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Structure-Based Traceless Specific Fluorescence Labeling of Smoothened Receptor†

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c8obxxxxxd

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ABSTRACT: Smoothened receptor (SMO) mediates Hedgehog (Hh) signaling pathway and plays a vital role in embryonic development and tumorigenesis. Visualization of SMO bears the potential to prompt new insights into its enigmatic mechanisms and associated disease pathogenesis. Based on recent progress in structural studies of SMO, we designed and characterized a group of affinity probes facilitating turn-on fluorescence labeling SMO at the ε-amine of K395. These chemical probes were derived from a potent SMO antagonist skeleton by conjugation of a small non-fluorescent unit *O*-nitrobenzoxadiazole (*O*-NBD). In this context, the optimal probes were evolved to be capable of efficiently and selectively lighting up SMO regardless of whether it is in micelles or in native membranes. More importantly, the resulting labeled SMO only bears a very small fluorophore and allows for recovery of unoccupied pocket by dissociation of the residual ligand module. These advantages should promise the probe to serve as a potential tool for monitoring SMO trafficking, understanding Hh activation mechanisms, and even diagnosis of tumorigenesis in the future.

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Introduction

G protein-coupled receptors (GPCRs) constitute the largest superfamily of membrane proteins that mediate transmembrane transduction of extracellular signals, including endogenous chemical ligands and exogenous stimuli.[1] Smoothened receptor (SMO) is a Frizzled (class F) GPCR that regulates the physiologically important Hedgehog (Hh) signaling pathway.[2-3] This pathway exhibits high-level activity in embryonic development of tissues and organs while maintaining only low-level activity in adult tissues.[4-5] Dysregulation of the pathway, usually constitutive activation by oncogenetic mutation, results in several human cancers, including basal cell carcinoma and medulloblastoma.[6-8] Thus, the SMO has been an intensively pursued target for anti-tumor agent developments.[9-12] Since 2012, two drugs, vismodegib (GDC-0449) and Sonidegib (LDE225), have been marketed.[13-14] Research on SMO has witnessed tremendous progress in structural studies.[15] To date several high resolution structures have emerged of 7-transmebrane domain (TMD) [16-17] and extracellular domain (ECD)[18-19] SMO as well as the very recent two-domain structures.[20-21] Nevertheless, many elements of SMO-related biology are not yet fully answered[5], highlighting the urgency for development of new research tools and alternative biophysical methodologies.

Fluorescence imaging has revolutionized to be a powerful approach to investigate biological processes virtually under the microscope.[22] Fluorescence microscopy of particular protein is highly dependent on precision introduction of fluorophores that minimally perturb the receptor function. Generally, protein labeling has been achieved through various well-developed methods.[23-24] Genetically encoded fluorescence tag is one of most prevailing and straightforward strategy to yield fluorescence labeling proteins. However, self-fluorescence fusion proteins and other chemoenzymatic tag strategy series, are based on the introduction of a relatively large fragment

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that are likely associated with unpredictable interference.[25-26] Besides, antibody- or nanobody-based technology suffers from unreliable accessibility of high-quality sources. Though the incorporation of unnatural amino acid (uaa) exemplifies the size advantage showing great potency in GPCR research, it requires expert manipulation and engineered expression systems.[27] In contrast, small molecule-based strategies can offer several advantages of which the most important are low-cost and the ability to readily label endogenous proteins without engineering.[28-29]

Fluorescent ligands, composed of ligand and fluorophore, have often been used in the imaging of protein-ligand interaction and ligand evaluation.[28, 30] To date, the most promising fluorescent probe for SMO is CP-bodipy, which is derived from the naturally occurring SMO ligand cyclopamine (CP).[31] CP-bodipy has been widely used in the discovery of drug-like agonists and antagonists.[32] However, CP was recently found to act as a dual-role ligand since it can bind to two domains as either an agonist or antagonist[19]; a characteristic that had confused our understanding of SMO biology. Here, in contrast to generally applied chemical probes, we turned to a ligand-directed grafting strategy to generate unoccupied fluorescent SMO, in which a fluorescent group could be specifically transferred to the receptor[33] whose function will be recovered. Presumably, this labeling strategy with less interference would allow investigations of more complex SMO biology.

