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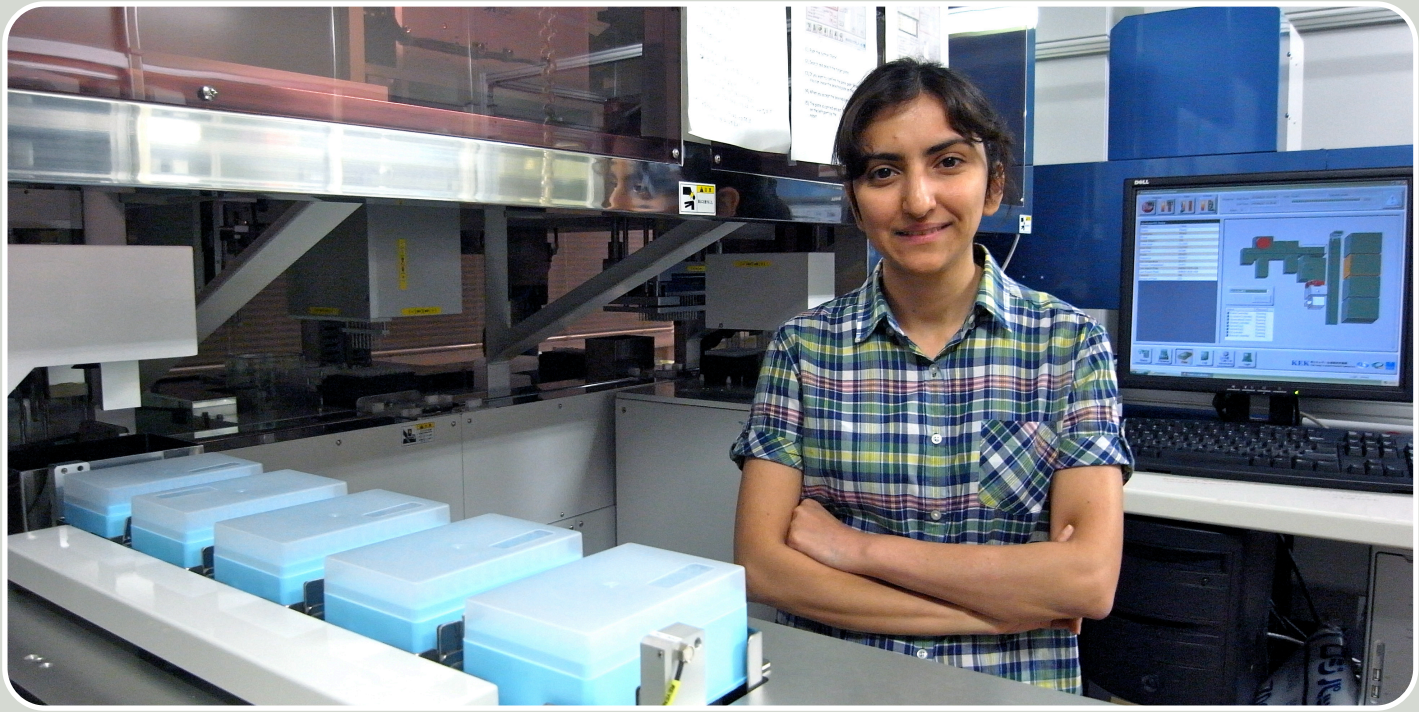
FEATURE STORY



Dr. Simin Rahighi of KEK solved the structure of a protein called the NEMO-ubiquitin complex, unlocking the mechanism of immune response in eukaryotic cells.

Understanding the immune response mechanism with protein crystallography

[Ubiquitin, NEMO, Ectodermal Dysplasia, Drug Design]

