Alignment (MSA) (it is known that evolutionary covariance information extracted from MSAs is required for accurate modelling of natural proteins by AF2[30,31], potentially in order to obtain a sufficiently accurate initial structure estimate). Especially given the relatively small numbers of problematic targets in each CASP, however, a deeper study on this subject is needed, and deep learning methods could help with this task.

4 | CONCLUSIONS

A key objective of CASP is to monitor progress in predictive performance on different kinds of target protein. Thus, a robust and objective classification of targets is essential. Although previous classification has benefitted from detailed consideration by experts in protein evolution, the new, purely automatic method introduced here provides a new labor-saving foundation for CASP-to-CASP comparisons. We show that it largely recapitulates previous classifications and, furthermore, may provide numerical estimates of difficulty beyond the current four classes, potentially facilitating future study of features correlating with target difficulty.

Much as a purely automatic division of targets into EUs would also be desirable, the CASP15 set illustrate why that seems not yet to be possible. For example, a satisfactory EU definition for the ABC transporter T1158 was only achieved by manual reference to a set of structures and an understating of the structure-function relationship of the target: none of the automated domain partitioning algorithms produced sensible results. Nevertheless, clear and objective guidelines were followed as far as possible relating, for example, to the gradients of the Grishin plots. Finally, it is worth noting that although consistent policy is followed for EU definition, the resulting sets may still differ from CASP to CASP as predictions improve. Thus, as more groups accurately capture domain packing there will be fewer instances of splitting and more where larger multi-domain units are retained as the EUs: this tendency towards larger EUs could tend to depress global quality metrics and should be borne in mind by future assessors.

Table 1. CASP15 tertiary structure prediction targets, their split into evaluation units (EUs) and classification to homology-based prediction classes. Canceled targets are highlighted in red; targets that were released as auxiliary structures for other prediction categories (ligand, oligo, protein-RNA complex) are in yellow.

Target	Number of struct. domains	Number of EUs	**EU boundaries**	**Residues in**
T1104	*1*	*1*	T1104-D1: 1-117	117
T1115				
T1115v1				
T1106s1	*1*	*1*	T1106s1-D1: 50-120	71
T1106s2	*1*	*1*	T1106s2-D1: 2-112	111
T1109	*1*	*1*	T1109-D1: 7-209, 216-226	214
T1110	*1*	*1*	T1110-D1: 7-227	221

Target	Number of struct. domains	Number of EUs	**EU boundaries**	**Residues in I**
T1112	*2*	*1*	T1112-D1: 1-460	460
T1113	*1*	*1*	T1113-D1: 1-192	192
T1114s1	*2*	*2*	T1114s1-D1: 20-79	60
			T1114s1-D2: 80-189	110
T1114s2	*2*	*1*	T1114s2-D1: 48-369	322
T1114s3	*1*	*1*	T1114s3-D1: 4-516	513
T1115	*3*	*2*	T1115-D1: 25-200	176
			T1115-D2: 201-272	72
T1118				
T1118v1				
T1119	*1*	*1*	T1119-D1: 7-54	48
T1120	*2*	*2*	T1120-D1: 8-125	118
			T1120-D2: 126-235	110
T1121	*2*	*2*	T1121-D1: 2-204	203
			T1121-D2: 205-381	177
T1122	*1*	*1*	T1122-D1: 4-237	234
T1123	*1*	*1*	T1123-D1: 33-258	226
T1124	*2*	*1*	T1124-D1: 7-384	378
T1125	*5*	*6*	T1125-D1: 327-460	134
			T1125-D2: 461-608	148
			T1125-D3: 609-797	189
			T1125-D4: 798-946	149
			T1125-D5: 947-1096	150
			T1125-D6: 1097-1162	66
T1127	*1*	*1*	T1127-D1: 6-210	205
T1127v2				
T1129s2	*1*	*1*	T1129s2-D1: 33-640	608
T1130	*1*	*1*	T1130-D1: 28-133, 139-195	163
T1131	*2*	*1*	T1131-D1: 1-161	161
T1132	*1*	*1*	T1132-D1: 5-102	98
T1133	*3*	*1*	T1133-D1: 4-427	424
T1134s1	*2*	*1*	T1134s1-D1: 2-230	229
T1134s2	*1*	*1*	T1134s2-D1: 10-313	304
T1137s1	*2*	*2*	T1137s1-D1: 20-169	150
			T1137s1-D2: 170-409	240
T1137s2	*1*	*2*	T1137s2-D1: 1-149	149
			T1137s2-D2: 150-343	194
T1137s3	*1*	*2*	T1137s3-D1: 1-149	149
			T1137s3-D2: 150-313	164
T1137s4	*3*	*3*	T1137s4-D1: 44-159	116
			T1137s4-D2: 160-394	235
			T1137s4-D3: 395-468	74
T1137s5	*2*	*2*	T1137s5-D1: 33-169	137
			T1137s5-D2: 170-390	221
T1137s6	*2*	*2*	T1137s6-D1: 1-151	151
			T1137s6-D2: 152-399	248
T1137s7	*1*	*1*	T1137s7-D1: 1-325	325
T1137s8	*1*	*1*	T1137s8-D1: 16-266	251
T1137s9	*1*	*1*	T1137s9-D1: 25-289	265
T1139	*2*	*1*	T1139-D1: 23-317	295

