Ligand binding site structure influences the evolution of protein complex function and topology

Citation for published version:
Abrusan, G & Marsh, J 2018, 'Ligand binding site structure influences the evolution of protein complex function and topology' Cell Reports. DOI: 10.1016/j.celrep.2018.02.085

Digital Object Identifier (DOI):
10.1016/j.celrep.2018.02.085

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published in:
Cell Reports

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Ligand Binding Site Structure Influences the Evolution of Protein Complex Function and Topology

Highlights

- Ligand binding site structure significantly influences protein function evolution
- MBS homomers have more similar ligand binding pockets than monomers and other homomers
- Cofactor and metal-binding MBS homomers have more conserved QS than other homomers
- MBS homomers are promising targets for developing antibiotics and multitarget drugs

Authors

György Abrusán, Joseph A. Marsh

Correspondence

gyorgy.abrusan@igmm.ed.ac.uk

In Brief

Homomers with ligand binding sites involving multiple protein chains (MBS homomers) evolve new functions slower than other homomers and monomers, and the ones binding cofactors/metals also have more conserved quaternary structure (QS). These complexes are likely to be promising targets for antibiotics and multitarget drugs.
**Ligand Binding Site Structure Influences the Evolution of Protein Complex Function and Topology**

György Abrusán1,2,* and Joseph A. Marsh1

1MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road, Edinburgh EH4 2XU, UK
2Lead Contact
*Correspondence: gyorgy.abrusan@igmmed.ac.uk
https://doi.org/10.1016/j.celrep.2018.02.085

**SUMMARY**

It has been suggested that the evolution of protein complexes is significantly influenced by stochastic, non-adaptive processes. Using ligand binding as a proxy of function, we show that the structure of ligand-binding sites significantly influences the evolution of protein complexes. We show that homomers with multi-chain binding sites (MBSs) evolve new functions slower than monomers or other homomers, and those binding cofactors and metals have more conserved quaternary structure than other homomers. Moreover, the ligands and ligand-binding pockets of homologous MBS homomers are more similar than monomers and other homomers. Our results suggest strong evolutionary selection for quaternary structure in cofactor-binding MBS homomers, whereas neutral processes are more important in complexes with single-chain binding sites. They also have pharmacological implications, suggesting that complexes with single-chain binding sites are better targets for selective drugs, whereas MBS homomers are good candidates for broad-spectrum antibiotic and multitarget drug design.

**INTRODUCTION**

The majority of genomic traits show dramatic changes with organismal complexity and genome size (Lynch, 2007). Due to their high effective population size, prokaryotes are characterized by small, dense genomes, where the amount of coding sequence typically takes up more than 90% of the genome. In contrast, in higher eukaryotes, the fraction of coding sequence takes up only a small fraction (1%–2%) of the genome, and its fraction changes gradually with genome size. A similar trend is present for many other traits: intron size, intron number, number of genomic parasites, codon bias, and recombination rate all show clear correlations with the size of the genome (Lynch and Conery, 2003), which is caused by the very different effective population sizes and, in consequence, strength of selection in species with large and small genomes (see Lynch, 2007 for an overview).

There is, however, a notable exception to this pattern: protein complexes. The majority of proteins do not function in isolation but form complexes, which can be either homomeric, i.e., are formed by several units of the same protein, or heteromeric, which are formed by several different proteins (see recent reviews by Levy and Teichmann, 2013 and Marsh and Teichmann, 2015). Whereas it is known that, during crystallization, non-physiological complexes are formed frequently, an implicit assumption of most studies on complex evolution is that the structure of protein complexes is adaptive and is a result of selection (Ali and Imperiali, 2005; Marianayagam et al., 2004; Nishi et al., 2013). This assumption is highly intuitive and is supported by numerous observations. For example, pathogenic mutations are highly enriched in protein interfaces (Sahni et al., 2015; Yates and Sternberg, 2013), quaternary structure is frequently conserved (Levy et al., 2006), protein complex assembly pathways tend to be evolutionarily optimized and conserved (Marsh et al., 2013; Wells et al., 2016), and different biological functions are strongly associated with different types of quaternary structure (Bergendahl and Marsh, 2017). However, Lynch (2013) has recently pointed out that, unlike most genomic traits, the frequency of protein complexes in different taxa, and their quaternary structure, do not show the same dramatic changes with the complexity of organisms (and in consequence, their effective population size) as most genomic traits and thus do not scale with the strength of selection. This suggests that stochastic processes may play a fundamental role in the evolution of complexes, particularly in the case of homomers (Lynch, 2013). Further support for this hypothesis comes from the observation that the global distribution of quaternary structure topologies in complexes with experimentally determined structures is largely consistent with the distribution expected from random combinations of evolutionary steps (Ahnert et al., 2015).