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Results & discussion

Design of SMO ligand-based *O*-NBD conjugated probes. We employed a ligand-directed grafting strategy to specific labeling of SMO. Generally, the affinity labeling probe comprises two fragments, one for the directing ligand that can recognize SMO precisely and the other for the transferred labeling group (Figure 1A). With careful investigation of the TMD ligand binding modes in X-ray crystallography (Figures S1 and S2), we hypothesized that residue K395 could be a potential modification site. The modification of this seemingly unsettled residue would presumably have less interference on TMD ligand binding. Importantly, K395, which locates in the outer edge of the binding pocket, also exhibits close spatial proximity to the TMD ligands.[21] Particularly, the distance was found to be within 4 Å between the nucleophilic ε-amine of K395 and the closest point in the corresponding co-crystallized ligand, including 4-fluorine in LY2940680 (Figure 1B), methyl in cyclopamine, hydroxyl group in AntaXV, pyridine nitrogen in SAG-1.5, and the nitro group in the TC114 (Figure S2).

To accommodate the amine chemistry of the selected lysine, we drew on the successful experience of *O*-NBD fragment to achieve fluorescence transmission.[34] As expected, *O*-NBD acted as a versatile 'turn-on' florescent widget for the S_NAr reaction with ε-amine of lysine under physiological conditions. Applied here is the common skeleton of LY2940680 and TC114 as the starting anchor fragment to prompt the specific transferring reaction of *O*-NBD. In our previous efforts in the digestion of the modular contribution of LY2940680 and in the development of optimal co-crystallized ligand TC114, we found that the substituents in the head benzoic fragment are highly tolerated. Initially, a trifluoromethyl group was removed in the initial design so as to simplify the synthesis (Figure 1C).[21, 35]

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Evolution of probes for rapid labeling of SMO. With the chosen ligand and labeling group, we set out to measure the linker. To this end, a range of linker lengths of one to five atoms was considered in the possible reaction orientation between a swinging lysine and the reactive *O*-NBD group. The linker was pre-installed in the benzoic moiety that could be integrated in a convergent synthetic route. Our synthesis commenced by the coupling of the secondary amine with hydroxyl-tethered benzoic acids, followed by the *O*-arylation with F-NBD in the presence of triethylamine and 4-dimethylaminopyridine at room temperature to give corresponding *O*-NBD conjugated probes (Scheme S1 and S2).

Labeling with designed probes was performed on detergent-solubilized SMO and monitored by the fluorescence increase (λ_{ex} = 488 nm; λ_{em} = 530 nm). The reaction was set in the physiological buffer (25 mM HEPES, pH 7.4, 25 °C) with 1:1 ratio of labeling compounds and protein (2.5 μM each). To determine the corresponding labeling rate, we performed both *in situ* fluorescence determinations using a microplate fluorescence reader and fluorescent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Under these conditions, we observed that probe **3** with a 3-atom linker reacted fastest, closely followed by probe **2** with 2-atom linker (Figures 2C, 2D and S3). According to our previous work, a phenyl ether analog would be more potent than benzyl ether.[35] Furthermore, phenol generated from probe **1** after the S_NAr reaction, would be a seemingly much better leaving group than alcohols from other probes even though the reaction of probe **1** still runs much slower, probably due to the steric hindrance of the crowded di-aryl ether that blocks the nucleophilic attack from K395. Probes **4** and **5** with longer linkers dramatically slowed the reaction, which correlated with the greater separation of the desired reaction partners. Overall, we observed a clear relationship between the linker lengths and the labeling efficiencies.

