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Moonlighting proteins – nature's Swiss army knives

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ABSTRACT

The human body is a complex biological machine with billions of cells and vast numbers of biochemical processes – but our genome only contains 22,000 protein-encoding genes. Moonlighting proteins provide one way to increase the number of cellular activities. Moonlighting proteins exhibit more than one physiologically relevant biochemical or biophysical function within one polypeptide chain. Already more than 300 moonlighting proteins have been identified, and they include a diverse set of proteins with a large variety of functions. This article discusses examples of moonlighting proteins, how one protein structure can perform two different functions, and how the multiple functions can be regulated. In addition to learning more about what our proteins do and how they work together in complex multilayered interaction networks and processes in our bodies, the study of moonlighting proteins can inform future synthetic biology projects in making proteins that perform new functions and new combinations of functions, for example, for synthesising new materials, delivering drugs into cells, and in bioremediation.

Keywords: *moonlighting proteins, multifunctional proteins, protein structure and function*

1. What are moonlighting proteins?

The human body is an amazingly complex machine with billions of cells that work together to perform a vast number of biochemical processes, from digesting

our food, carrying oxygen, transporting nerve impulses, providing force to move our muscles, healing wounds, fighting off infections, sensing our surroundings through taste, touch, sight, and so on. Each of these processes requires hundreds or thousands of protein machines to serve as the structures and also to synthesise the components, convert energy from our food to the chemical energy needed to perform the functions, break down old components and regulate all these processes and coordinate with other components, cells, and organs. The vast number and variety of functions led many to predict that the human genome sequence would contain hundreds of thousands or even millions of protein-encoding genes. Surprisingly, the actual number is only around 22,000 genes! How can this discrepancy be explained? Part of the explanation lies in the ability of a gene to encode multiple versions of a protein through the process of alternative splicing. More recently it has become apparent that many individual proteins can also serve multiple, sometimes completely unrelated functions. These 'moonlighting proteins' are able to perform more than one physiologically relevant biochemical or biophysical function using only one polypeptide chain[1]. In this subset of multifunctional proteins, the multiple functions are not due to gene fusion events during evolution or the result of multiple proteolytic fragments from a large precursor protein that perform different functions. Several hundred moonlighting proteins have been identified, and they include a diverse set of proteins with a large variety of functions[2]. Several examples are given in Table 1 and discussed below.

As an example, I will describe the first moonlighting protein that I worked on. Phosphoglucose isomerase (PGI) has been studied for over 100 years as an enzyme in glycolysis, a central biochemical pathway in sugar metabolism that is found in the cell cytoplasm in almost all species on Earth. (Interestingly, the only known exceptions are some obligate intracellular parasites that do not have a complete glycolysis pathway.) PGI has been extensively characterised through enzyme activity assays, ligand and inhibitor binding assays, mutagenesis, and structure determination. The X-ray crystal structures determined by my research group and others clearly contain an active site pocket where the substrate binds and undergoes conversion to product[3]. In many ways, PGI is a classic example of an enzyme. However, there is more to this protein than first suspected. Searching for papers about PGI leads to articles about proteins that appear to belong to a very different class of protein. Neuroleukin is an extracellular nerve growth factor that supports the survival of embryonal neurons[4,5]. Autocrine motility factor is also an extracellular protein that binds to target cells and causes them to change their motility[6]. Differentiation and maturation mediator is another extracellular protein that causes premature B cells to mature into antibody secreting white blood cells[7]. The surprising thing is that these three proteins are all the same protein as PGI! The intracellular PGI enzyme that functions in glycolysis in almost all species has been adopted in some mammalian cell types to serve a second type of function as a secreted, extracellular signalling protein.