In this paper, using ligand binding characteristics of proteins present in the PDB, we test whether the acquisition of novel functions/ligands depends on protein quaternary structure and whether it depends on the structure of the ligand binding pocket. We hypothesized that changes in the assembly and organization of protein complexes can result in a rapid emergence of novel functions without dramatic changes in the tertiary structure if the functional sites are formed by more than one protein in the complex (thus changes in assembly might be a source of evolutionary innovations). Whereas protein-ligand interactions have been studied for decades and are of fundamental importance...
in drug discovery (where it is generally assumed that similar binding sites bind similar ligands; Klabunde, 2007), large, PDB-scale analyses of protein-ligand interactions have so far characterized such interactions only at a protein domain or fold level (Furnham et al., 2016; Ji et al., 2007; Kinjo and Nakamura, 2009; Nath et al., 2014), and we are not aware of any study that tested whether there are general relationships between complex formation, complex evolution, and ligand binding. We find that the structure of the ligand binding pocket has a profound influence on the evolution of protein function. In the case of complexes where the residues of a binding site are restricted to a single protein chain, the characteristics of ligand binding in protein complexes and monomers do not differ qualitatively, suggesting that, in the case of these complexes, the “Lynch conjecture” is likely to be correct and neutral processes are very important in the evolution of quaternary structure of such complexes. In contrast, in homomers with small-molecule binding sites involving residues from several protein chains, we observe clear differences that contradict our initial hypothesis: they acquire novel ligands/functions much slower than monomers and bind chemically more similar ligands. In addition, in the case of homomers with multi-chain binding metals or cofactors, we observe a markedly lower variability in quaternary structure (unit number) than in homomers with single-chain binding sites (SBSs). We also find that the ligands of homomers with multi-chain binding sites (MBSs) show significantly lower structural variability than monomers or other homomers, are significantly enriched in nucleobase-containing ligands, and frequently perform metabolic functions.

RESULTS

The Structure of Ligand Binding Site Influences the Evolution of Function of Complexes

We use ligand binding and the presence of binding pockets as a proxy of protein function because it allows comparisons on a PDB-wide scale and is based on experimental data; thus, one can compare homologs without the assumption of functional similarity. To test whether the evolution of protein function is significantly influenced by quaternary structure, we used the BioLIP database (Yang et al., 2013), a semi-manually curated database of protein-ligand interactions. BioLIP contains only ligands that are likely to be biologically relevant (and thus are not artifacts of crystallization, such as solvents used in structure determination) and also provides an annotation of the ligand binding sites (unit number) than in homomers with single-chain binding sites (SBSs). We also find that the ligands of homomers with multi-chain binding sites (MBSs) show significantly lower structural variability than monomers or other homomers, are significantly enriched in nucleobase-containing ligands, and frequently perform metabolic functions.

Cofactors and Metals Binding Multiple Protein Chains Influence the Evolution of Quaternary Structure

Next, we tested whether the observed differences in binding result in differences in the variability of quaternary structure, as defined by the number of subunits in a complex. We found that, in the case of proteins with MBS cofactors or metals, quaternary structure evolves differently in MBS and SBS complexes (Figures 2K, 2L, S2E, and S2F), but surprisingly, in MBS complexes binding only small molecules, there is no such effect (Figures S2K and S2L). In homomers with codactors/metals, MBS complexes show a much higher conservation of unit number than in SBS complexes (Figures 2K and S2), whereas in heteromers, the pattern is the opposite: the structure of such MBS heteromers appear to change faster than SBS heteromers (Figures 2L and S2F). Besides contributing to catalysis (Fischer et al., 2010), certain cofactors and metals are known to stabilize the tertiary structure of proteins; classic examples include the heme of myoglobin and cytochrome-c. Our results strongly suggest that MBS cofactors and metals also influence the formation and evolution of protein complexes. In MBS homomers, their effect is most likely stabilizing, resulting in slower
evolution of quaternary structure, and selection is likely to influence the assembly and composition of such homomers more than of SBS homomers. In contrast, in MBS heteromers, they seem to contribute to evolutionary innovations (Figure 2L).

