Monitoring Changes in the Oligomeric State of a Candidate Endoplasmic Reticulum (ER) Ceramide Sensor by Single-molecule Photobleaching

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Sphingolipids are vital structural components of cellular membranes and provide a rich reservoir of bioactive molecules that regulate a multitude of physiological processes (1, 2). Ceramides are essential precursors for sphingolipid production. They are synthesized de novo by N-acylation of sphingoid bases, a reaction catalyzed by ceramide synthases on the cytosolic surface of the ER (3, 4). In mammals, the bulk of newly synthesized ceramides is converted to sphingomyelin (SM) by a SM synthase (SMS) in the lumen of the trans-Golgi (5, 6). Efficient production of SM requires a cytosolic ceramide transfer protein (CERT) that mediates delivery of ER ceramides to the trans-Golgi (7). Besides constituting the backbone of all sphingolipids, ceramides have been implicated as potent mediators of cellular stress pathways, cell cycle arrest, and apoptotic cell death (1, 8). Indeed, various anti-cancer regimens have been reported to cause a transient increase in endogenous ceramide levels through de novo synthesis, leading to G0/G1 arrest and apoptosis (9–12). Knockdown of ceramide transfer protein (CERT) sensitizes different types of cancer cells to cytotoxic agents (13). Reciprocally, decreased levels of endogenous ceramides by overexpression of glucosylceramide synthase potentiates cellular multidrug resistance (14). This implies that cells need to monitor ceramide levels closely to avoid jeopardizing their viability during sphingolipid biosynthesis. How this is accomplished remains to be established.

Identification of the Golgi-resident SM synthase uncovered a multiplicity of SMS-encoding genes in the human genome (6, 15). The best-conserved member of this so-called SMS family, SMSr/SAMD8, does not synthesize SM but produces trace amounts of the SM analogue ceramide phosphoethanolamine in the lumen of the ER (16–18). Remarkably, acute disruption of SMSr catalytic activity in human cancer cells induced mitochondrial apoptosis by causing a rise in ER ceramides and their mislocalization to mitochondria (16, 19). We found that SMSr-catalyzed phosphoethanolamine production, although required, was not sufficient to suppress ceramide-induced cell death and that SMSr-mediated ceramide homeostasis is critically dependent on the N-terminal sterile α-motif or SAM domain of the enzyme (19). Based on these results, we postulated a primary role of SMSr in monitoring ER ceramide levels to protect cells against the intrinsic danger of sphingolipid bio-

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‡¶2 The abbreviations used are: ER, endoplasmic reticulum; SAM, sterile alpha motif; DGK, diacylglycerol kinase; meGFP, monomeric enhanced GFP; PPL-PEG-RGD, poly-L-lysine-polyethylene glycol-arginine-glycine-aspartate; SM, sphingomyelin; SMSr, sphingomyelin synthase-related protein; TIRF, total internal reflection fluorescence; PFA, paraformaldehyde; PLL-PEG-RGD, poly-L-lysine-polyethylene glycol-arginine-glycine-aspartate.
synthesis. Uncovering the mechanism by which SMSr senses and controls ER ceramides has become a major focus of our ongoing work.

SAM domains are wide-spread protein modules that participate in diverse functions, ranging from mediating protein–protein interactions to lipid and RNA binding (20, 21). BLAST searches revealed that the SAM domain of SMSr is closely related to the SAM domain of diacylglycerol kinase δ (DGKδ), a central regulator of lipid signaling at the plasma membrane. The biological activity of DGKδ is controlled by its SAM domain, which mediates the formation of helical polymers that sequester the enzyme in the cytosol to prevent its mobilization to the plasma membrane until pathway activation (22–24). Interestingly, we found that residues critical for homo-oligomerization and controls ER ceramides has become a major focus of our ongoing work.

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A major drawback of accessing the subunit stoichiometry of membrane-bound proteins using bulk biochemical approaches is that it requires disruption of the membrane environment, which may abolish native protein complexes and induce artificial protein assemblies. In addition, as these approaches rely on ensemble measurements, they average over a large quantity so that information on the assembly state of particular protein subpopulations is lost. With the advent of single-molecule fluorescence microscopy techniques, it has become possible to systematically analyze the oligomeric state of individual membrane-bound proteins with minimal perturbation of the biological system (27). Notably, single-molecule photobleaching has emerged as a valuable tool to extract the stoichiometry of protein receptors, ion channels, and transporters at the plasma membrane of living cells (28). Provided that every subunit is tagged with a fluorescent label, the number of subunits within a multimeric protein complex can be determined by counting the discrete steps of photobleaching in the fluorescence emission time traces of single fluorescent particles. However, few studies have extended the application of single-molecule photobleaching to analyze membrane protein oligomerization in internal cellular organelles, and we are unaware of any reports in which this technology has been applied to analyze protein assemblies in the ER.