Target	Number of struct. domains	Number of EUs	**EU boundaries**	**Residues in**
T1145	*4*	*2*	T1145-D1: 4-102	99
			T1145-D2: 103-635	533
T1146	*1*	*1*	T1146-D1: 29-307	279
T1147	*1*	*1*	T1147-D1: 12-103	92
T1148				
T1150	*1*	*1*	T1150-D1: 3-351	349
T1151s2	*1*	*1*	T1151s2-D1: 28-111	84
T1152	*1*	*1*	T1152-D1: 1-46	46
T1153	*1*	*1*	T1153-D1: 3-297	295
T1154	*4*	*2*	T1154-D1: 30-234	205
			T1154-D2: 235-1069	835
T1155	*1*	*1*	T1155-D1: 5-108	104
T1157s1	*3*	*3*	T1157s1-D1: 1-661	661
			T1157s1-D2: 662-757, 1005-1022	114
			T1157s1-D3: 758-1004	247
T1157s2	*4*	*3*	T1157s2-D1: 1-106	106
			T1157s2-D2: 107-323	217
			T1157s2-D3: 324-464	141
T1158	*5*	*2*	T1158-D1: 48-234, 347-394, 409-615, 861-974	556
			T1158-D2: 235-346, 692-860, 975-1296	603
T1158v1-4				
T1159	*2*	*1*	T1159-D1: 1-160	160
T1160	*1*	*1*	T1160-D1: 5-33	29
T1161	*1*	*1*	T1161-D1: 1-48	48
T1162	*1*	*1*	T1162-D1: 4-28, 59-196	163
T1163	*1*	*1*	T1163-D1: 8-191	184
T1165	*6*	*6*	T1165-D1: 2-595	594
			T1165-D2: 596-1319	724
			T1165-D3: 1320-2008	689
			T1165-D4: 2049-2130	82
			T1165-D5: 2621-3000	380
			T1165-D6: 2181-2620	440
T1169	*7*	*4*	T1169-D1: 1-345	345
			T1169-D2: 1302-2735	1434
			T1169-D3: 378-699, 1223-1301	401
			T1169-D4: 700-1222	523
T1170	*3*	*2*	T1170-D1: 4-243	240
			T1170-D2: 244-315	72
T1173	*2*	*2*	T1173-D1: 1-62	62
			T1173-D2: 63-204	142
T1174	*2*	*2*	T1174-D1: 1-216	216
			T1174-D2: 217-338	122
T1175	*1*	*1*	T1175-D1: 1-312	312
T1176	*1*	*1*	T1176-D9: 1-138, 139-170	170
T1177	*2*	*1*	T1177-D1: 1-223	223
T1178	*1*	*1*	T1178-D1: 17-291	275
T1179	*1*	*1*	T1179-D1: 2-253	252
T1180	*2*	*1*	T1180-D1: 1-404	404
T1181	*3*	*2*	T1181-D1: 1-88	88
			T1181-D2: 89-688	600

Target	Number of struct. domains	Number of EUs	**EU boundaries**	**Residues in**
T1182	*2*	*1*	T1182-D1: 21-544	524
T1183	*2*	*1*	T1183-D1: 1-195	195
T1184	*1*	*1*	T1184-D1: 34-63, 73-101, 106-171	125
T1185s1	*1*	*1*	T1185s1-D1: 4-71	68
T1185s2	*2*	*1*	T1185s2-D1: 11-66, 84-349	322
T1185s4	*1*	*1*	T1185s4-D1: 20-200, 222-244, 251-280	234
T1186				
T1187	*1*	*1*	T1187-D1: 3-166	164
T1188	*2*	*1*	T1188-D1: 25-597	573
T1189				
T1190				
T1191				
T1192				
T1193				
T1194	*1*	*1*	T1194-D1: 7-167	161
T1195	*1*	*1*	T1195-D1: 3-279	277
T1196	*1*	*1*	T1196-D1: 9-351	343
T1197	*1*	*1*	T1197-D1: 16-277	262

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