In the case of complexes without cofactors or metals, the evolution of quaternary structure does not seem to evolve differently in SBS and MBS complexes, despite the clear differences in their ligand binding patterns (Figures S2G–S2L). However, in the case of such heteromers, the lack of increasing trend with sequence divergence (i.e., the fact the more distant sequences do not have more different quaternary structure; Figure S2L) suggest that, besides biological factors, the observed patterns are also significantly influenced by biases in the PDB. This could be caused by incomplete crystals or the recent availability of cryoelectron microscopy (cryoEM) structures, which are typically much larger than the structures obtained with X-ray crystallography.

**Complexes with MBSs Have Chemically Less Variable Ligands and Binding Sites**

What mechanism could be responsible for the slower functional change seen in MBS homomers? There are two fundamentally different possibilities. First, MBSs may be more generic and flexible, and in consequence, they may be able to process a broader spectrum of ligands. Examples of this are certain membrane proteins, receptors, and regulatory proteins (Pabon and Camacho, 2017). It has been shown recently that, in the case of a multidrug resistance protein, the conformational variability of its bipartite binding site is the most likely cause of its very broad substrate specificity (Johnson and Chen, 2017). However, flexible binding sites and broad substrate specificity are also present in some SBS homomers (Hvorecny et al., 2017) and even monomers (Fong et al., 2017). Alternatively, the functions and ligands of MBS homomers may be more conserved than of SBS homomers.

We tested which of these hypotheses is true by comparing the chemical characteristics of ligands and the structural
Figure 2. Evolution of Ligand Binding and QS

We use the ability to bind ligands of homologous proteins (scaled with sequence similarity) as the measure of their functional similarity.

1) Evolution of ligand binding of protein complexes

1. General relationships between ligand binding and QS in homomers, heteromers, and monomers.

A) Homomers with MBSs show a much slower functional divergence than monomers. This pattern is caused by two separate processes: (1) the binding pockets of small-molecule ligands diverge significantly less rapidly than the pockets of monomers and (2) MBS homomers have a higher fraction of cofactors, which are frequently identical and have highly conserved binding pockets plus fewer metal ions.

B) In homomers with binding sites restricted to a single chain, the ability to bind the ligands of homologs changes qualitatively similarly to monomers.

C) MBS heteromers do not show the same pattern as MBS homomers.

3) Evolution of QS

K) Homomers, with cofactors or metals

Legend continued on next page.
variability of the binding sites. We tested the chemical similarity of ligands of homologous structures using the ChEBI (Chemical Entitites of Biological Interest) ontology database (Degtyarenko et al., 2008; Hastings et al., 2016). ChEBI provides a manually curated hierarchical ontology, conceptually similar to gene ontology (GO), that is based on chemical structure (Figure 3A). We used the number of different terms between chemical ligands, excluding nucleic acids, and peptides (Figure 3A). Our analysis shows that, in the case of MBS homomers, the difference between ligands of homologs is smaller than between ligands of monomers, whereas in the case of SBS complexes, the difference is somewhat larger or not significant (Figures 3B–3E). Excluding cofactors from the analyzed ligands does not change the pattern dramatically (Figure S3), although it does influence the most diverged homologs.

Ligand binding frequently results in conformational changes of proteins, and we also tested the structural variability of the binding site by comparing the root mean square deviation (RMSD) of the significant hits (with \(Z\) score > 2) of homologs with sequence similarity between 30% and 49%. Because RMSD depends on the size of the superposed structures, we used the size of the matching pocket as a covariate. We expected that hits to binding sites in proteins with more different ligands and binding sites will have higher RMSD. As predicted, we found a significant difference between MBS and SBS homomers (Figure 4A), but we found no consistent difference in heteromers (Figure 4B), irrespectively whether cofactors were included or excluded from the dataset. Additionally, using those proteins where ligand binding (holo) and ligand-free (apo) structures are both present in the PDB, we tested whether ligand binding results in a larger conformational change in MBS homomers than SBS homomers (measured as the RMSD of the ligand binding pocket of the holo structure in the apo structure). We did not find a clear difference between the two (Figure 4D). Our results based on pocket comparison support the findings based on chemical similarity: on average, the structural variability of the binding sites in MBS homomers is lower than SBS homomers, and the cases where flexibility is assumed to result in ligand promiscuity are most likely a minority.