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To further accelerate the labeling process, we modified the directing anchor fragment of the probe. Probe **6** was built by re-introducing the trifluomethyl group to the benzoyl head of probe **2**. Meanwhile, given the important role of bioisosteres in drug evolution, we designed probes **7**-**10** by introducing nitrogen in the benzoic and phthalazine moiety (Scheme S1-S3).[36] The efficiency of probes **6-10** were evaluated as above (Figures 2C, 2E and S4). Contrary to the expectation that the addition of a trifluoromethyl group to the benzoyl would dramatically enhance the antagonism of SMO, an even lower labeling rate was observed for probe **6**. This contradiction reflected the inconsistency of ligand efficacy and labeling efficiency. Similarly, we observed that nitrogen introduction to the probe generally improved the labeling reaction, though their corresponding precursors showed deteriorated potency in luciferase reporter assay (Figure S5). As shown, introducing a single N atom to 3-position of the benzoyl (**7**) or to 4-position of the phthalazine (**9**) notably increased the labeling rate compared to probe **2**. A further replacement of phenyl in probe **9** by 3-pyridyl, generated probe **10** with a slight improvement. In contrast, replacing 3-pyridyl in **7** by a pyrazinyl group (**8**) largely decreased the labeling rate.

Confirmation of specificity of SMO labeling by probe 9. Most GPCRs are known for their low expression and susceptibility to an unnatural membrane environment. A fusion partner is usually required to improve both expression and stability. Here we applied the construct that has successfully yielded high resolution crystal structure of two-domain SMO, co-bound with TC114.[21] In this construct, a flavodoxin (FLA, MW 16 KD) fusion protein added 4 extra lysines in the intracellular loop 3 (ICL3) so that there are 19 lysines in total, making it even more challenging to specifically target K395 while keeping others unlabeled.

To determine the labeling specificity, we designed two K395 mutant constructs (K395A and K395R). All the SMO constructs, together with adenosine A2A receptor (another GPCR that does

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not specifically bind to SMO ligands such as LY2940680), were treated with probe **9**. The only bright band in the fluorescent SDS-PAGE corresponded to the original SMO, while all others were blank, indicating the specific labeling on K395 (Figures 3A, 3B, S6 and S7). Not surprisingly, pre-treating SMO with 10 equivalents of TC114 significantly obstructed the labeling process of probe **9** (Figures 3C and S8). Moreover, digestion-MS/MS analysis also confirmed that the NBD modified site was indeed K395 (Figure 3D). In addition, probes **2**, **6**, **7**, **8**, **10** underwent selective labeling on K395 with different efficiencies as well (Figure S9).

Specifically labeling SMO on membrane using probe 9 and 10. To test the feasibility of probe **9** and **10** to light up SMO in native membrane environments, an on-membrane labeling experiment was then carried out. The membrane with over-expressed SMO was treated with 0.1, 1, and 10 μM of probe**s**, followed by an SDS-PAGE analysis (Figure 4). The fluorescence results showed that both probe **9** and **10** could selectively label SMO at the concentration of 1 μM and no other protein bands were clearly observed even at higher concentration. However, we also observed the side reaction between the probe and the membrane lipid with primary amine. This might be ascribed to the high hydrophobicity (ClogP ~ 5) and the linear shape (similar to cholesterol) of the probe which makes it highly detained in the lipid part. To this end, the two probes might not be applicable to target SMO in live cell. The on-membrane-labeled SMO protein was later purified and analyzed by HPLC with size exclusive columns and UV/fluorescence detectors (Figures S10 and S11), providing another operation to obtain the purified fluorescence labeling SMO protein.

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Conclusion

In summary, we have developed a series of turn-on probes for site selective labeling on SMO. Guided by rich crystal structures of SMO, the probe was designed based on the interaction between the target residue and ligand. The labeling was enhanced by carefully regulating properties of the linker and the anchor ligand. The strategy has generated labeling probes with high-selectivity and convenient wash-free determination. This method worked well not only with purified SMO protein but also for on-membrane labeling. As fluorescence group transferring reagents, the ligand moieties of these probes were able to dissociate or be replaced by other SMO ligands, providing the opportunity for further treatment of the SMO receptor. Taking advantage of a small fluorescence group, these probes can serve as useful tools for mechanistic study of SMO conformational change in vitro. The strategy exhibits promising prospects for various types of probes labeling on SMO receptor, such as ^{19}F probes for NMR study, radioactive isotopes or biotin tag for immunology study. However, probes in this context seemed to be not applicable to live-cell imaging at present owing to undesired reactions with biological membrane components along with modest stability in aqueous buffer (Figure S11), further optimization is still on the way.