But is this ability to do two things in two places just a one-off quirk of PGI? Our previous model of how the genome encodes proteins was that one gene

Table 1 *Examples of moonlighting proteins*

Protein name	One function	Another function
Phosphoglucose isomerase (PGI)	Enzyme in glycolysis	Extracellular signalling protein
Delta2 crystallin (ducks)	Arginosuccinate lyase in urea cycle	Crystallin
Epsilon crystallin (birds and reptiles)	Lactate dehydrogenase	Crystallin
Zeta crystallin (camels and frogs)	Quinone oxidoreductase	Crystallin
Zeta crystallin (elephant shrews)	Aldehyde dehydrogenase	Crystallin
Pyruvate kinase	Enzyme	Cell surface receptor
Glutathione S-transferase	Enzyme	Cell surface receptor
Triosephosphate isomerase	Enzyme	Cell surface receptor
Fructose-bisphosphate aldolase	Enzyme	Cell surface receptor
SMC3	Binds to chromosomes	Extracellular matrix component
Enolase	Enzyme in glycolysis	Receptor for plasminogen
Aconitase	Enzyme in the citric acid cycle	RNA binding protein
Ribosomal protein S3	Part of ribosome	Transcription factor
Ribosomal protein L13a	Part of ribosome	Transcription factor
Ribosomal protein L10a (plants)	Part of ribosome	Inhibits virus reproduction
Oestrogen receptor	Transcription factor	Part of signalling pathway

evolved to encode one protein that has one function. Do other proteins besides PGI also moonlight, or perform more than one function? The first examples found of moonlighting proteins were some of the crystallins. Crystallins are proteins that are highly expressed in the lens of the eye and provide its structure and transparency. Piatigorsky and co-workers[8,9] discovered that the major crystallin in the lens of the duck eye, called the delta2 crystallin, is arginosuccinate lyase. Arginosuccinate lyase is better known as an enzyme that is found in almost all species where it functions in the urea cycle and is needed for the biosynthesis of the amino acid arginine. In other species, different proteins were adopted to be crystallins. The epsilon crystallin found in swans, ostriches, geese and other birds as well as reptiles like crocodiles[10,11] is the same protein as lactate dehydrogenase, another enzyme that is found in almost all species. The zeta crystallin in camels, llamas, guinea pigs and frogs is quinone oxidoreductase[12,13], and the eta crystallin in elephant shrews is aldehyde dehydrogenase[14]. The adoption of different proteins to serve as crystallins in a few

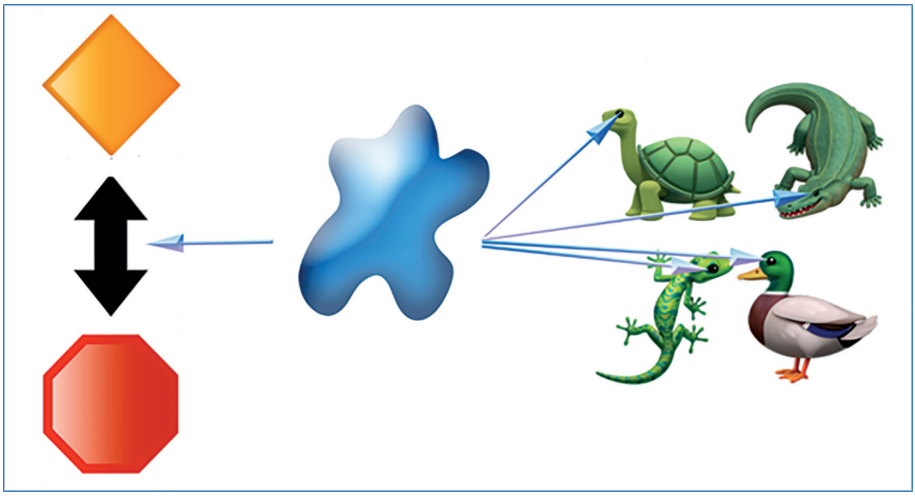

Figure 1 *A moonlighting protein can have two very different functions. Several of the taxon-specific crystallins are enzymes that perform a catalytic function converting a molecule into a different molecule. They have a second function in the lens of the eye. Different enzymes were adopted to perform a second function in different species.*

dozen different species has led to calling them the 'taxon specific crystallins'. In each of these cases, the protein is still capable of catalytic activity as an active enzyme in other cell types, but the substrates are not available in the lens (Figure 1).

