論文の内容の要旨

A screening method for identifying SUMOylated proteins
based on reconstitution of split fluorescence protein fragments
(二分割蛍光タンパク質の再構成に基づく
SUMO 化タンパク質同定のためのスクリーニング法)

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SUMOylation is a reversible post-translational protein modification process, in which a Small Ubiquitin-related Modifier (SUMO) covalently binds to a target protein, forming an isopeptide bond between a C-terminal glycine residue of the SUMO and a lysine residue in the target protein. Several SUMO family members were discovered from eukaryotic cells. Mammalians possess at least 3 SUMO isoforms called SUMO1, SUMO2, and SUMO3. The modification by SUMO isoforms regulates multiple vital cellular processes such as gene expression, chromatin structural changes, and DNA repairs. Thus, SUMOylation is essential for cell sustainability. Due to the important role of SUMO, identifying novel SUMOylated proteins is prospective for elucidating new SUMO-related biological mechanisms. In conventional methods, immunoprecipitation methods followed by MS analysis were used to screen SUMOylated proteins. However, immunoprecipitation methods include a cell-disruption process, which is not appropriate for screening SUMOylated proteins because SUMO is possibly removed from target proteins during cell lysis. Another one is the yeast two-hybrid method, where cell-disruption process is not included. Hoever, SUMO and a target protein should be recruited in the nucleus to verify the interaction. Thus, the proteins that hardly move into the nucleus are not appropriate as targets for investigating SUMOylation. To overcome these limitations, I aimed at establishing a new screening method for mammalian SUMOylated proteins. Using the present method, we aimed to

identify unreported SUMOylated proteins, especially targeted by SUMO2.

In the present screening system, reconstitution of split fluorescence protein fragments was used to detect SUMOylation because the reconstitution technique has advantages of enabling to detect protein-protein interaction under live-cell condition and having no limitation of protein species. A yellow fluorescence protein, Venus, was divided into two non-fluorescent fragments (VN and VC). VN fragment was genetically fused to a SUMO2 protein (VN-SUMO2). VC fragment was genetically fused to a library protein (VC-library), DNA sequences of which were randomly extracted from a mouse cDNA library. If the VC-library protein is modified by the VN-SUMO2 in living cells, the VN and VC fragments come close to each other, resulting in the reconstitution of fluorescent Venus, by which SUMOylation can be detected as fluorescent signal under live-cell condition (Fig.1). To verify SUMOylation of many library protein species, retrovirus harboring various VC-library DNAs were prepared and infected with the NIH3T3 (mouse embryo fibroblast) cells stably expressing VN-SUMO2, resulting in co-expression of each VC-library protein with VN-SUMO2 in each cell. The cells, which emitted fluorescence, were sorted by a fluorescence-activated cell sorter (FACS), the equipment enabling to sort target fluorescent cells according to the fluorescent intensities. Subsequently, the sorted cells were separated into single clones. Then each library cDNA was amplified from the isolated cell clones and the DNA sequences were analyzed. Finally, SUMOylated protein candidates were identified through the above procedure (Fig.2).

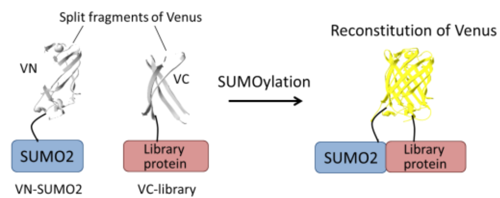

Fig.1 The principle of detecting SUMOylation under a live-cell condition using reconstitution of split fluorescent protein fragments
A yellow fluorescent protein Venus is dissected into two non-fluorescent fragments (VN and VC). Each fragment is fused to SUMO2 and a library protein respectively (VN-SUMO2 and VC-library). When VC-library protein is modified by VN-SUMO2, the two fragments are brought into proximity, resulting in reconstitution of the fluorescent Venus.

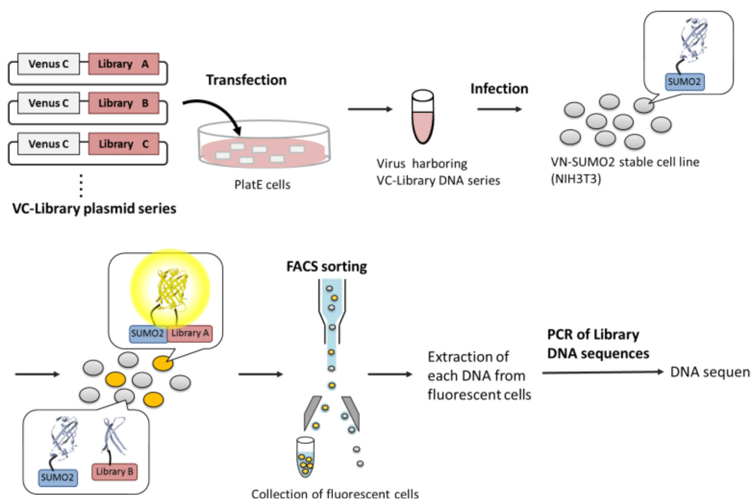

Fig.2 Schematic of screening for mammalian SUMOylated proteins based on reconstitution of split Venus fragments.
Library DNAs are inserted into virus infection vectors with DNA of VC fragment and transfected into PlatE cells. The produced virus harboring VC-library DNAs are added to NIH3T3 cells stably expressing VN-SUMO2. The fluorescent cells harboring reconstituted Venus are sorted by FACS. Each library DNA is extracted from each fluorescent cell and analyzed, by which SUMOylated protein candidates are identified.