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Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

The authors (FX, SZ and HT) are thankful to Shanghai Municipal Government, ShanghaiTech University for startup financial support, and National Key R&D Program of China (2018YFA0507000) for support. This work was also partially funded by the National Natural Science Foundation of China (No. 21672147 to HT and 21708026 to FZ). We thank J. Liu, Q. Shi and L. Yang for assistance in the membrane preparation, protein purification and NMR data collection respectively at the Core Facility of iHuman Institute. We thank Y. Zhao for the generous donation of NIH-3T3 cell line for the assay. We thank Z.-J. Liu, D. Wu and D. Liu for their insightful discussion and assistance in manuscript preparation.

NIH3T3 cell line for the assay was generously provided by Dr. Zhao Yun from Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. HEK293 cells for the expression of engineered protein was purchased from ATCC. Both of the cell lines were maintained at the core facility of iHuman Institute, ShanghaiTech University.

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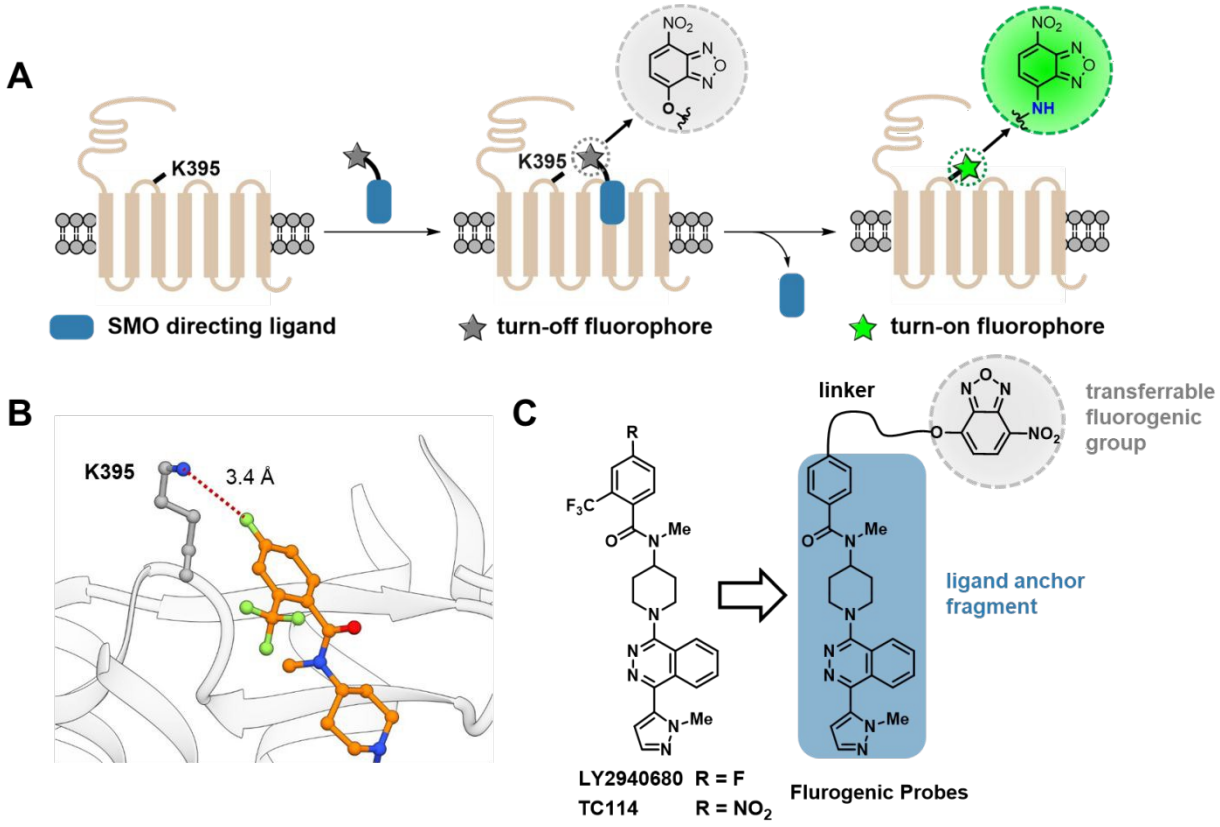

