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Editorial overview: Engineering and design: New trends in designer proteins

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Julia Shifman is a faculty member at the Department of Biological Chemistry, the Hebrew University of Jerusalem. Julia received her Ph. D. from the University of Pennsylvania and performed her postdoctoral training in California Institute of Technology. She has been in the field of computational protein design from its very beginning, focusing on design of protein–protein interactions (PPIs) Julia investigates mechanisms of PPI evolution and works on design of protein-based inhibitors for various disease-related target proteins.

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Dr. Niv Papo is a molecular biologist focusing on protein engineering and cancer imaging and therapy. He joined the Ben-Gurion University faculty in 2011. Dr. Papo completed his PhD at the Weizmann Institute of Science in the field of antimicrobial peptides, a post doctorate fellowship in the field of molecular chaperones and a post doctorate training at Stanford University in protein engineering. Dr. Papo's current research focuses on the development of new mono- and multi-specific proteins and protein-small molecule conjugates that promise to aid in both the diagnosis and treatment of cancer.

With the constant growth of the protein design community, each year brings new designer proteins with applications in such diverse areas as drug design and delivery, synthetic biology, materials science, biosensing and imaging. The past year has seen a number of advances in the design methodology where combining computational and directed evolution approaches proved to be particularly advantageous. This issue addresses several challenging questions in protein engineering where recent breakthroughs have been reported.

A number of recent studies prove that various natural protein effectors could be converted into high-affinity and high-specificity inhibitors or activators of various target proteins. The review by Zhang et al. describes how such engineered molecules could be used to modulate cell signaling either by binding to extracellular domains of membrane proteins or by disrupting intercellular protein–protein interactions (PPIs). Examples include SH2 variants that modulate phospho-dependent signaling in cells and that can be used to fine-tune signal transduction to prevent undesired cell-fate changes. In discussing therapeutic applications, Zhang *et al.* cover the engineering of a variety of proteins, including: natural phospho-dependent interactions to modulate cell signaling; modulators of specific E3 ligases and deubiquitinases (DUBs) in the ubiquitin proteasome system (UPS) to obtain improved therapeutics with fewer side effects; and the extracellular domains and ligands of membrane proteins as promising non-immunoglobulin therapeutics. The evolved natural effectors could bind to their targets with very high affinity and specificity and lack many shortcomings of conventional antibodies.

As another alternative to antibodies, Könning et al. introduce engineered nanobodies as potential binding agents. These single-domain antibodies are found uniquely in camelids (camels, llamas and alpacas), being the smallest naturally occurring antibodies known. Nanobodies consist of only the heavy-chain C_H3 and C_H2 domains, together forming the Fc region, and the VHH domain, which is comprised solely of a single heavy chain. Since VHH is the only part of the protein that mediates antigen binding, it can be separated from the Fc region to produce a small protein, the nanobody, that can be evolved to bind to and inhibit various targets with high affinity, similarly to conventional monoclonal antibodies. The advantage of nanobodies is their small size that allows them to penetrate tissues much better compared to conventional antibodies, thus making them superior drugs and imaging agents.

While protein binders could be now engineered from a variety of various scaffold proteins, delivery of such binders to the target area in the human

body remains the main obstacle in protein-based drug design. One of the most challenging problems facing the protein design community is targeting proteins that are localized in the human brain. The challenge derives from the inability of many therapeutic molecules to permeate the blood brain barrier (BBB), whose physical, transport, and metabolic properties impede this passage. Goulatis and Shusta review methodologies developed to overcome this problem by targeting various vesicle-mediated transcellular BBB pathways and using BBB-targeting vectors that can be exploited for noninvasive drug delivery to the brain. These pathways include fluid-phase endocytosis, absorptive-mediated endocytosis and, of particular importance for drug delivery, receptor-mediated transport in the form of caveolae- or clathrin-mediated mechanisms. Goulatis and Shusta describe current protein engineering efforts to best utilize the available pathways, particularly receptor-mediated systems, for drug delivery. In such systems, engineered high-affinity and high-specificity antibodies or peptides for the desired target receptors are usually employed as targeting vectors.

Compared to soluble proteins, membrane proteins have been traditionally more challenging targets in protein design due to the sparse structural information available on this type of proteins and challenges in their expression and purification. Nonetheless, significant advances have been made in the design of outer membrane proteins (OMPs) that function as barriers between the exterior and interior of the cell. In general, OMPs serve not only as gateways to the cell but also as cellular control centers passing 'information' through membrane protein signaling networks to the cellular machinery. Both these functions are exploited in research aimed at understanding basic concepts in cell biology and at the development of therapeutic strategies. The review of Slusky focuses on the engineering of OMP β-barrels, with emphasis on structural changes and deconstruction, design of chemical functionalities, and creation of new folds. The reviewed studies clarify the structural roles of the β-barrel components and discuss the development of β-barrel-based biomolecules for therapeutics and diagnostics.