By using the present method, I screened SUMOylated protein candidates. When the NIH3T3 cells stably expressing VN-SUMO2 were infected with the retrovirus harboring various VC-library DNAs, some cells showed higher fluorescent intensities than control cells (Fig.3A), which indicated that the reconstitution of fluorescent Venus occurred by SUMOylation in the cells. The fluorescent cells were then sorted by FACS and incubated. The fluorescent intensities of the sorted cells showed successful separation of fluorescent cells from non-fluorescent cells (Fig.3B). Subsequently, the DNA sequence analysis of the sorted fluorescent cells resulted in screening 38 protein species. Of the proteins, Rpl37a and Lmna, which have been reported as modified by SUMO2 in mammalian cells, were included, which suggested that the present screening method enables to detect SUMOylated proteins. This also suggested the possibility that unreported SUMOylated proteins were included among the other 36 protein candidates. To discover unreported SUMOylated proteins, we next verified SUMOylation of the candidate proteins by performing immunoprecipitation and Western Blotting analysis. The candidate proteins and SUMO2 were respectively tagged with V5 and Myc. Then, the V5-tagged candidate proteins were expressed in NIH3T3 cells with or without Myc-SUMO2 co-expression and immunoprecipitated with anti-V5 antibodies. The collected proteins were subjected to gel electrophoresis, in which the proteins were separated according to the size. Subsequently, the proteins were reacted with anti-Myc antibodies and anti-V5 antibodies. If the candidate proteins are modified by Myc-SUMO2, signals from anti-Myc antibodies can be detected. In addition, when the V5-tagged candidate proteins are reacted with anti-V5 antibodies, not only the V5 signals corresponding to the unmodified candidate proteins, but also the upshifted V5 signals corresponding to the SUMOylated candidate proteins can be detected. Several candidate proteins were examined whether SUMOylation occurs or not by the above immunoprecipitation and Western Blotting analysis. Of the candidates examined, I found SUMOylation of “Atac2”, which is known for a histone acetyltransferase (Fig.4). When V5-tagged Atac2 was co-expressed with Myc-SUMO2, the immunoprecipitated proteins with anti-V5 antibodies showed reactivity to anti-Myc antibodies. The Myc signals were detected

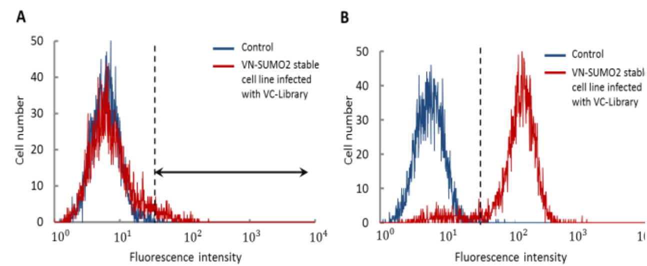

Fig.3. FACS sorting of the fluorescent cells.
FACS analysis of fluorescence intensities of VN-SUMO2 stable cell lines with or without infection VC-library DNAs. (A) Comparison of control cells with the library-DNA-infected cells before FAC sorting. The region shown as an arrow contains the cells with higher fluorescence intensities tha control cells. (B) Comparison of control cells with the library-DNA-infected cells after FACS sortin The fluorescent cells included in the target region were repeatedly incubated and sorted by a FAC four times. The data show the fluorescence intensity of the finally-obtained cells.

around 130 kDa and 150 kDa. In the same size of the 130 kDa Myc signals, V5 signals were detected when the immunoprecipitated proteins were reacted with anti-V5 antibodies, which suggested that Atac2 was SUMOylated. Hence, Atac2 was identified as a novel SUMOylated protein by the present study.

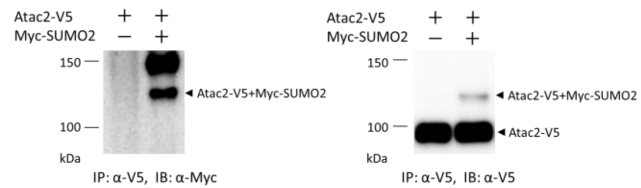

Fig.4 Detection of SUMOylation of Atac2
NIH3T3 cells were transfected with V5-tagged Atac2 in the absence or presence of Myc tagged SUMO2 co-expression, followed by immunoprecipitation with anti-V5 antibodies. The immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western Blotting with anti-Myc antibody (Left figure) and anti-V5 antibody (Right figure).

For characterization of Atac2 SUMOylation, further analyses were also conducted, including a mutation experiment to identify the SUMOylation site, an *in vitro* SUMOylation assay to confirm the SUMOylation site, and several experiments to examine what the 150kDa Myc signals detected in the immunoprecipitation and Western Blotting analysis was originated from.

In conclusion, I established a new screening method for mammalian SUMOylated proteins based on reconstitution of split fluorescence protein fragments. The present screening method has several advantages compared to the conventional screening methods. Unlike the immunoprecipitation-based screening method, the protein-SUMO interaction can be detected under a live-cell condition. In addition, unlike the yeast two-hybrid screening method, any proteins are appropriate as a target for investigating the SUMOylation because the locations where SUMOylation of target proteins occurs are not limited in nucleus. Thus, target proteins can be modified by VN-SUMO2 in the locations where the target proteins are modified by endogenous SUMO-2, which indicates that SUMOylation of target proteins occurs under relatively-natural condition. Besides, combination of the FACS sorting with the reconstitution technique enables fast collection of the fluorescent cells harboring SUMOylated library proteins with reconstituted Venus. Because of the above-mentioned advantages, the present screening method is prospective for discovering novel SUMOylated proteins. Actually, Atac2 was revealed to be a novel mammalian SUMOylated protein by the present method. The SUMOylation function of Atac2 remained to be examined, however further analysis will bring about a new insight into the SUMO-related biological mechanisms.