Another subclass of moonlighting proteins was hinted at by the discovery that another intracellular enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), is found on the extracellular surface of streptococci, a bacterial genus that includes the cause of strep throat[15]. Why is an intracellular enzyme out on the surface of a cell where its substrate is not found? Over the past few decades it was found that dozens of intracellular enzymes, as well as other proteins that function inside the cell in protein folding, synthesis of proteins, or binding to DNA, are placed on the cell surface where they perform a completely different function. Some aid in the uptake of nutrients, but the majority found so far are involved in interactions with other cell types or other species. In humans, at least four intracellular enzymes, pyruvate kinase, glutathione S-transferase, triosephosphate isomerase, and fructose-bisphosphate aldolase are displayed on the membrane of the sperm to interact with the surface of the egg[16–18]. Many bacterial species, both disease-causing pathogens and 'good' or 'probiotic' species, use intracellular enzymes on the cell surface to bind to host proteins and host cells. In some cases, these interactions help the bacteria colonise the gut or other tissues. Other intracellular/surface proteins are used by bacteria that infect us to bind to a protein in our bodies called plasminogen. Plasminogen is an inactive enzyme, a zymogen, but binding to the surface of the bacteria helps it become modified by other enzymes to become the active protease plasmin, which can chew up many kinds of proteins. Plasmin is used as a tool by these pathogenic bacteria to help break down and invade our tissues.

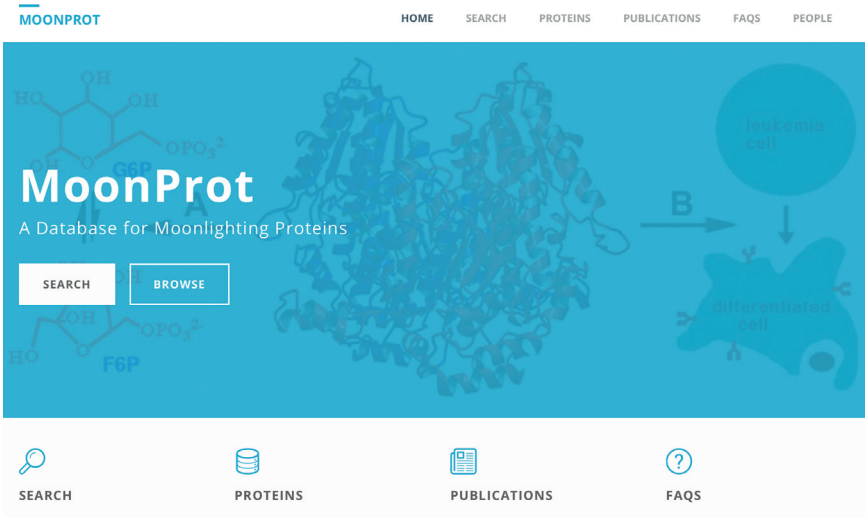

Figure 2 *The MoonProt Database includes information about over 300 moonlighting proteins. The database (located at www.moonlightingproteins.org) includes the proteins' names, amino acid sequences, links to protein structures in the Protein Data Bank (when available), descriptions of the different functions, and references to papers describing the experiments that demonstrated the proteins have those functions. The database home page shown above contains links for a list of all the included proteins (PROTEINS), frequently asked questions (FAQs) and lists of publications (PUBLICATIONS) about moonlighting proteins. There is also a search function so that a user can find known moonlighting proteins from a chosen organism (such as* Escherichia coli*) or with a selected function or protein name. The home page also includes a brief description of moonlighting proteins (not shown).*

Many other proteins have been found to have more than one function. Over 300 are listed in our online MoonProt Database[2] (Figure 2). Many combinations of functions are observed, such as enzymes that also function as parts of cytoskeletal fibres or components of the ribosome machine or of the proteasome. There are also channels that span the cell membrane to enable transport of ions that have a second function of interacting with and regulating other channels. SMC3 (structural maintenance of chromosome protein 3) binds to chromosomes in the cell's nucleus and is also secreted to form the extracellular matrix between cells[19–22]. Several chaperone proteins, which help proteins fold inside the cell, are also secreted to bind to cell surface receptors on other cells. Moonlighting proteins are not only diverse in function, but they are also found in all branches of the tree of life – bacteria, archaea, mammals, reptiles, birds, fish, worms, insects, plants, fungi, protozoans and even viruses.