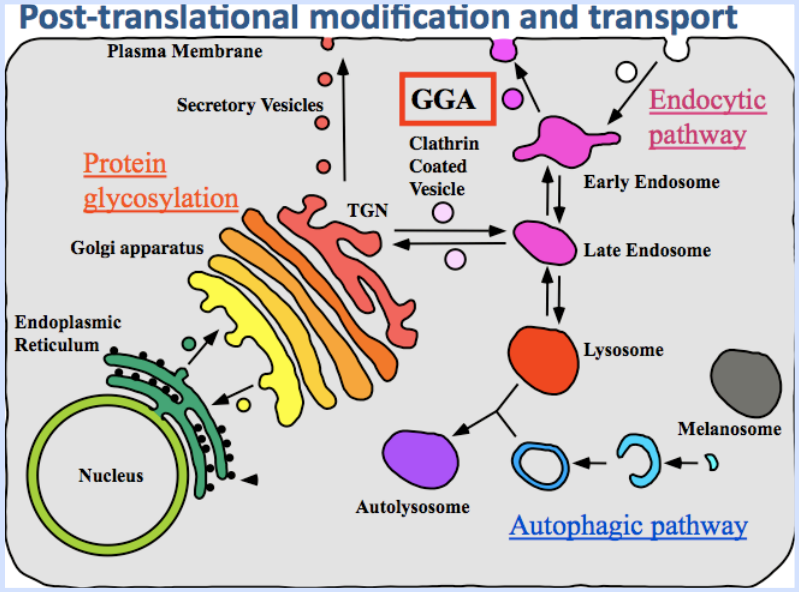
Scientists at KEK Photon Factory are exploring protein lifecycles—the production, modification, and transportation of proteins—using synchrotron radiation X-ray crystallography. Here, read a story about a regulatory protein called ubiquitin binding to a helical protein NEMO, and how a better understanding of the protein will help drug designers to control immune response in our body.

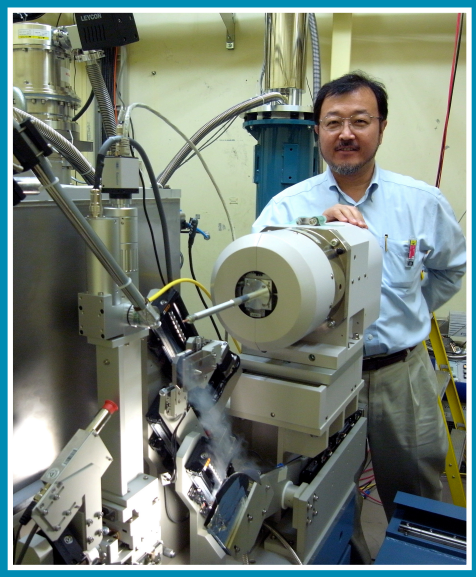
A hundred trillion cells. That is the number of eukaryotic cells in the human body. Each living cell is an enormously elaborate system designed for survival and growth. Each cell houses genetic material, the mechanisms to generate, modify, and transport proteins. Additionally, each cell orchestrates its own metabolism, including cell division, and the lifecycle of proteins. One interesting function of a cell—one which is particularly important to understand if you are a drug designer—is the regulation of responses to external stimuli.

The life of a protein molecule starts with protein synthesis. This is the first stage of the protein lifecycle. Here, genetic information in deoxyribonucleic acid (DNA) is copied to produce a ribonucleic acid (RNA) strand. This process is called transcription. The RNA strand then undergoes post-transcriptional modification, where it becomes messenger RNA (mRNA). The mRNA binds to a large protein complex and RNA, called a ribosome, which decodes the mRNA information and creates a protein. This process is called translation. The synthesized protein generally undergoes further modification with other types of proteins, called modification proteins. This is the process of post-translational modification. When the modification is done, proteins are transported to their proper destinations.

Cells decide the type of proteins to produce depending on their current requirements. In particular, when the cell regulates the immune response to external signals such as viruses, a protein complex called the nuclear factor kappa B, or NF-kappaB, controls the transcription of DNA. NF-kappaB is the key player in regulating the immune response to infectious diseases. The incorrect regulation thereof is linked to tumors, inflammatory

↓After a protein is generated in the process of translation, it undergoes various additional modifications to gain the functionality necessary to do its job. Afterwards, it is transported to its destination.