The bottleneck of protein design lies in experimental construction and testing of tens to hundreds of protein variants in order to optimize the design. A new approach has recently emerged where this process could be greatly accelerated through the use of next generation sequencing (NGS). In such an approach, high-throughput functional screening or selection (HTS) of thousands of single mutants is followed by NGS and the most frequently observed mutations are combined to produce the protein with the best fitness. A similar approach could be also used to construct protein fitness landscapes and to study protein evolution. Wrenbeck et al. review the progress in this methodology and summarize examples of its application to design of membrane proteins, enzymes and binders with optimized affinity and specificity. Future application of this methodology to various biological problems is likely to produce many interesting studies in the fields of protein evolution and design.

One attractive direction in protein design is designing small-ligand binding proteins that could serve as either biosensors or enzymes. To facilitate such designs, better understanding of architecture and evolution of ligand binding is needed. In recent years, development of efficient computational methods enabled large-scale studies that compare ligand binding sites in different proteins. These studies revealed that both convergent and divergent evolution of ligand binding sites occur in nature. The review by Rajmanovich describes the existing computational approaches for binding site comparison and summarizes recent findings on the evolution of binding sites. Importantly, recent studies have revealed that evolutionarily unrelated proteins can bind to the same ligand by using either the same or a different set of residues and that small differences in binding sites could result in drastic effects on binding affinity and specificity.

On a related topic, Yang and Lai discuss recent progress in the rational design of ligand-binding proteins. The development of new computational methods and improvements in HTS have facilitated design of a number of proteins that bind various small molecules and metal ions. The authors also describe studies where natural ligand-binding proteins have been computationally converted to recognize new ligands while retaining their original biological functions. Despite the advances that have been made in the design of ligand-binding proteins, Yang and Lai remind us of the limited success in designing such proteins with purely computational methods while combined computational/directed evolution approaches produce better results. The authors suggest several strategies for improving computational methodology for ligand-binding protein design.

Engineering of fluorescent proteins for *in vivo* imaging is probably the most successful example of protein engineering. Among various fluorescent proteins, red-fluorescent proteins (RFPs) are particularly suited to whole-animal imaging, as longer wavelength light is less scattered by tissue and causes lower phototoxicity. Yet, the photophysical properties of RFPs remain inferior to those of green and yellow fluorescent proteins, thereby confining the use of RFPs to a limited range of biological applications. While most efforts in fluorescent protein engineering have been achieved using directed evolution approaches, structure-based design emerged as an attractive strategy for RFP design. Eason et al. present recent advances in rational structure-based design of RFPs aimed at improving their brightness, monomerization, maturation, and photostability. The authors also discuss strategies for future engineering of RFPs for various applications.

Recent efforts in synthetic biology have been directed at manipulating protein networks inside living cells. One attractive strategy for controlling protein activity inside cells is to use light as an on/off switch. Photoswitchable proteins can be engineered by fusing a natural effector protein (inhibitor or an activator) to a photo-switchable domain. The main difficulty in the design of such photoswitchable proteins lies in finding a particular sequence that functions in the light but not in the dark (or vice versa). The search for such sequences requires the development of complicated selection procedures that involve both positive and negative selection. Brechun et al. summarize advances in the design of photoswitchable proteins and describe applications of such molecules in protein re-localization in the cell, effector recruitment, protein clustering, and protein degradation.

The final review is devoted to design of repeat proteins, *i.e.* proteins containing repeating structural units that have evolved through gene duplication and accumulation of diverging mutations. In nature, repeat proteins serve a variety of functions, including ligand recognition, molecular assembly, and signaling. Due to the possibility of incorporating multiple functions into repeat proteins, such proteins are an attractive target for protein engineering. Attempts over the past 15 years to rationally design repeat proteins have been fraught with difficulties. However, a better understanding of repeat protein architecture and assembly and an improvement in computational methods have led to the recent successful designs of a number of different repeat proteins. In their review, Parmeggiani and Huang introduce the reader to approaches for repeat protein design including sequence consensus design and structure-based design with or without a template. They discuss future directions in repeat protein design, such as complete parametrization in the design, design of unstructured proteins, and modular control of repeat proteins.