The PDB is not an unbiased database, as its composition is significantly influenced by the priorities of the research community. Thus, the observed differences could also be caused by biases in the PDB rather than real biological differences, if MBS homomers have more ligands. To rule this out, we determined the ligand diversity of sequences in the PDB, measured as the total number of ligands of the homologs of a sequence divided by the number of homologs it has (Figure S4). Note that, because we ignored structural differences between ligands, high diversity does not necessarily mean high structural variability (for example ATP and ADP are different ligands, although are structurally very similar) but simply the high number of non-identical compounds, irrespectively whether they are small modifications of each other or radically different; thus, this measures research effort and not structural variability. The results show that proteins with MBSs have higher number of ligands per sequence, indicating that the higher similarity of ligands and lower structural variability of MBS homomers are not caused by their lower ligand diversity in the PDB (Figure S4).

Functional Analysis of MBS Homomers
To test whether MBS homomers have characteristic functions, we performed a Gene Ontology enrichment analysis and also an enrichment analysis of the ChEBI terms of their ligands. We found that, whereas the molecular functions of MBS homomers are diverse, several functions are significantly enriched in comparison to all ligand binding homomers, including acyl-coenzyme A (CoA) dehydrogenase activity, transaminase activity, thiamine pyrophosphate binding, cofactor binding, transport membrane transport, ion binding, transporter activity, and other functions frequently involving cofactor binding (Figures 5A and S5; Table S1). Next, we tested whether the chemical composition of the ligands of MBS homomers is biased toward certain chemical groups by a ChEBI structural term enrichment analysis (Experimental Procedures). We found that the ligands that are present in ChEBI (of the almost 20,000 ligands present in BioLiP at the time of writing, only ~3,100 are present in ChEBI) show clear structural biases: the most enriched chemical structures are
nucleobase-containing ligands, frequently containing ribonucleotides (Figure 5B; Table S2). It has been long known that many protein cofactors are derivatives of nucleotides (Petsko and Ringe, 2008), and it has been suggested that this pattern is ancient and might have originated in the RNA world (Ji et al., 2007; White, 1976). However, excluding cofactors from the analyzed ligands does not change the pattern qualitatively. To assess the biochemical role of these ligands, we also performed an enrichment analysis of the ChEBI role ontology terms, which indicates that the most significantly enriched terms are metabolites, including fundamental metabolites (Figure 5C; Table S3), irrespectively whether cofactors are included or not. This suggests that MBS homomers are performing central, mostly metabolic, and probably ancient functions.

General Evolutionary and Structural Characteristics of Complexes with Single- and Multi-chain Binding Complexes

The patterns described above could also potentially emerge if MBS homomers are significantly younger and faster evolving than SBS monomers or homomers, which could result in the observed lower chemical variability, as younger protein families are expected to have less diverse functions (Osadchy and Kolodny, 2011). To test this, we compared the age of proteins that are part of MBS and SBS complexes, using the human protein age dataset provided by Capra et al. (2012, 2013; Experimental Procedures). We found no significant difference in the ages of MBS and SBS complexes (Figure S6). In fact, MBS complexes are somewhat more enriched in the oldest age groups, which is in agreement with the higher frequency of cofactor binding in these groups (Figure 1).

Ligand binding frequently results in conformational changes of both proteins and their ligands (Stockwell and Thornton, 2006), and flexibility has been shown to be essential for the proper functioning of ligand-binding proteins (Petsko and Ringe, 2008). Therefore, because differences in flexibility could have significant consequences for ligand binding, we compared the flexibilities of subunits from MBS and SBS complexes using a simple method based upon relative solvent

**Figure 3. Homomers with MBSs Are Characterized with Chemically More Similar Ligands**

(A) Example of ChEBI ontology similarity between two different chemical compounds (dopamine and guanine). Shared ontology terms are highlighted with red; dopamine and guanine specific terms are highlighted with blue and green, respectively. The difference in their chemical composition was measured as the proportion of the ligand specific and all terms, i.e., (blue + green)/all terms: (15 + 17)/49 = 65%.