Figure. 1. Rational design and synthesis of SMO ligand-derived labeling probes. (A) General scheme for the SMO labeling using ligand directed residue modification. The directing ligand of SMO is indicated as blue rectangle. Labeling and labeled groups are indicated as grey and green stars, respectively, that indicated off and on states. (B) K395 in the ECL2 of SMO is within about 3.4 Å of the 4-fluorine of LY2940680 from the crystal structure (PDB: 4JKV). (C) Schematic illustration of SMO antagonist derived probes.

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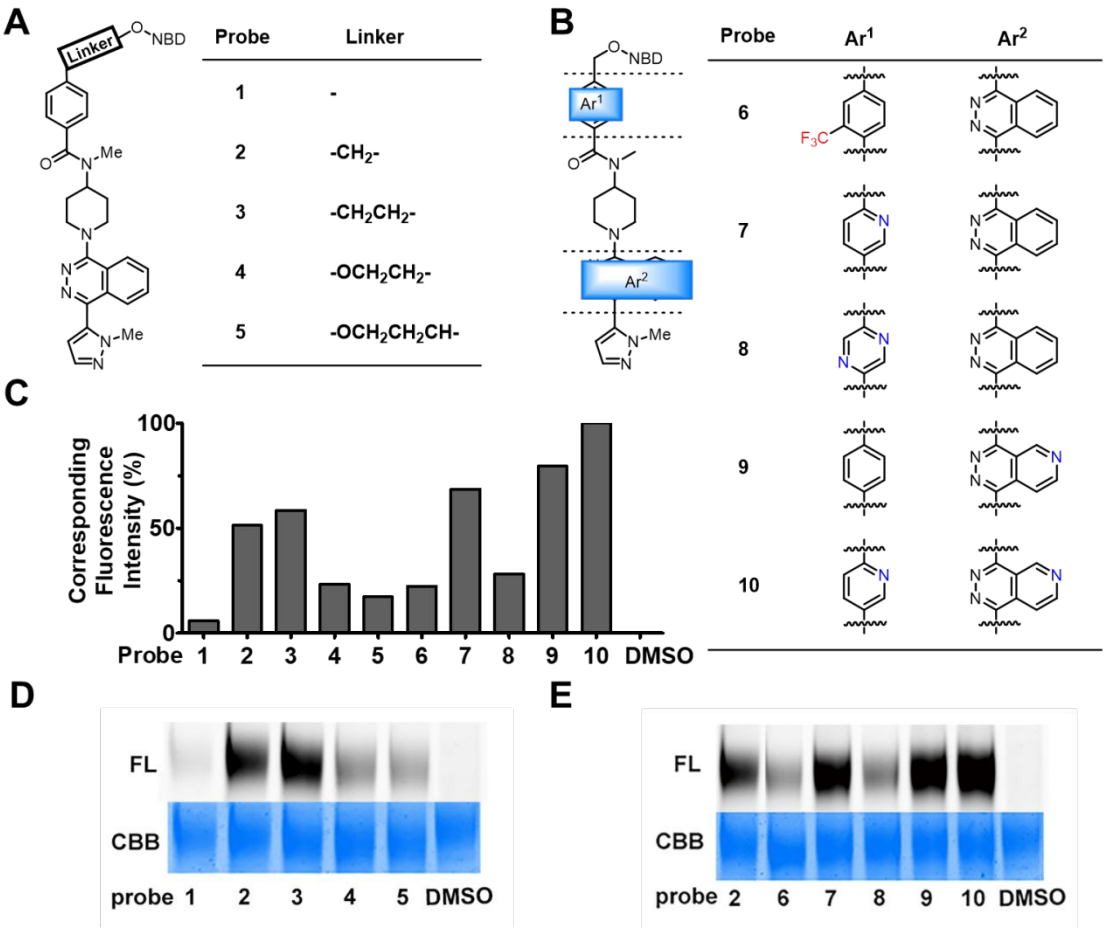