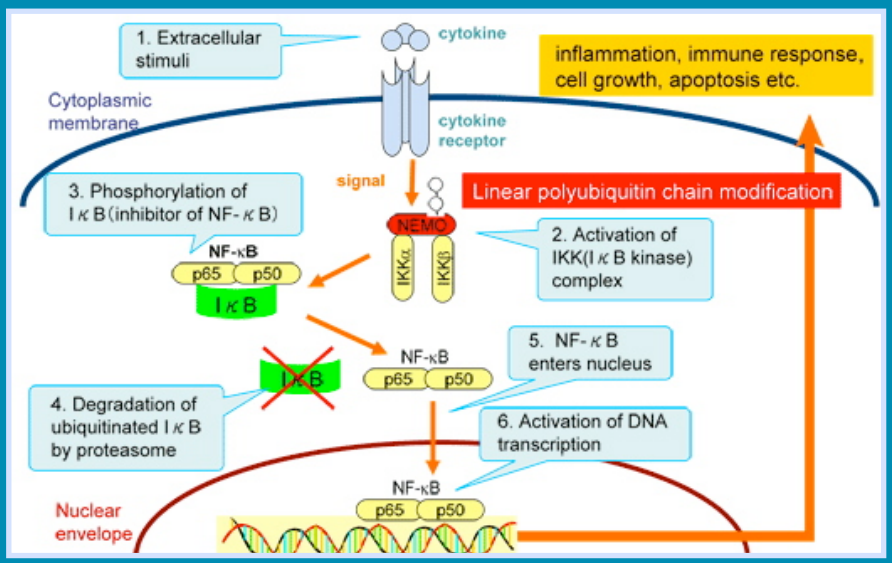


↑Prof. Sohichi Wakatsuki of KEK, the director of both the Photon Factory and the Structural Biology Research Center at KEK, stands behind the newly built X-ray crystallography beamline at KEK's Photon Factory. The beamline started operation in late May 2010.

diseases, and improper immune developments.

The dream of the structural biology scientists at KEK is to understand the lifecycle of proteins using crystallography. According to Prof. Soichi Wakatsuki, the director of both the Photon Factory at KEK and the Structural Biology Research Center at KEK, the job of a structural biologist is to look at structures and try to understand the function of these structures. Understanding the protein modification and transportation process has been the primary goal of an international team of over 30 structural biology laboratories from 8 countries for over ten years.

"After post-translational modification, proteins are released to do their work. The destination of a protein is sometimes written into the sequence of the protein, while other times protein is modified to be dictated its destination," explains Wakatsuki. "The system that coordinates the delivery of proteins is very complex, because not all required information can be written in genes. These are things we would like to understand at the atomic level."



A model of ubiquitin signaling in the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB). It was known that when ubiquitins bond to the NF-kappaB essential modifier (NEMO), the inhibitor kappa B kinases (I-kappaB) are released, activating the NF-kappaB. The NF-kappaB then penetrates deep inside the nucleus, and promotes transcription of DNA related to immune system response.

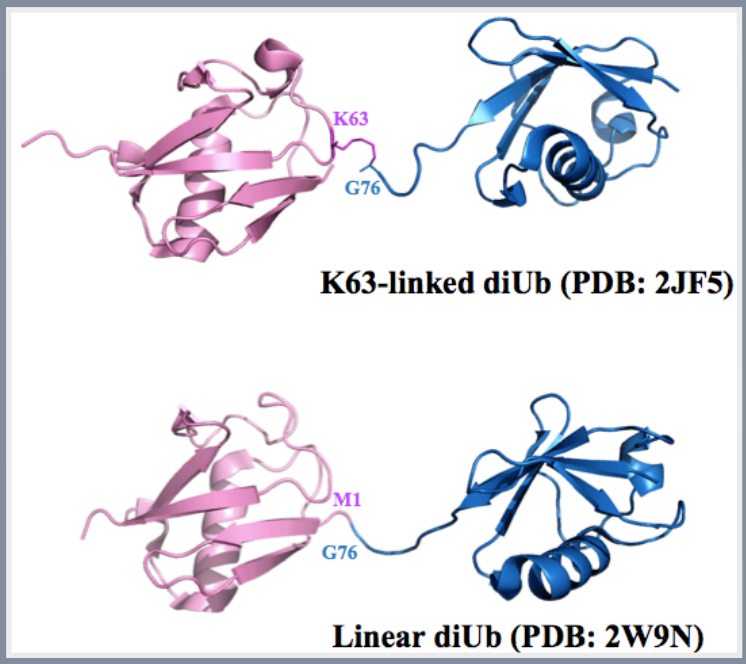
Post-translational modification and the ubiquitin world

In post-translational modification, the cell can add new functions to newly generated proteins. According to Wakatsuki, this modification is analogous to a person putting on makeup, and it can involve many different types of make-up processes. Such processes include structural change, chemical alteration of the amino acids in the protein, the addition of functional groups, or the addition of other proteins.