2. How can one protein machine do two different jobs?

Proteins have evolved over billions of years so that their three-dimensional structure is precisely arranged to perform a function. An enzyme has a pocket lined with a

very specific sequence of amino acids in just the right places to bind to a substrate and catalyse a reaction. Proteins that perform other functions have different shapes, for example, a fibre such as collagen in our bones and cartilage often has a long, extended structure compared to the globular shape of most enzymes, hormones and transport molecules, *e.g.* hemoglobin. So how can one protein structure perform more than one function? There are several solutions to this problem. For many of the crystallins, the overall structure and physical properties of the protein is great for both functions – no significant change in structure was needed to enable the enzyme to be adopted to perform the role of a crystallin. For the intracellular/cell surface proteins, the ancestral protein structure contained the pocket where an enzymatic chemical reaction takes place, and during evolution another part of the surface of the protein gained the features needed for binding to host cells or other proteins. This site of interaction can involve a very small percentage of the amino acids that make up a protein structure. In a study of an enolase enzyme from *Streptococcus pneumoniae* that also binds to plasminogen, Ehinger and co-workers[23] found that out of the 434 residues making up the whole protein, a small region of the protein surface consisting of only nine amino acids was sufficient for binding to plasminogen. If we look at the structure of PGI, the amino acids that were conserved during billions of years of evolution show us the location and identity of the amino acids required for the original function of the protein[3]. They cluster around the active site pocket, where they interact with bound substrate, promote the catalytic activity, and help form the shape of the active site pocket. But in this large protein of over 500 amino acids per

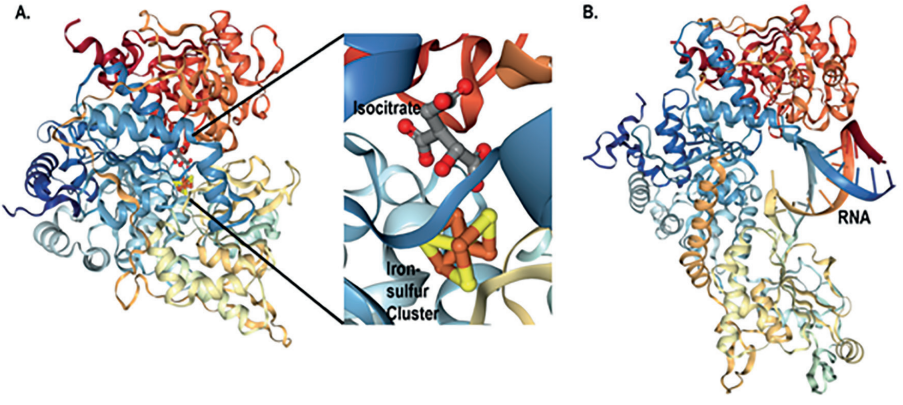

Figure 3 *Aconitase undergoes a large conformational change when it changes functions. (A) Aconitase is shown as a ribbon diagram. The insert illustrates the binding sites for the substrate of the enzyme's catalytic reaction (isocitrate, shown as a ball-and-stick diagram with grey sticks representing covalent bonds and red spheres representing oxygen atoms) and the iron-sulfur cluster (shown with orange and yellow bonds), which aids in the reaction. (B) If the concentration of iron in the cell decreases, the iron-sulfur cluster leaves its binding site, and the protein undergoes a large conformational change. Notice that the red end of the molecule is farther away from the yellow end than in part A. The extended protein can now bind to RNA and regulate the expression of several proteins encoded by the nucleotide sequence of the RNA molecule.*

subunit, there are many surface features of the protein that are not needed for the enzyme function. In fact, a comparison of PGI proteins from different species shows that entire alpha helices and surface loops have been added or subtracted, and these proteins are still capable of catalysing the PGI reaction[3]. A single protein structure can easily accommodate both an active site pocket and a small protein interaction site.