(B and C) The chemical properties of ligands of homomers with MBSs (B) change less with sequence divergence than the properties of ligands of monomers or other complexes, but not in heteromers (C).

(D and E) Ligands of homomers with SBSs (D) show a weak but consistently higher variability in the chemical properties of ligands than monomers, while there is no clear difference between SBS heteromers (E) and monomers. Bars represent averages; whiskers 95% confidence intervals; **p < 0.005; *p < 0.05; t tests, with Benjamini-Hochberg correction for multiple testing. See also Figure S3.
stronger selection in prokaryotes, complexes where the topol- et al., 2015; Figure 6 C). We hypothesized that, due to much frequencies of homomers and heteromers, with homomers be- mational changes upon binding.

frequencies of the higher flexibility of MBS complexes is unclear; one possibility is that it is the consequence of the somewhat larger ligands of MBS complexes, which may require larger confor- mational changes upon binding.

accessible surface area ($A_{\text{rel}}$). Essentially, $A_{\text{rel}}$ is the ratio of the solvent accessible surface area observed for a polypeptide chain (ignoring intermolecular interactions) to the value expected for a folded protein of the same molecular weight (Marsh and Teichmann, 2011). Previously, $A_{\text{rel}}$ parameter has been shown to correlate very well with several more complex measures of protein flexibility as well as the magnitude of conformational changes that occur upon binding. Thus, it provides a simple way to analyze the flexibilities of protein complex subunits on a large scale. Interestingly, we observe that MBS complexes are generally more flexible than SBS complexes (Figure 6A). Previously, it was demonstrated that the subunits of protein complexes, heteromers in particular, are more flexible in their unbound states than monomers (Marsh and Teichmann, 2014) and that subunits of complexes with different symmetry types have different flexibilities (Marsh and Teichmann, 2014), i.e., cyclic homomers are more flexible than dihedral homomers. A comparison of symmetry types of homomers nevertheless indicates that the higher flexibility of MBS homomers is not a byproduct of biases in symmetry: MBS homomers are not consistently enriched among complexes with cyclic symmetry (Figure 6B). The biological function of the higher flexibility of MBS complexes is unclear; one possibility is that it is the consequence of the somewhat larger ligands of MBS complexes, which may require larger conformational changes upon binding.

Prokaryotes and eukaryotes are characterized with different frequencies of homomers and heteromers, with homomers being much more frequent in prokaryotes (Lynch, 2012; Marsh et al., 2015; Figure 6C). We hypothesized that, due to much stronger selection in prokaryotes, complexes where the topology is more influenced by stochastic processes, i.e., SBS complexes (Figure 5D), supporting the hypothesis that SBS complexes are subject to weaker selective constraints.

**Pathogenic Mutations Are Most Enriched in the Binding Sites of Heteromers**

Finally, we tested whether quaternary structure and the structure of binding pockets influences the pathogenicity of mutations in the binding sites. Proteins with different quaternary structure have different baseline levels of pathogenic mutations, which increase from monomers through homomers to heteromers, with MBS complexes having higher frequencies of pathogenic mutations than SBS complexes, both in homomers and heteromers (Figure 7; baseline is indicated with a red horizontal line). Mutations in binding sites are known to be more pathogenic than other mutations, and our findings confirm this; in most complex types, the frequency of pathogenic mutations is significantly higher than the baseline level (*p < 0.05; **p < 0.005; tests of proportions). The highest frequencies of pathogenic mutations are in the binding sites of heteromers (Figure 7B), which in the case of small-molecule binding MBS heteromers, is close to 30% of mutations. In the case of metal-binding MBS homomers, the total number of pathogenic mutations is very low, altogether 5, which results in low statistical power (Table S4).

