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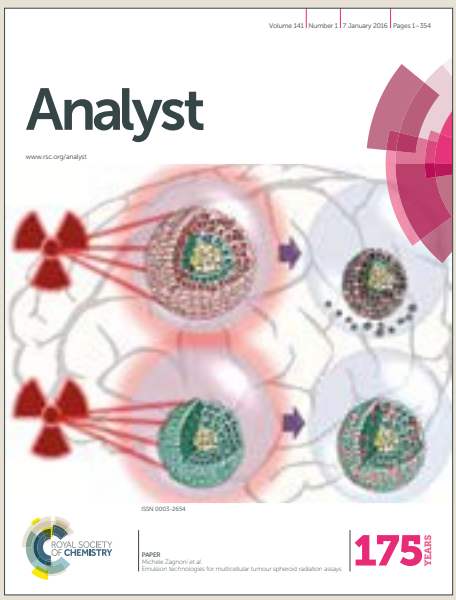

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Fast and sensitive quantification of protein SUMOylation using dual luciferase biosensor

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Herein a convenient strategy was developed to quantify protein SUMOylation. The amount of target proteins and SUMO proteins conjugated to them could be quatified through luciferase-based bioluminance detection and the relative bioluminance was used to evaluate the SUMOylation degree of target proteins.

SUMOylation is a post-translational process which modify target proteins via the covalent linkage of small ubiquitin-related modifier (SUMO) to target proteins[1-3]. When SUMOylation occurs, SUMO protein is cleaved at the C-terminal to expose a diglycine motif by SENP (sentrin/SUMO-specific protease) enzymes[4]. The processed SUMO protein is then transiently passed to ubc9 (ubiquitin-conjugating 9), the only SUMO-conjugating enzyme, for covalently linking to the SUMO binding site on targets[5]. SUMO attached on the targets could induce protein conformation change or influence the interaction between substrates and other cellular proteins[2, 3], and consequently, lead to the regulation of protein function and control of various physiological and pathological processes, including gene transcription[6], DNA repair[7], cell cycle regulation[8], cancer development[9] and Parkinson's disease[10]. Since SUMOylation is a dynamic process which represents cellular responses in different conditions, detection of protein SUMOylation in living cells instead of simple reaction systems such as purified proteins in tubes is quite necessary. Therefore, good approaches for SUMOylation detection and quantification are urgently required for studying and understanding this protein modification.

Several methods have been developed to detect SUMOylation[11]. The most widely used strategy involves detection of the appearance of higher molecular weight bands, which indicate the SUMOylation form of target proteins by Western blot assays[12]. This method is time-consuming, poorly sensitive, and occasionally leads to false negative or inconclusive results for weakly SUMOylated targets[13]. Another approach, Mass spectrometry-based strategy, enables accurate quantification of SUMOylation[12], but the artificial construction of a special proteomic database and complicated data analysis were required by this method[13] and added the utilization difficulty. Recently, fluorescent signal based single molecular detection has also been utilized to determine the SUMOylation level[14, 15]. Using this method, fluorescent molecules covalently linked to functional protein tags fused with SUMO proteins are determined at the single-molecule level. However, these measurements largely rely on total internal reflection fluorescence (TIRF) microscope to ensure the single fluorescent molecule signal is visible, and restricted its application. Therefore, considering the substoichiometric level of SUMOylation[16], a sensitive, quantifiable and easily operated method for SUMOylation detection is highly desired.

Luciferases (Luc) are a class of oxidative enzymes which catalyze luciferin substrate to produce bioluminescence. Several luciferases have been found to show a linear relationship between the enzyme concentrations and the resulting luminance, also, these enzymes do not require post-translational processing for enzymatic activity[17, 18]. These properties make luciferases excellent genetic reporters. Precise quantifications of Luc-fused proteins are readily achieved by measuring the catalyzed luminance with a luminometer. As such, excellent detection sensitivity up to femtogram level can be provided which is much higher than that obtained through Western blot analysis[19]. Furthermore, the detection accuracy can be greatly improved via the combined use of two different luciferases, for example, the firefly (*Photinus pyralis*) luciferase (PLuc, PL), which catalyzes oxidation of beetle luciferin[17] and Renilla (*Renilla reniformis*) luciferases (RLuc, RL), which catalyze luminescent reactions by utilizing the coelenterate luciferin[18]. In this system, one luciferase is applied to report the experimental value, whereas the other luciferase generates a control value to reflect the

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background. The activities of two luciferases can be measured sequentially from different luminescent reactions within the same sample because of the substrate differences. Using this system, the target protein quantity can be accurately reported in terms of the relative activity of two luciferases, thereby minimizing the experimental variability caused by the transfection efficiency, cell viability, cell lysis efficiency, or other sources of variability.