In this study we report on the application of single-molecule photobleaching to monitor changes in the oligomeric state of SMSr. Using cell spreading surface-active coating agents in combination with total internal reflection fluorescence (TIRF) microscopy allowed us to image GFP-tagged SMSr proteins as single fluorescent spots in the ER of intact HeLa cells and record photobleaching traces of hundreds of individual fluorescent protein assemblies. Our analysis reveals that the N-terminal SAM domain of SMSr drives self-assembly of the protein in ER-resident oligomers and that curcumin promotes this process. Overall, these results open up new avenues to investigate the mechanism by which SMSr controls ceramide levels in the ER.

Results

Imaging of ER-resident Monomeric Enhanced GFP (meGFP)-SMSr—To study the oligomeric state of the ER-resident protein SMSr by single-molecule photobleaching analysis, we fused meGFP to the N terminus of the protein. In meGFP, the tendency of commonly used eGFP to dimerize at high concentrations was eliminated by substituting hydrophobic residues at the dimerization interface with positively charged residues (29). For an accurate determination of the stoichiometry of SMSr oligomers, meGFP-tagged SMSr was expressed from a plasmid in HeLa cells in which expression of the endogenous protein was eliminated using CRISPR/Cas9 technology. As shown in Fig. 2, meGFP-SMSr primarily localized to the ER of transfected HeLa-SMSr cells. Removal of the N-terminal SAM domain caused a redistri-

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bution of meGFP-SMSr from the ER to the Golgi. The addition of a C-terminal KKXX motif (KKSA) proved sufficient to target meGFP-SMSrΔSAM to the ER (Fig. 2). Together, these results are in line with our previous finding that the SAM domain serves a critical role in retaining SMSr in the ER (19) and that addition of an N-terminal meGFP-tag does not interfere with this function.

Single-molecule photobleaching analysis of fluorescent membrane proteins that reside in the ER is challenging because of the complex three-dimensional architecture of the organelle. Even at the plasma membrane, there is a chance that distinct protein complexes lie within a single diffraction-limited spot. Because there are stacks of ER membranes on top of each other, there is a higher chance that multiple complexes appear as a single fluorescent spot so that the number of bleaching steps does not provide accurate information on their assembly state. Indeed, for cells grown on the bare glass coverslip, we could not reliably pick individual fluorescent spots (Fig. 3B, left panel). In addition, the fluidity of the ER bilayer (30) and dynamic rearrangements of the ER network in live cells contribute to a high mobility of the fluorescent proteins, whereby their movement out of the focus plane can be easily misinterpreted as a bleaching step. To cope with these challenges, we imaged meGFP-SMSr in chemically fixed cells. We tested glutaraldehyde, paraformaldehyde (PFA), and methanol-based fixation protocols. This revealed that PFA fixation gave the best signal-to-noise ratio (data not shown). To reduce the density of individual fluorescent spots, cells were seeded on coverslips coated with the surface-active copolymer poly-L-lysine-polyethylene glycol-arginine-glycine-aspartate (PLL-PEG-RGD). This copolymer, which contains a poly-L-lysine backbone for binding to the glass coverslip and protein adsorption-resistant poly-ethylene glycol side chains, is functionalized with RGD peptides that mediate cell adhesion through integrin receptors (Fig. 3A) (31). With a very low fluorescence background, PLL-PEG-RGD-coated surfaces have been applied for single molecule detection of protein complexes in the plasma membrane (32). The PLL-PEG-RGD coating induced extensive cell spreading, resulting in large peripheral regions with a spot density of ER-resident proteins amenable for single-molecule photobleaching analysis (Fig. 3B, middle panel). The increased adhesion also resulted in a flattening of the cells, which diminished the overall background caused by out-of-focus illumination. For single molecule detections, cells were illuminated with 488-nm laser light and visualized by TIRF microscopy (Fig. 3B, right panel). This further improved the signal-to-noise ratio and allowed us to readily pick hundreds of individual fluorescent spots per cell. Therefore, in all successive experiments, fluorescent spots were imaged in PFA-fixed cells grown on PLL-PEG-RGD-coated coverslips using TIRF microscopy.