**DISCUSSION**

Our findings indicate that the structure of ligand binding site, i.e., whether it is formed by residues of a single chain or by multiple chains, has profound consequences for the evolution of protein function and, in the case of cofactor or metal-binding proteins, also for quaternary structure. In the case of SBS complexes, the change in ligand binding follows qualitatively the same
patterns as in monomers, indicating that, in the case of such complexes, ligand binding is influenced little by quaternary structure and the evolution of quaternary structure is likely to be more influenced by stochastic processes, as it was suggested by Lynch (2013; Figure 2). Additionally, among all complex types, the baseline frequency of pathogenic mutations is the lowest in SBS complexes (Figure 7), further supporting this hypothesis. In contrast, in complexes that bind metals and cofactors, the quaternary structure of both MBS homomers and MBS heteromers evolves at a significantly different rate than in SBS complexes. Surprisingly, we found no evidence that changes in quaternary structure are a source of evolutionary innovations in

Figure 5. Functional Characteristics of MBS Homomers, in Relation to All Homomers included in the Analysis

(A) Scatterplot of significantly enriched GO molecular function terms, summarized and visualized with REVIGO. Related terms form clusters that are labeled with the most significant term of the cluster. The size of the circles corresponds to the number of proteins in the term; colors indicate significance. (See also Figure S5 and Table S1 for all enriched terms and exact significances.) MBS homomers have diverse functions, including acyl-CoA dehydrogenase activity, transaminase activity, thiamine pyrophosphate binding, cofactor binding, transmembrane transport, ion binding, or transporter activity, which frequently involve binding of cofactors.

(B) Graph of significantly enriched ChEBI structural ontology terms. White nodes are not significant; the intensity of red corresponds to significance (see Table S2 for exact p values). Most enriched structural terms are related to nucleobases/nucleotides.

(C) Graph of significantly enriched ChEBI role ontology terms. (See Table S3 for exact p values.) The enrichment shows that the ligands of MBS homomers are typically involved in metabolism.
homoigomers (at least when considering ligand binding): both
ligand binding and the ligands of MBS homoigomers change sig-
nificantly less with sequence evolution than in monomers (Figures 2A and 3A). The higher variability of quaternary structure (i.e.,
unit number) in cofactor-binding SBS homoigomers (Figure 2K)
also suggests that purifying selection is more important in
shaping the quaternary structure of MBS homoigomers than of
SBS homoigomers.

In the case of cofactor-binding heteromers, we see the oppo-
site pattern: the unit number of MBS complexes changes faster
than monomers (Figure 7), and the qua-
ternary structure of such homoigomers is still frequently conserved (Levy et al., 2008; see also Figures 2 and S2). How-
ever, in addition to adaptive forces, the nonlinear change of tertiary structure with sequence similarity may contribute
to the pattern. Typically, above ~40% sequence identity, the tertiary structure
of homologous proteins differs little (see Abrusán and Marsh, 2016 for examples),
and structural similarity declines signifi-
cantly only below ~30% sequence iden-
tity. In fact, structure is so much more
conserved than sequence that homol-
gy-based tools for tertiary structure prediction like Rosetta
(Kim et al., 2004) or I-TASSER (Yang et al., 2015) can frequently
build reliable predictions based on templates with as low as
15% sequence identity. Thus, similar quaternary structure of homologs might be, to some degree, the byproduct of their
similar tertiary structure: similar building blocks result in similar complexes.

The observation that ligand structure and ligand pocket struc-
ture changes much less with sequence in MBS than SBS homoigomers is likely to have important consequences for drug design:
the development of broad-spectrum antibiotics and antiviral
drugs; for polypharmacology (Bolognesi and Cavalli, 2016);
and also drug repositioning. Our findings suggest that antibiotics
targeting MBS homoigomers are likely to be broader spectrum than antibiotics that target SBS homoigomers. In addition, it is likely that
evolving resistance to drugs that target MBS homoigomers is more
difficult, at least where the evolution of resistance is due to the
accumulation of mutations, like in rapidly evolving retroviruses.
The traditional aim of drug design has been designing drugs
that bind specifically to one target protein (monopharmacology)
to achieve maximum specificity and minimize side effects.
However, it has become clear that many diseases, like cancers
and psychiatric diseases, have polygenic causes, and in such