Depending on the necessary functions, proteins need to make up with different set of modification molecules. For example, in regulation of the NF-kappaB proteins, a modification process called ubiquitination plays a significant role. During ubiquitination, one or more regulatory proteins called ubiquitin bond to the target protein. Ubiquitin, so named because its existence is ubiquitous, is a very simple protein, composed of only 76 amino acids. However, this simple structure gives rise to some complex chemistry, as a ubiquitin molecule has the ability to bond to another ubiquitin molecule in eight different ways.

Until recently, only seven types of ubiquitin-ubiquitin bonding were known. The last amino acid in the ubiquitin molecule, the tail, was known to bind to any of the seven lysines—6th, 11th, 27th, 29th, 33rd, 48th, or 63rd amino acids—in another ubiquitin molecule. Then, in 2008, a group from Osaka University discovered a new type of connection which they called the linear chain. In a linear chain, the head of one ubiquitin molecule bonds with the tail of another.

The number of links in a ubiquitin chain could be, theoretically, any number. With the addition



Ubiquitin is a simple protein, consisting of just 76 amino acids. Seven lysines (marked with the letter K) in the ubiquitin molecule can link to the tail amino acid of another ubiquitin. A K63 linkage (top), and the head amino acid links to the tail of another ubiquitin forming a linear di-ubiquitin (bottom). The linear di-ubiquitin was thought to be functionally similar to the K63 linked di-ubiquitin because two structures are similar.

of the head-to-tail linear linkage, the number of possible combinations of multiple ubiquitins linked quickly grew. Approximately 27,000 papers have already been published on ubiquitin so far. “The ubiquitin world is new, and the horizon is wide open and far away,” says Wakatsuki.

Finding NEMO… with ubiquitin

While exploring the ubiquitin world, Dr. Simin Rahighi of KEK, a postdoc at Wakatsuki’s laboratory, made several major findings, eventually uncovering the biological importance of the linear di-ubiquitin molecule. Through a combination of hard work, and a little bit of chance, Rahighi was able to develop a very clear picture of the NF-kappaB essential modifier (NEMO) binding to a linear di-ubiquitin, in which two ubiquitin molecules are connected by a head-to-tail bond. This structure of the NEMO-di-ubiquitin complex amazed the community and also stirred up a controversy.

The strand of ubiquitin is folded up into a very small volume in a particular way. It happens that, in this folded configuration, the head of the protein is very close to the K63 amino acid (‘K’ stands for lysine, while the number stands for the address of the amino acid). Because of the geometrical proximity, the shape of the head-to-tail linear di-ubiquitin is very similar to that of the K63 linked di-ubiquitin. Since protein function is strongly influenced by protein shape, many people, including Wakatsuki, thought that the biological function of these two di-ubiquitin structures would be very similar.

The shapes may be similar, but the two di-ubiquitins—linear and K63 linked—greatly differed in the difficulty of actually producing them. The K63 lysine linkage, where the tail of one ubiquitin binds to the K63 amino acid of another, is not spontaneous. It does not simply happen when you bring the parts close together. In fact, creating the bond requires complicated chemical manipulations including three different enzymes.

On the other hand, a di-ubiquitin with linear linkage was much simpler to synthesize. The head-to-tail structure could be created through simple genetic engineering. Instead of linking after the protein synthesis, the two DNA sequences of ubiquitin are first connected before the protein synthesis so that it produces a linearly linked di-ubiquitin. “Many years of development in genetic engineering now allow us to freely change DNA sequences,” says Wakatsuki. “In fact, most of proteins used in biological experiments are now produced by genetic engineering.”

The idea now was to take a shortcut to finding the biological function of the K63 linked di-ubiquitin. Instead of trying to produce the difficult K63 linked di-ubiquitin, Wakatsuki and Rahighi thought, why don’t they produce similar shaped linear di-ubiquitin and see how it interacts with other interesting proteins such as NEMO?