Figure 2. Probe evolution in linker and anchor unit. (A) Chemical structures of probes **1-5** with linker length from one to five atoms. (B) Chemical structures of probes **6-10** showing modification in ligand skeleton. (C) Normalized fluorescence (λ_{ex} = 488 nm; λ_{em} = 530 nm) intensity of SDS-PAGE of the FLA-SMO construct treated with probes **1-10**. (D) SDS gel of the FLA-SMO construct treated with probes **1-5**. The gel was photographed under a fluorescence detector and CBB staining. (E) SDS-PAGE of a FLA-SMO construct treated with probes **6-10** as well as probe **2**.

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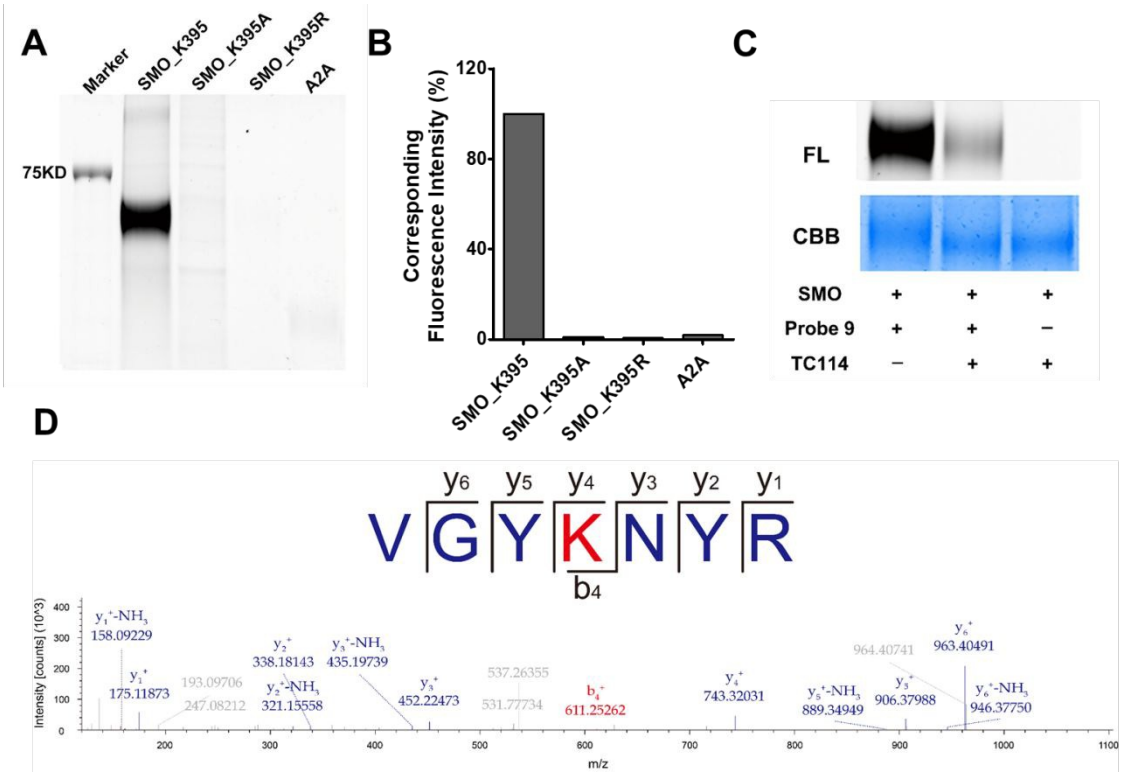

Figure 3. Selective labeling on K395 of SMO. (A) SDS gel of the FLA-SMO construct (SMO_K395), the K395A mutant (SMO_K395A), the K395R mutant (SMO_K395R), and A2A treated with probe **9**. The gel was photographed under a fluorescence detector (λ_{ex} = 488 nm; λ_{em} = 530 nm). (B) Normalized fluorescence intensity of SDS-PAGE of FLA-SMO constructs and A2A treated with probe **9**. (C) SDS-PAGE of FLA-SMO construct (SMO_K395) treated with probe **9** and/or TC114. The gel was photographed under a fluorescence detector or CBB staining. (D) Digestive MS analysis.