In this study, we report a quantifiable, sensitive, and easily operated method for protein SUMOylation detection based on the dual luciferases reporter system. As shown in Fig. 1, Flag-PL-protein X and HA-RL-SUMO1 fusion proteins were expressed in mammalian cells. In the case of SUMOylation occurs, the RL-SUMO1 covalently link to the Flag-PL-protein X. After immunoprecipitation(IP) of Flag-tagged proteins, only the Flag-tagged target protein and attached RL-SUMO1 remained in the final sample. We demonstrated that the amount of attached SUMO proteins can be quantified by measuring the activity of RLuc fused with SUMO molecules. Meanwhile, the amount of target protein in the same sample can be quantified by measuring the activity of PLuc, and the relative activity of two luciferases (RLuc/PLuc) can effectively quantify the SUMOylation degree of target proteins and significantly improve experimental accuracy.

The GTPase-activating protein RanGAP1, a well-known SUMOylation substrate[20], was used in this work as a model target to validate the effectiveness of our SUMOylation detection strategy. As the first step, plasmids expressing the Flag-PL-RanGAP1 fusion protein and the HA-RL-S1 fusion protein were constructed. Both fusion proteins can successfully express in HEK293T cells and catalyze their corresponding luciferin substrates to produce a high bioluminance (Fig. S1). To verify whether the fused luciferases affect SUMOylation of target proteins, immunoprecipitation assays were performed. HEK293T cells were co-transfected with pFlag-RanGAP1, pMyc-Ubc9 and pHA-RL-S1. At 30h post-transfection, cells were harvested and immunoprecipitated according to regular SUMOylation assay protocol. RanGAP1-RL-S1 band which is about 120kD can be detected using both anti-SUMO1 antibody and anti-HA antibody in the precipitated sample (Fig. 2A, Lane 3), indicating that the fused RLuc on SUMO1 did not affect SUMOylation of target protein. Similarly, Both PL-RanGAP1-S1 band which is about 145kD (Fig. S2, Lane 2) and PL-RanGAP1-RL-S1 band which is about 175kD (Fig. S2, Lane 3) can be detected in the precipitated samples, implying that the fused PLuc on RanGAP1 protein did not interfere with its SUMOylation. All these results above make our Luc-based strategy feasible.

Then the Luc-based method was performed. To minimize the influence of background luminescence, Flag-PL and HA-RL were used as negative controls. Four groups of cells were transfected with pFlag-PL + pHA-RL, pFlag-PL + pHA-RL-S1, pFlag-PL-RanGAP1 + pHA-RL, and pFlag-PL-RanGAP1 + pHA-RL-S1 respectively (all the groups were transfected with pMyc-Ubc9). 30h post-transfection, cells were harvested and immunoprecipitated with anti-Flag antibody and eluted by Flag peptide. Then the luminescence produced by both PLuc and RLuc of each group were measured. PLuc activity reported the protein amount of precipitated Flag-PL or Flag-PL-RanGAP1. RLuc activity reported protein amount of HA-RL or HA-RL-S1 covalently linked to Flag-tagged proteins. To eliminate the deviation caused by the expression level differences of target protein in each group, the relative luciferase activity (Rel.Luc.Act) of the two luciferases (RLuc/PLuc) was calculated (Fig. 2B). Rel.Luc.Act of four groups were 10.848(±0.425), 611.164(±47.437), 7.476(±0.677) and 17068.840(±550.868). The value of the group pFlag-PL-RanGAP1+pHA-RL-S1 is much higher, and the other three control groups also showed low signals. Considering that HA-RL has no interaction with either Flag-RanGAP1 or Flag-PL-RanGAP1(Fig. 2A, Lane 1 and S2, Lane 1), these signals maybe caused by background luminance. Thus, the Rel.Luc.Act of groups transfected with pHA-RL-S1 were normalized to the Rel.Luc.Act of the group transfected with pHA-RL to get a final value to indicate the degree of SOMOylation of the target proteins. As shown in Fig. 2C, the control protein Flag-PL had a final value of 56.424 (±5.470) and was set as baseline. The final value of the experimental protein Flag-PL-RanGAP1 protein was 2293.647(±236.792), which is much higher than that of Flag-PL, thereby indicating SUMOylation of RanGAP1 occurred.