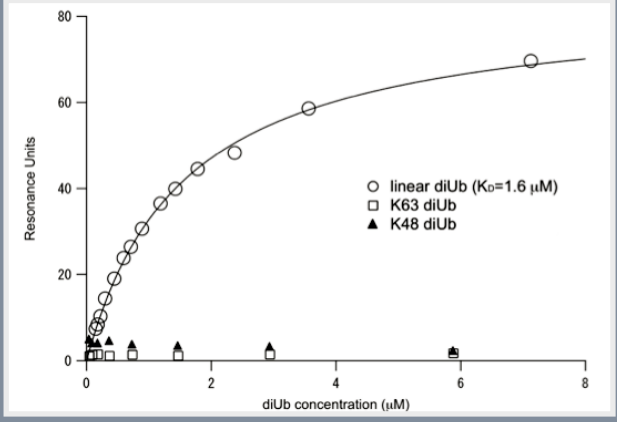
With this new strategy, Rahighi set off to see how a linear di-ubiquitin would interact with NEMO. The first step was a test using a measurement method called surface Plasmon resonance (SPR), which measures the degree of resonance between two molecules. The results surprised everyone. The resonance curves showed that linear di-ubiquitin strongly binds to NEMO, while the K63 linked di-ubiquitin did not.

Crystallizing the ubiquitin-NEMO complex

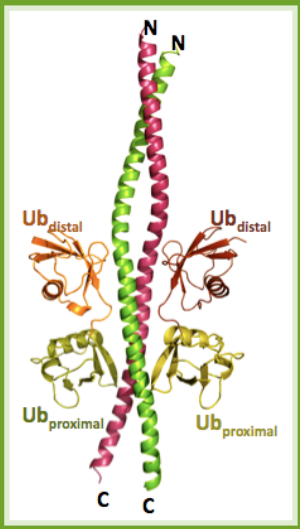
Excited by these results, Rahighi began trying to grow crystals of NEMO in order to discover the structure of NEMO. This was in October 2006. Just six months later, she was able to solve the NEMO structure. Next, she launched into a new project: growing crystals of the NEMO and linear di-diubiquitin complex.

Growing clean, flawless crystals with exact periodic structure is the key to crystallography, and yet it is very difficult with small, dynamic biomolecules. Rahighi tested a hundred different detergent additives, and hundreds of different growing conditions. Eventually, she succeeded, and brought her crystals to the KEK Photon Factory. There, she shot the crystals with X-ray beam, and examined the resulting diffraction patterns. “I was able to get good diffraction data that were supposed to be sufficient for my analyses,” says Rahighi.

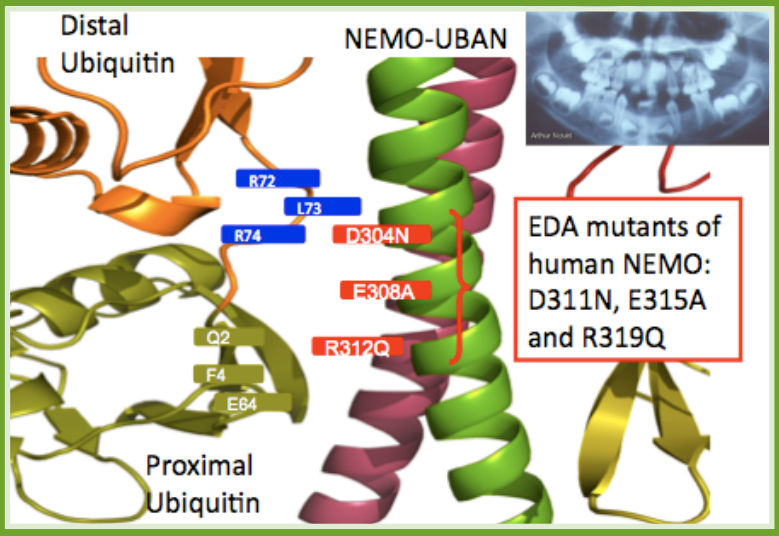
Then, she ran into a problem. Despite the diffracting quality crystals and good data, Rahighi was unable to solve the structure. To solve a structure, one needs two bits of information: the intensity and the phase of the diffracted X-rays. Current detectors can record the intensity, but not the phase. Phase was something scientists needed to infer from some known or produced reference structure. Rahighi tried different reference structures, but



The degree of resonance between NEMO and different types of di-ubiquitin were measured by looking at the reflection of light from the surface of NEMO-ubiquitin crystals. Contrary to everyone’s expectation, the linear di-ubiquitin and the K63 linked di-ubiquitin gave very different results.