Figure 6. Structural and Evolutionary Char-
acteristics of Single- and Multi-chain Bind-
ing Complexes
(A) Subunits forming MBSs are significantly more
flexible than subunits with single-chain sites
(Wilcoxon tests).
(B) The frequency of single- and multi-chain binding sites in different symmetry groups of homoigomers. The
symmetry groups are A, asymmetric; C, cyclic; C2, two-fold dimeric; C2h, two-fold symmetric with >2 subunits; and D, dihedral (tests of proportions).
(C) The frequency of homoigomers is higher among
prokaryotes, whereas heteromers are more frequent in eukaryotes (tests of proportions).
(D) The frequency of complexes with MBSs is
higher in prokaryotes than in eukaryotes, particular-
ly in the case of heteromers (tests of proportions).
On (B)–(D), whiskers represent 95% confidence intervals. On all panels, **p < 0.005 and *p < 0.05.
diseases, drugs that target several proteins simultaneously (multi-target or promiscuous drugs) can be much more efficient than single-target drugs, despite their more complex and less understood pharmacology (Peters, 2013; Roth et al., 2004). Because similar binding pockets in general bind similar ligands (Klabunde, 2007), protein families with many MBS homomers are likely to be good candidates for the development of multi-target drugs, particularly in cancers, due to the specific metabolic characteristics of many cancers (Cairns et al., 2011). Finally, our results may be also relevant for drug repositioning (Li and Jones, 2012), because binding site similarity is a requirement for repositioned drugs. An example is the retroviral drug nelfinavir, which binds HIV protease, a homomer with a MBS, and is currently being repositioned as a (promiscuous) cancer drug (Koltai, 2015).

**EXPERIMENTAL PROCEDURES**

**Data Sources and Data Preprocessing**

The sequences of proteins having a structure in the PDB were downloaded from Uniprot, and we used the Uniprot mappings between sequences and PDB structures. To identify homologous protein pairs, we performed an all versus all BLAST search on these sequences, with an e-value cutoff $10^{-4}$, the sequence pairs with significant BLAST hits were realigned with Muscle (Edgar, 2004), and their global sequence similarity was determined. Chimeric protein sequence pairs with significant BLAST hits were realigned with Muscle irrespectively of the length of the chains. Defined as structures with chains from minimum two different proteins, sequences were classified as monomers. Heteromer PDB structures were defined as a heteromer if it has at least two different proteins; else, if it has at least one homomeric PDB entry, it was classified as a homomer, and the remaining sequences were classified as monomers. Heteromer PDB structures were defined as structures with chains from minimum two different proteins, irrespectively of the length of the chains.

**Searches for Ligand Binding Sites**

We used ProBiS (Konc and Janežič, 2010, 2017) to identify ligand binding pockets in homologous proteins, which identifies binding sites by local structural search and compositional similarity. We performed all searches between monomeric protein pairs; i.e., we searched all structures of the target protein with all ligands of all structures of the query protein (Figure 1E). In the first step, the biological units of every PDB entry were preprocessed: their atoms and chains were renumbered so that every atom and protein chain was unique in the structure, and from the target protein structures, all ligands were removed, whereas from the query structures, only waters. Due to the limitations of PDB format, structures with more than 99,999 atoms in the asymmetric unit, as the ligand annotation of these entries is incomplete in BioLiP. In the case of entries where the asymmetric unit contains the biounit differences between the two are incorporated into the BioLiP annotation; see Yang et al. (2013) for details.

From the ligands of BioLiP, we used cofactors, metal ions, and small molecules. Cofactors were determined as ligands that are annotated as cofactors in the ChEBI database (Hastings et al., 2016) or in Fischer et al. (2010). Small molecules were defined as every ligand that is not a cofactor, metal ion, nucleic acid, or a peptide.

**Figure 7. The Frequency of Pathogenic Mutations Is Different in Different Complexes**

(A–D) Complexes with (A) small molecules, (B) cofactors, (C) metal ions, and (D) all ligands. The baseline level of pathogenic mutations is highest in MBS heteromers and lowest in monomers. In most complex types, the frequency of pathogenic mutations of the binding sites is higher than the baseline level, particularly in the case of heteromers. **p < 0.005; *p < 0.05; tests of proportions; whiskers represent 95% confidence intervals.
ProBiS in finding matches and whether there are systematic differences between homomers, heteromers, and monomers by determining the fraction of significant hits between homologous structures that are known to bind the same ligand (Figure S7). We found that the efficiency of ProBiS is high and is not influenced qualitatively by the size or number of chains in a PDB entry (Figure S7).