Other moonlighting proteins use much more complex ways to perform two different functions. In a few cases, the protein undergoes a large conformational change to perform a second function. Aconitase, which catalyses a reaction in the citric acid cycle, undergoes a very large movement of domains to become an RNA binding protein[24] (Figure 3). In addition, many intrinsically disordered proteins (IDPs), do not have a completely folded three-dimensional structure until they bind to another molecule (protein, DNA, *etc.*). Some IDPs perform multiple functions by folding into different shapes with different partners. The mechanics by which one protein can perform two functions is an ongoing area of discovery.

3. How can a moonlighting protein do each job at the right time and place?

Another current area of research is how the proteins perform the right amount of each function in the right place and at the right time. Too little or too much activity can result in disease. In some cases, a single moonlighting protein can perform multiple functions at the same time, in other cases the different functions are regulated so that a protein performs only one function at a time and can switch to a different function when conditions within the cell change. Sending the protein to different places where there are different molecular partners also can be involved in performing different functions, for example the cytoplasm where there might be a substrate, the nucleus where it can interact with DNA, or outside the cell where it can interact with other cell types. How many of the proteins are targeted to these different locations is not completely understood, but regulation of a moonlighting protein's functions and locations often involves post-translational modifications (PTMs).

PTMs are estimated to be used to regulate the functions of over half of all proteins. These can involve many types of alterations to the polypeptide chain, including addition or removal of a functional group like a phosphoryl, acyl, or acetyl group. Even a relatively small change like addition of a phosphoryl group can have significant changes in the conformation of a protein and in its ability to bind to other molecules. In some cases, a PTM also serves as a signal like a zipcode (postal code) to target the protein to another location in the cell. Several proteins that are part of the ribosome machine that makes proteins have another function in the cell nucleus. When ribosomal protein S3 (rpS3) in bacteria becomes phosphorylated, it leaves the ribosome to participate in DNA damage repair and also to act as a transcription factor[25,26]. Another ribosomal protein, L13a, moves to join a multiprotein transcription factor in the nucleus when it is phosphorylated[27]. In plants, L10a (rpL10a) also moves to the nucleus where it helps prevent reproduction of a virus[28,29]. A different

kind of PTM, addition of a fatty acid palmitoyl group, causes the oestrogen receptor to leave the nucleus and move to the cell membrane where it communicates with a signalling pathway[30].

As described above for PGI and other moonlighting proteins, one of the most common ways to switch between functions is for an enzyme that has one function inside the cell to be secreted to function as a signalling molecule or to become attached to the cell surface to function as a receptor. In most cases, these intracellular/surface moonlighting proteins share physical chemical characteristics with other cytoplasmic proteins[31], so how these proteins are selected for secretion, how they are transported across the cell membrane, and how they become attached to the cell surface are unknown and are important current areas of research.

4. What if a moonlighting protein is involved in a disease?

As more and more proteins are being found to moonlight, it is becoming clear that this group of proteins includes many proteins involved in common diseases, including cancer, diabetes, heart disease, arthritis, and other autoimmune diseases. When one of our proteins has two or more functions, figuring out which function is involved in disease and how to target the correct function can be important. Targeting the entire protein can inhibit both functions and result in toxicity and side effects (Figure 4). For example, we might want to