Protein SUMOylation can be rapidly reversed by some de-SUMOylating enzymes such us SUMO specific proteases (SENPs)[21]. N-Ethylmaleimide (NEM) is an irreversible inhibitor of all cysteine peptidases, including SENPs[22]. Therefore, NEM is usually used in SUMOylation detection assays to block de-SUMOylation. To further assess our SUMOylation detection method, we evaluated whether de-SUMOylation could be detected by our system when NEM was not used. In Western blot assays, the SUMOylation form of both RanGAP1 and PL-RanGAP1 is hardly observed when NEM was not used to block de-SUMOylation (Fig. 3A and S3). As shown in Fig. 3B, using our Luc-based detection method as previously described, we deter-mined the SUMOylation degree of RanGAP1 without treatment of NEM to be 246.021 (three repeats). When treated with NEM, the SUMOylation degree of RanGAP1 rise to 2154.154 (three repeats), thereby indicating that the de-SUMOylation process was inhibited.

SUMO protein is synthesized as inactive precursors initially and then cleaved by protease to expose their COOH-terminal diglycine motif[4]. Then, the activated SUMO protein is transferred to the SUMO conjugating enzyme Ubc9 which can transfer SUMO protein to the target proteins, making Ubc9 highly required for an efficient SUMOylation[5]. In the detection experiment via Western blot analysis, both RanGAP1-S1 band and PL-RanGAP1-RL-S1 band are much weaker without the ex-ogenous Ubc9 (Fig. 3C and S4). As shown in Fig. 3D, using our Luc-based detection method, we determined that the SUMOylation degree of RanGAP1 with and without the exogenous Ubc9 are 2321.424 and 304.016 (three repeats), which is consist with the results of Western blot and previously report, further confirming the feasibility of our approach.

Sensitively detecting the changes of SUMOylation degree of target protein under different conditions is the key technology for researching its regulation and molecule mechanisms. To

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evaluate whether the changes of SUMOylation degree could be sensitively detected by the proposed method, we detected the dose effects of co-transfected SUMO1 proteins on the SUMOylation of RanGAP1. In the detection experiment via Western blot analysis, the dose effect of both SUMO1 and RL-S1 were confirmed (Fig. 4A and S5). Subsequently, using our strategy, from all three replicates, the average SUMOylation degrees of RanGAP1 co-transfected with 0.1, 0.2, and 0.4 μg of HA-RL or HA-RL-S1 were calculated to be 434.436, 1126.095, and 2254.485, respectively (Fig. 4B), indicating our strategy can sensitively quantify the changes of SUMOylation degree under different conditions, thus, can be used for research on the regulation and molecular mechanism of protein SUMOylation.

The differences of expression levels among different proteins make it complicated to compare their SUMOylation degree. The regular method is scanning the gray-level of non-SUMOylated band and SUMOylated band of each protein after Western blot assay, which is time consuming and insensitive. To verify whether our Luc-based strategy can quantify the SUMOylation level of different proteins, the tumor suppressor p53[23], another well-known SUMOylation substrate was employed. In the experiment via Western blot analysis, both p53-S1 band and PL-p53-RL-S1 band were extremely weak, even difficult to observe (Fig. 4C and S6), indicating low sensitivity of Western blot detection strategy, also making it hard to scan and quantify. As shown in Fig. 4D, using our Luc-based SUMOylation detection method, we determined that the SUMOylation degree of the negative control PL, p53 and RanGAP1 was 46.496, 387.880 and 2142.277 (three repeats). These quantitative results enable the comparative analysis of SUMOylation degree of different proteins, and also prove that our strategy could avoid false negative or inconclusive results for weakly SUMOylated targets effectively.