**Determination of Ligand Chemical Similarity and Binding Site Variability**

We used the obo ontology files downloaded from the ChEBI database (Degtyarenko et al., 2008; Hastings et al., 2016) to compare the chemical similarities of ligands. For every ligand, its entire structural ontology graph was computed from the obo files using a recursive algorithm, using the “is_a” tag, and the average of the shared/different terms was determined for every possible ligand pair of the homologous sequences.

Structural variability of the binding sites of homologs was performed using the RMSD of the significant hits (Z score ≥ 2), using the sequence pairs with 50%–30% identity. We used the size of the match as a covariate because RMSD is not a size-independent measure; it was measured as the number of aligned ProBiS vertices. We performed similar searches between the ligand binding (holo) structures and ligand-free (apo) structures of the same proteins when they were both available in the PDB. In these searches, no significance cutoff was used. Variability in the structure of the binding sites was measured as the average RMSD of the best hits, using the size of the match as covariate.

**Gene Ontology and ChEBI Ontology Enrichment**

From the UniProt-CrossRef annotation of proteins that are present in the PDB, we extracted the list of GO terms associated with a particular protein. Next, for every protein, we extracted the entire hierarchy of its GO terms, i.e., all parents up to the highest level “molecular function” term, and determined the enrichment of GO terms in MBS homomers with GeneMerge (Castillo-Davis and Hartl, 2003), using all homomers in the PDB as the background set. Significances were corrected for multiple testing with the Benjamini-Hochberg method (a.k.a. false discovery rate [FDR]), and the list of significantly enriched terms was submitted to the Revigo server (Supak et al., 2011) to remove redundancies, summarize, and visualize the results.

ChEBI term enrichment was determined as follows. We determined the nonredundant list of ligands in MBS homomers and in all homomers, containing only a single instance of every ligand. For every ligand, we identified its parental terms using the ChEBI structural ("is_a") and role ("has_role") ontologies. The enrichment of terms was also calculated with GeneMerge, which is a generic tool for term-enrichment analysis of ranked lists. Enriched terms (after correction for multiple testing with FDR) were visualized with a method conceptually similar to the graphical output of GOrilla (Eden et al., 2009), with directed graphs and using color coding to indicate the significance of the enrichment; the intensity of red indicating significance. Graphs were drawn using GraphViz.

**Evolutionary and Structural Characteristics of Complexes**

The evolutionary age of human proteins was based on the ProteinHistorian database (Capra et al., 2012), using the Princeton Protein Orthology (PPOD)/PANTHERv7 dataset, generated with asymmetric Wagner parsimony. The flexibility of subunits of proteins was determined as described in Marsh and Teichmann (2014); the symmetry type of homomorphic complexes is based on the classification of PDB.

**Estimation of the Frequency of Pathogenic Mutants**

The list of disease-causing mutations was downloaded from Ensembl (variation dataset) and was mapped to the PDB structures of human proteins or their homologs if the sequence similarity between the human protein and its homolog was higher than 90% and the mutated amino acid was identical. The set of putative neutral mutations is based on ExAC variants (Lek et al., 2016) that map to structures of the PDB using a similar procedure as described above. The frequency of pathogenic mutants—both baseline and binding—was determined for every protein type independently as the number of disease mutations divided by the sum of disease and neutral mutations.

**Analysis Tools and Statistics**

All analyses and statistical tests (except GO and ChEBI enrichment) were performed with in-house Perl scripts and R. Protein structures were visualized with UCSF Chimera (Pettersen et al., 2004).

**DATA AND SOFTWARE AVAILABILITY**

Supplemental data are available from Edinburgh Datashare (https://datashare.is.ed.ac.uk/handle/10283/3024).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and four tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.085.

**ACKNOWLEDGMENTS**

We thank Janez Konc for help and suggestions on ProBiS usage and Jonathan Wells and the anonymous reviewers for helpful comments on the manuscript. We acknowledge the use of the Eddie3 computing cluster of the University of Edinburgh and thank for the support from the Research Computing staff. This work was supported by the Medical Research Council (Career Development Award MR/M02122X/1 to J.A.M.).

**AUTHOR CONTRIBUTIONS**

G.A. conceived the project, performed the analyses, and wrote the first version of the manuscript. J.A.M. contributed to the analysis and the writing of the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

Received: July 5, 2017

Revised: January 17, 2018

Accepted: February 22, 2018

Published: March 20, 2018

**REFERENCES**


