The Decline and Death of The Protein Empire: Molecular Investigations in Ubiquitination Pathway

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It was early 1990s. There were sporadic reports in the newspapers about the greatest boxing legend suffering from a disease which had a fancy name. It struck my imagination as a little kid who had just started learning primary school biology. As a child, I was intrigued both by the man and the disease where you lose motor control of your limbs. I couldn't fathom what that meant for a champion whose greatest strength were his hands which now kept having tremors. The man was Muhammad Ali and in 1984 he was diagnosed with post-traumatic Parkinson's disease attributed to his near-fatal boxing matches in the late 1970s. During that time, the cause and general idea of the disease were sparse but the fact that a world-famous sportsperson was afflicted by it made headlines and curiosity peaked among the public.

Fast forward 2016. I was in the fifth year of my PhD studies when Muhammad Ali died. His was a long and painful battle with Parkinson's. In the last thirty years, the disease has acquired some recognition in general public and a lot has been done for the patients to ease the pain as the disease progresses in later stages since no cure has yet been found. Around 10 million people are living with Parkinson's and the scientific community has a lot of interest in studying the underlying causes of the disease. The battle has only just begun.

Our bodies are remarkably evolved structures, which have an intricate network of organ systems, working towards maintaining the balance necessary for survival. The cells that make-up the tissues, and eventually the organs, have mechanisms to produce, degrade, recycle, and transport various molecules such as proteins, lipds, etc. essential for their holistic functioning. These molecules are responsible

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for catalysing and participating in various chemical reactions which keep turning the wheel of life. Just as physicists study the sub-atomic particles to understand the physical phenomenon in the universe, we biologists, using various techniques, understand how proteins work inside cells. They are crucial to find how cells trigger diseases such as Parkinson's.

One such family of proteins is essential in maintaining the homeostasis inside a cell by degrading non-essential proteins just like a garbage disposal system. The balance between the production and degradation of proteins is highly crucial since the accumulation of non-required proteins hamper the normal cellular pathways. Life on earth evolved ways to utilise minimum energy to produce maximum products and hence not waste unnecessary efforts in producing proteins that are not required. These proteins, which target other proteins for degradation, are called Ubiquitin E3 Ligases. The word 'ubiquitin' derives from the Latin word 'ubique' meaning 'everywhere' as it is found ubiquitously throughout all organisms. Ubiquitin is a small protein which was first discovered in 1975. It found its enormous importance through the work of AvramHershko, Aaron Ciechanover, and Irwin Rose who in the 1980s showed that ubiquitin attached itself to proteins which were meant to be degraded and shuttled them to a barrel-like structure, called proteasome, where the proteins were chopped up and recycled. For their seminal work, the trio received the 2004 Nobel Prize in Chemistry and opened avenues hitherto unknown in the ubiquitination pathway.

Ubiquitination involves a cascade of enzymatic reactions which begins with a large protein called the Ubiquitin Activating Enzyme or E1. It brings the single ubiquitin and positions it onto a second player called the Ubiquitin Conjugating Enzyme or E2. The third player involved in this degradation process is the Ubiquitin E3 Ligase. Finally, the E3, laden with the ubiquitin, approaches a target protein and loads the ubiquitin molecule on top of it. This process happens multiple times and the target proteins have a chain of ubiquitins on top of them. Various E3s have designated E2 partners and their crosstalk determines the specificity of the ubiquitin attachment.

As you can imagine, the specificity of this machinery is near astronomical. To keep the cycle churning efficiently, there are around at least thousand different E3 ligases working to capture degradable targets inside cells. Drug companies had their eyes latched onto these E3 ligases since targeting them for drugs meant eradicating diseases which involved the rogue or anomalous behavior of proteins. It was a haven for pharmaceutical dreams. The holy grail for biologists. Finally, we had begun our journey in understanding mysterious and incurable diseases just like Parkinson's, Huntington's, etc. In 1998, a gene called Parkin was discovered to be the causative agent in autosomal recessive Parkinson's disease. Twenty years later, we now know that Parkin is an E3 ligase which targets specific proteins for degradation but in spite of the crystal structure of Parkin where we can visualise the protein we are miles away from designing any therapeutics against the disease.

Our structural biology and biochemistry-driven laboratory works on a group of E3 ligases in humans called RING E3 ligases. These are enzymes with a domain called RING hence acquiring their particular name. Over the last decade, information has slowly gathered regarding the structural and biochemical map of RING ligases. They follow the basic ubiquitination 3-enzyme cascade as described above. There are two E1s, about 30 E2s, and about 1600 RING E3s encoded by the human genome. E3s target a huge number of substrate proteins downstream of the cascade and

the specificity is tightly controlled by E2-E3 pairs. The enzymes may work on a similar catalytic principle but our studies have shown that the E2-E3 interaction is not universal as it was previously thought to be. Each E3 has its own signature and, unless we establish the biochemistry, simple brute force targeting of enzymes might lead to fruitless attempts at blocking cellular pathways.

We have employed multi-protein complex crystallisation and subsequent X-diffraction of the crystals to determine the structures of E3 and E2. This enabled us to visualise the interacting interfaces of the proteins. On close inspection, we visualised minute yet significant differences in the interactions with already published data. This led us to employ various biophysical and biochemical methods to identify if the differences in structure led to any difference in their enzymatic properties. And indeed replacing single amino acids in not just E3s but also in E2s created massive differences in the enzyme activity and their binding affinities with each other. Engaging isothermal calorimetric titrations, fluorescence anisotropy, circular dichroism, and other biophysical techniques, we elucidated the thermodynamic parameters and structurally dynamic nature of E2:E3 interactions. We found that among the RING E3 ligases there were sub-groups determined by the absence or presence of the particular amino acids at the E2-E3 interfaces noted from our crystal structures. Any mutation of these crucial amino acids led to the abrogation of E3 activity hence proving that even though the cognate E2 partner of E3s may be same, but the subtle changes in the interfaces of the E2-E3 leads to huge differences which were earlier unknown. This was the most exciting and challenging part of our study.

So, not only does our study promotes the inculcation of all available methods to understand enzymes, but to also keep an open mind about proteins being highly dynamic and specialszed molecules even though they might seem to work in a similar manner in a particular cellular pathway. On one hand, crystallography helps us visualise proteins with their partners and helps us see the amino acid architecture, while on the other, it is true that a structural view is just one snapshot of a protein in a particular orientation. Proteins are in constant molecular movement inside cells. If we get a hundred or even more snapshots of a protein pair only then can we equate our findings to a real-world scenario. But it can be very difficult to generate such a large amount of structures and hence using biochemical techniques is crucial in augmenting visualised molecular architecture with the chemical phenomenon. Our studies showed us an insight into the evolutionary selection of proteins based on their requirements inside a cell. A pattern can be formulated as to why certain enzymes retained particular aspects of their structure and others lost them. It all comes back to the nature's beautiful design of utilising the minimum energy pathway.

In a nutshell, progressive steps towards developing drugs in diseases require the concurrent knowledge from both basic and applied sciences. Our work in understanding the basic tenets of the molecular mechanisms of enzymes involved in the protein degradation pathway will build a solid foundation for future experimental design involving specific protein targeting keeping therapeutics in mind. It has been 34 years since Ali was diagnosed with Parkinson's. A lot has been discovered, experimented, and investigated since then but there is a lot to be done to reveal the molecular mysteries inside living cells and it can only be achieved with continuous in-depth analysis of the interacting proteins at the molecular level.