All of above results demonstrate that our Luc-based strategy can sensitively characterize the degree of SUMOylation in a quantitative manner. The luciferase assay is extremely sensitive, and provides a detection sensitivity of 10^{-16} g. Therefore, much smaller amounts of samples and experimental materials are required compared with that used in conventional Western blot assays. Besides, the detection of SUMOylation bands takes days to achieve non-quantitative results when Western blot analysis is used. By contrast, luciferase assay can be accomplished within minutes, and the relative luciferase activity, which represented the average SUMOylation degree of target proteins, exhibited good reproducibility. The final value of SUMOylation degrees reflects the relative amount of target proteins and attached SUMO proteins, thereby minimizing the experimental variability caused by differences in the target protein amount, cell viability, transfection efficiency, or other extraneous influences. Thus, the proposed method provided higher accuracy.

However, there are still some issues which should be taken into consideration when using this technique. The proposed method requires plasmids transfection for over-expression of luciferase-fused SUMO-1 and target proteins. Subsequently, it cannot be used for detection of endogenous SUMOylation. Besides, potential contamination caused by non-covalently binding SUMO-1 or endogenous SUMOylated proteins on targets is also noteworthy. Purification of SUMOylated protein through IP is a critical step in our detection process, as well as in other method studies[14]. Since IP is usually performed in non-denaturing buffer, it's difficult to remove non-covalently contaminants completely. It has been reported that protein-protein interaction is usually ionic strength and PH dependent.[24, 25] Hash condition such as low PH, high salt concentration and denaturing buffer may be helpful to prevent non-covalent bindings. However, IP of target proteins by anti-Flag antibody is also a non-covalent affinity process, and hash conditions would weaken the interaction between antibody and targets and affect the purification of SUMOylated proteins. Besides, hash conditions may also lead to loss of luciferase activity, which is essential for following detection. The use and development of good peptide tag and its specific antibody with high affinity, luciferase with high stability and appropriate condition which could remove non-covalently bindings to target but do not affect IP purification or luciferase activity would be great helpful to solve this problem, but still need to be investigated. However, our technique works well on SUMO-1-modified proteins such as RanGAP1, and the results of our method correlated well with traditional WB assay, indicating that potentially existed non-covalently bindings did not obviously affect the detection results. Comparing with Western blot assay, our technique shows great advantages in sensitivity, quantification and convenience, but also limitations in distinguishing potentially non-covalently binding interactions and mixed SUMO chains. We believed that our technique could be complementary to WB and the combinational use of our technique and WB would facilitate SUMOylation research.

In summary, we developed a novel strategy for quantifying the degree of protein SUMOylation based on a dual luciferase reporter system. This method is highly sensitive, easily operated and accurately quantifiable by eliminating the influence caused by protein expression levels, transfection efficiency and other sources of variability. In theory, this method could be expanded to any type of ubiquitin-like modification, including ubiquitination[26] and NEDDylation[27] via genetic fusion of the modifiers with luciferase. Therefore, this strategy has the potential to be a powerful tool to investigate and understand the roles and molecular mechanisms of protein modifications in physiological processes and human disease.

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Notes and references

1. F. Golebiowski, I. Matic, M. H. Tatham, C. Cole, Y. L. Yin, A. Nakamura, J. Cox, G. J. Barton, M. Mann and R. T. Hay, *Sci Signal*, 2009, **2**.
2. E. S. Johnson, *Annual review of biochemistry*, 2004, **73**, 355-382.