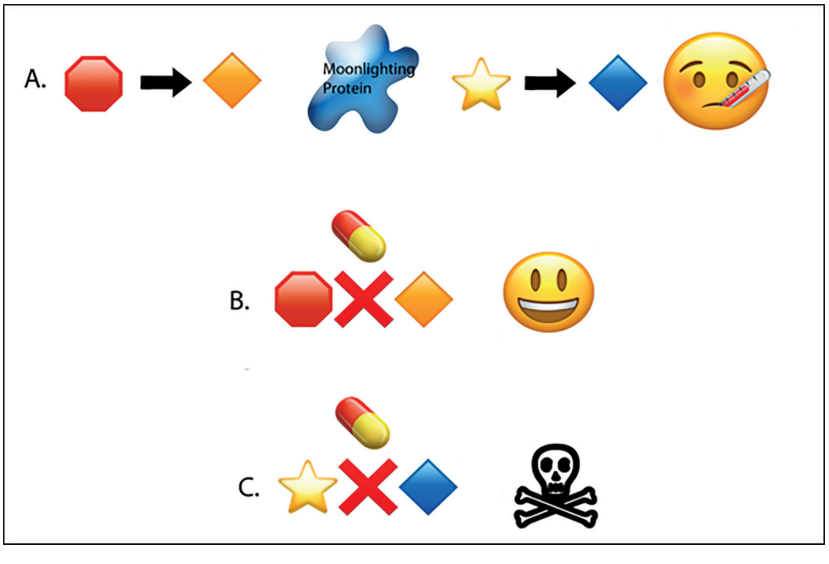

Figure 4 *Understanding all the functions of a moonlighting protein is important when targeting that protein with a drug to treat disease. (A) One of the functions (octagon to yellow diamond or star to blue diamond) of a moonlighting protein might be involved in a disease. (B) Inhibiting one function with a drug might lead to reduction in disease symptoms. (C) If the drug also inhibited the other function of the moonlighting protein, it could lead to side effects or toxicity. It is important that a drug targets the correct function.*

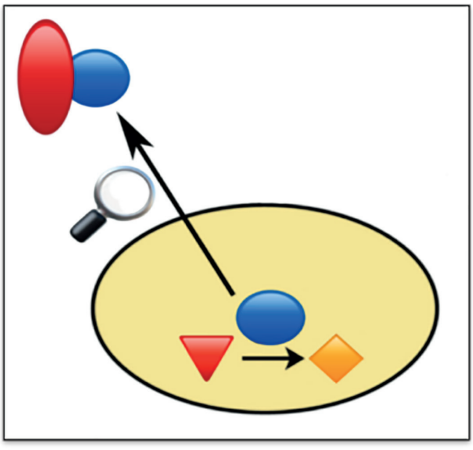

Figure 5 *There are still many questions about how the multiple activities of moonlighting proteins are regulated. Moonlighting proteins may perform their different functions in different cellular locations. Dozens of cytoplasmic proteins are known to be secreted to perform a second function outside of the cell. The secretion system that selects and transports a portion of the pool of a cytoplasmic protein to the outside is unknown.*

prevent PGI's cell signalling function in breast cancer metastasis, but blocking its enzyme function in glycolysis would be deadly to the patient.

On the other hand, understanding all the functions of a moonlighting protein in a pathogen, as well as how it is targeted to different parts of the cell and how different functions are regulated could provide clues to new ways to eliminate pathogens and treat disease. For example, many pathogens secrete moonlighting cytoplasmic enzymes to interact with host tissues and interfere with the host's immune system. Finding out how this secretion mechanism works could lead to a way to prevent those proteins from being secreted and might lead to better treatments of infections (Figure 5). With the increasing serious global health problem of so many bacterial species becoming resistant to current antibiotics, we need new information about these aspects of bacterial life to develop new types of antibiotics to combat them.

5. What's next?

Studies of moonlighting proteins are broadening our understanding of the immense possibilities of what proteins can do and increases our understanding of the complex multilayered protein interaction networks and processes in our bodies. There is still a great deal to learn. Further information about their structures, functions, regulation and roles in disease is needed for developing new ways to combat bacterial infections and finding less toxic treatments for cancer and other diseases. Our knowledge of moonlighting proteins can also be important for future studies in another way. The growing field of synthetic biology includes creating new proteins that can perform new functions and new combinations of functions, for example, for synthesising new materials, delivering drugs into cells, and in bioremediation. How to make these new proteins can be informed by the structures, mechanisms, and regulation of moonlighting proteins that have naturally been modified, reused and repurposed for additional functions.

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