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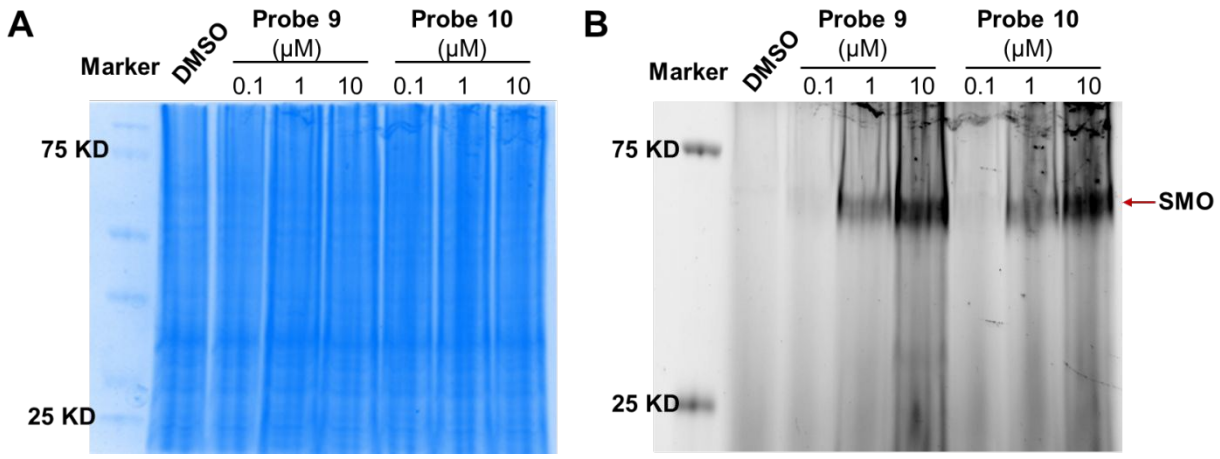

Figure 4. Probe 9 and 10 selectively labeled SMO on membrane. SDS-PAGE of membrane with over-expressed SMO after being treated with 100 nM, 1 μM and 10 μM probe **9** or probe **10** at 4 °C. The SDS gel was visualized by CBB staining (A) or a fluorescence detector with the excitation wavelength at 488 nm and the emission wavelength at 530 nm (B).

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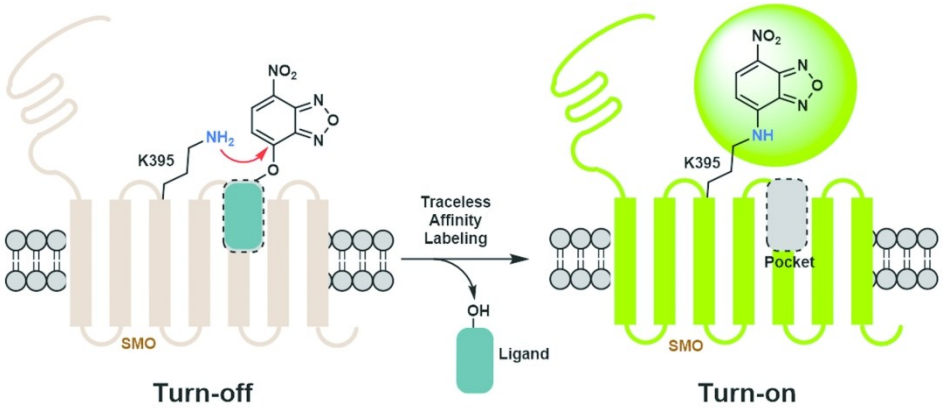

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