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DOI: 10.1039/C6AN02479C

3. S. Creton and S. Jentsch, *Cell*, 2010, **143**, 848-U329.
4. R. T. Hay, *Trends in cell biology*, 2007, **17**, 370-376.
5. E. S. Johnson and G. Blobel, *Journal of Biological Chemistry*, 1997, **272**, 26799-26802.
6. A. Verger, J. Perdomo and M. Crossley, *Embo Rep*, 2003, **4**, 137-142.
7. S. Bergink and S. Jentsch, *Nature*, 2009, **458**, 461-467.
8. X. D. Zhang, J. Goeres, H. Zhang, T. J. Yen, A. C. G. Porter and M. J. Matunis, *Mol Cell*, 2008, **29**, 729-741.
9. K. Il Kim and S. H. Baek, *Mol Cells*, 2006, **22**, 247-253.
10. K. D. Sarge and O. K. Park-Sarge, *Trends Biochem Sci*, 2009, **34**, 200-205.
11. R. S. Hilgarth and K. D. Sarge, *Methods in molecular biology*, 2005, **301**, 329-338.
12. M. H. Tatham, E. Jaffray, O. A. Vaughan, J. M. P. Desterro, C. H. Botting, J. H. Naismith and R. T. Hay, *Journal of Biological Chemistry*, 2001, **276**, 35368-35374.
13. I. Matic and R. T. Hay, *Methods in molecular biology*, 2012, **832**, 239-247.
14. Y. Yang and C. Y. Zhang, *Angew Chem Int Edit*, 2013, **52**, 691-694.
15. Y. Yang and C. Y. Zhang, *Analytical chemistry*, 2014, **86**, 967-972.
16. R. T. Hay, *Mol Cell*, 2005, **18**, 1-12.
17. J. R. Dewet, K. V. Wood, D. R. Helinski and M. Deluca, *Proceedings of the National Academy of Sciences of the United States of America*, 1985, **82**, 7870-7873.
18. J. C. Matthews, K. Hori and M. J. Cormier, *Biochemistry-Us*, 1977, **16**, 85-91.
19. T. M. Williams, J. E. Burlein, S. Ogden, L. J. Kricka and J. A. Kant, *Anal Biochem*, 1989, **176**, 28-32.
20. J. Joseph, S. H. Tan, T. S. Karpova, J. G. McNally and M. Dasso, *J Cell Biol*, 2002, **156**, 595-602.
21. E. T. H. Yeh, *Journal of Biological Chemistry*, 2009, **284**, 8223-8227.
22. J. D. Gregory, *Journal of the American Chemical Society*, 1955, **77**, 3922-3923.
23. A. Jakobs, J. Koehnke, F. Himstedt, M. Funk, B. Korn, M. Gaestel and R. Niedenthal, *Nature methods*, 2007, **4**, 245-250.
24. J. H. Jensen, *Curr Pharm Biotechno*, 2008, **9**, 96-102.
25. K. Tsumoto, D. Ejima, A. M. Senczuk, Y. Kita and T. Arakawa, *J Pharm Sci-Us*, 2007, **96**, 1677-1690.
26. J. Herrmann, L. O. Lerman and A. Lerman, *Circ Res*, 2007, **100**, 1276-1291.
27. J. Jones, K. Wu, Y. Y. Yang, C. Guerrero, N. Nillegoda, Z. Q. Pan and L. Huang, *J Proteome Res*, 2008, **7**, 1274-1287.

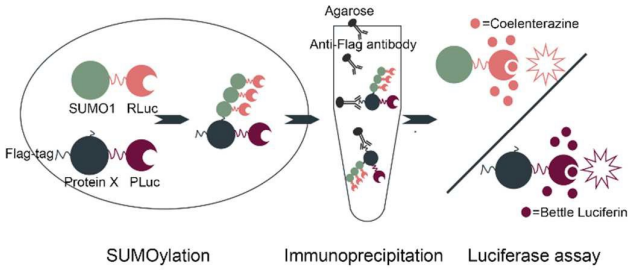

Fig. 1 Working principle of the luciferase-based SUMOylation detection strategy. Flag-PLuc-protein X and HA-RLuc-SUMO1 were expressed in mammalian cells to allow SUMOylation. After isolation of Flag-tagged proteins through immunoprecipitation, PLuc and RLuc activities were determined to report the amount of protein X and the amount of RLuc-SUMO1 attached on protein X respectively. The relative activity of the two luciferases was used to evaluate the SUMOylation degree of protein X.

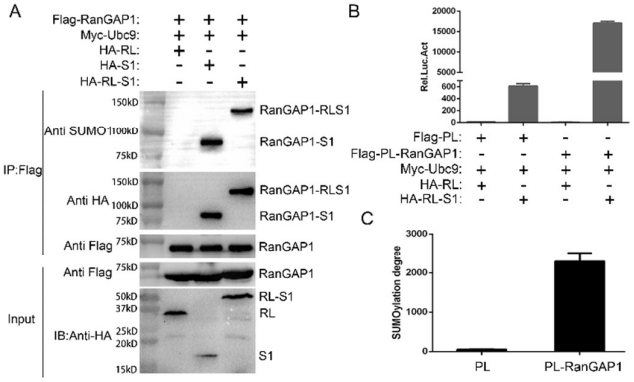

Fig. 2 Detection of the SUMOylation of RanGAP1 via Western blotting analysis and luciferase analysis. (A) Detection of the SUMOylation of Flag-RanGAP1 with HA-RL, HA-S1 and HA-RL-S1, respectively, by Western blotting assay. (B, C) Detection and quantification of the SUMOylation of RanGAP1 protein by luciferase assay. Lysates of cells co-transfected with different plasmids were treated as previously described, and the luminance produced by PLuc and RLuc was measured. (B) Relative activities of the RLuc and PLuc reporters. (C) SUMOylation degrees of Flag-PL and Flag-PL-RanGAP1 were calculated from figure B. Error bars show the standard deviation of three experiments.