Rahighi's work found two linear di-ubiquitins binding to one NEMO dimer (the two central spirals).



The linear di-ubiquitin-NEMO picture can explain the ectodermal dysplasia (EDA). Three residues in EDA patients' NEMO are known to be mutated, and are all involved in bonding to linear di-ubiquitin. The mutation prohibits the ubiquitins from bonding to NEMO.

none worked. The results always had a discontinuous or unexpected electron density. This was the beginning of her yearlong expedition into trials of various phasing methods.

"The approach was basically try-and-error," says Rahighi. "It was very frustrating. I knew I was so close to the goal, yet it was still so far." In her experiment, she tried introducing heavy atoms into protein crystals, got diffractions, but signals from heavy atoms were not sufficient for phasing. She also tried mutating her protein complex in various ways, none of which worked for her until she came across one rather uncommon approach: surface entropy reduction (SER).

SER is an experimental method, in which some of the amino acid residues located at the surface of the protein are mutated to lower the surface entropy. In particular, the residues in NEMO proteins that have long side chains are mutated to shorter ones. This in turn reduces the entropy—randomness—of the system, making it easier to crystallize.

With SER, the structure of the resulting NEMO-ubiquitin complex could be solved, and showed a clear binding between two linearly linked di-ubiquitins and the NEMO molecule. Up to this point, scientists knew that NEMO bind to linear ubiquitin chains much stronger than to K63 linked ubiquitin chains, but did not know why. The crystallography gave a definitive answer to this question.

The most accurate NEMO-ubiquitin structure

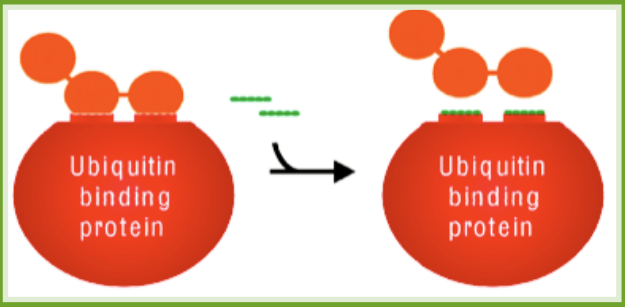
At first, Rahighi's finding was met with skepticism. A model of NEMO-ubiquitin structure had already been published by a group at the Weill Cornell Medical College in the journal Molecular Cell in January 2009. Based on nuclear magnetic resonance (NMR) spectroscopy, they believed the NEMO-ubiquitin structure consisted of a single di-ubiquitin binding to one NEMO dimer.

Their di-ubiquitin-NEMO picture did not agree with Rahighi's crystalline structure in which two di-ubiquitins binding to either side of one NEMO dimer. She did some more research, and found several pieces of medical evidence suggesting that the linear di-ubiquitin picture was the most accurate model of NEMO and ubiquitin bonding. For example, her model could explain the effects of a severe disease called ectodermal dysplasia (EDA). Three residues in EDA patients' NEMO were known to be mutated, and are all involved in binding with linear di-ubiquitin. If any of the three residues are mutated, then binding would not occur. The malfunctioning of the binding of the residues would entirely prevent the activation of NF-kappaB.

Rahighi's results can be applied to drug design. For example, when an immune response in our body is causing damage, as is the case with hay fever, one can suppress the level of response by cutting away the linear ubiquitin chain from NEMO in only the area of inflammation. "In drug design, drug delivery to the target area is extremely important," says Wakatsuki. "We also study genetic modification of protein transport to bring about speedy drug delivery."

"We study basic science, which provides basis for further practical investigations. It is very nice to find something that is useful to our health," says Rahighi. "Crystallography is the most powerful tool we have to study molecules, and I enjoy using it to see biosystems at the atomic level."

At the KEK Photon Factory, a new beamline BL-1A just began operation last month, producing brilliant, low-energy X-ray beam. This enables structural biology scientists to solve the phase problem using anomalous signals from sulfur atoms in natural protein crystals. Rahighi continues to explore the ubiquitin world with the new beamline.



Using the newly discovered NEMO-ubiquitin structure, scientists can design drugs to control the immune response.

Related Link:
Structural Biology Research Center Photon Factory

Related Issue:
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Paper:
Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation.

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