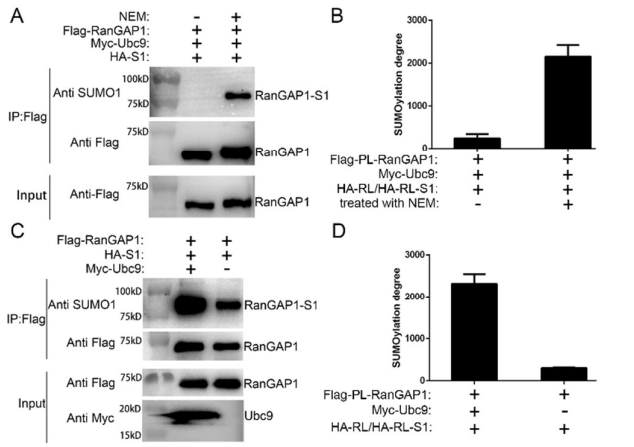

Fig. 3 Detection of the SUMOylation of RanGAP1 protein with or without NEM treatment and exogenous Ubc9 using Western blotting analysis and luciferase reporter system. (A) Detection of the SUMOylation of Flag-RanGAP1 by HA-S1 treated with or without NEM via Western blotting analysis. (B) Quantification of the relative degrees of SUMOylation Flag-PL-RanGAP1 with or without NEM treatment by luciferase-based strategy. (C) Detection of the SUMOylation of Flag-RanGAP1 by HA-S1 co-transfected with or without pMyc-Ubc9 via Western blotting analysis. (D) Quantification of the relative degrees of SUMOylation Flag-PL-RanGAP1 co-transfected with or without pMyc-Ubc9 by luciferase-based strategy. Error bars show the standard deviation of three experiments.

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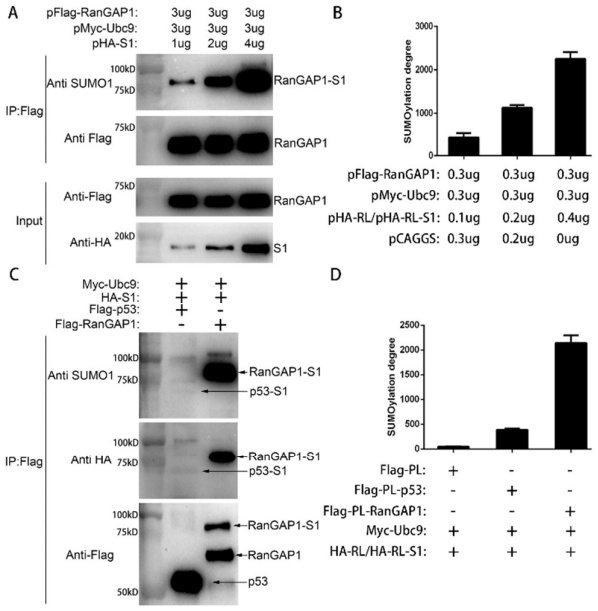

Fig. 4 Detecting dose effects of co-transfected SUMO1 proteins on the SUMOylation of RanGAP1 and omparative analysis of the SUMOylation of p53 and RanGAP1 protein via Western blot analysis and luciferase analysis. (A) Detection of the SUMOylation of Flag-RanGAP1 co-transfected with 1, 2 and 4 μg of pHA-S1 by Western blot assay. (B) Quantification of the relative degrees of SUMOylation of RanGAP1 co-transfected with 0.1, 0.2 and 0.4 μg of pHA-RL or pHA-RL-S1 by luciferase-based strategy. Error bars show the standard deviation of three experiments. (C) Detection of the SUMOylation of Flag-p53 and Flag-RanGAP1 by HA-S1 via Western blot analysis. (D) Quantification of the relative SUMOylation degree of PL, p53 and RanGAP1 by luciferase-based strategy. Error bars show the standard deviation of three experiments.