Single-molecule Photobleaching of ER-resident meGFP-SMSr—To determine the subunit stoichiometry of ER-resident SMSr oligomers, we imaged peripheral regions of meGFP-SMSr-expressing cells where fluorescent spots with optimal circularity can be readily found at a density ranging between 10 and 100 per 10 × 10 μm² area (Fig. 4A). At these densities we could obtain enough spots for statistical analysis while keeping the probability low such that distinct protein complexes would lie within a diffraction-limited spot. Depending on cell morphology and meGFP-SMSr expression levels, we were able to pick 50–400 individual fluorescent spots per cell. Emission intensities of the fluorescent spots were extracted from the
acquired movies as described under “Experimental Procedures” (supplemental Video 1). Most fluorescent spots were bleached within 5–10 s of illumination. Typically, the fluorescence intensities of these spots decreased in several discrete steps, indicating photobleaching of individual GFP molecules (Fig. 4C). Fluorescent spots that were highly mobile or that did not show any distinct photobleaching behavior were excluded from the analysis. Intensity plots of the selected spots revealed between 1 and 4 discrete bleaching steps, with a typical distribution of \( \frac{1}{10} \) step, \( \frac{3}{10} \) step, \( \frac{1}{10} \) step, and \( \frac{4}{10} \) step (see below). Occasionally, we also encountered spots that bleached in five discrete steps. As these spots occurred at a very low frequency (<1% of total spots; data not shown), we decided to include them in the category of four steps (~4%).

As the distribution of bleaching steps may fluctuate with fluorescent protein expression levels, we next analyzed photobleaching traces of fluorescent spots in three cells expressing meGFP-SMSr at different levels (Fig. 5, A and B). Based on the relative fluorescence intensities, expression levels of meGFP-SMSr in these cells were estimated to vary one order of magnitude. Although the density of fluorescent spots between the cells differed greatly, all of the analyzed individual spots showed a very similar distribution in bleaching steps (Fig. 5C). This led us to conclude that the assembly state of ER-resident meGFP-SMSr complexes is largely independent of the expression level of the fluorescent protein. Thus, single-molecule photobleaching appears to be a suitable approach for imaging SMSr oligomers in the ER.

**Self-assembly of meGFP-SMSr into ER-resident Oligomers Is Critically Dependent on Its SAM Domain—**Co-immunoprecipitation and chemical cross-linking studies revealed that SMSr can self-assemble into trimers and hexamers and that SMSr homo-oligomerization is mediated by the N-terminal SAM domain of the protein. Although removal of SAM completely abolished the formation of SMSr trimers and hexamers, SMSr SAM retained the ability to form homo-dimers. To verify that the SAM domain drives homo-oligomerization of SMSr in the ER of intact cells, we next analyzed photobleaching traces of fluorescent spots in cells expressing meGFP-SMSr and meGFP-SMSrSAM. Even though removal of SAM causes a redistribution of the bulk of meGFP-SMSr to the Golgi, cells expressing meGFP-SMSrSAM still contained residual amounts of the fluorescent protein in the ER that were sufficient to allow a meaningful comparative analysis (Fig. 6A). In two independent experiments, we counted the bleaching steps of a total of 1221 fluorescent spots in meGFP-SMSr-expressing cells and of 606 fluorescent spots in meGFP-SMSrSAM-expressing cells. In each experiment we found that removal of the SAM domain reduced the maximum number of bleaching steps from four to two (Fig. 6B). In addition, the fraction of fluores-
recent spots that was bleached in one step increased from ~45% to ~75%. To verify that these changes were due to a loss of SAM and not to an overall reduction in the ER-associated pool of the fluorescent protein, we also compared photobleaching traces of fluorescent spots between cells expressing meGFP-SMSr and meGFP-SMSrΔSAM proteins that carry a C-terminal KXXX ER-retrieval motif (Fig. 2). Again, we observed that SAM removal reduced the maximum number of bleaching steps from four to two and increased the fraction of fluorescent spots with a single bleaching step (Fig. 7). From this we conclude that SMSr self-assembles into ER-resident oligomers and that this process is critically dependent on the protein’s N-terminal SAM domain.

Curcumin Promotes meGFP-SMSr Homo-oligomerization in the ER—We recently observed that curcumin, a drug known to deregulate ceramide and calcium levels in the ER (25, 26), promotes self-assembly of SMSr into hexamers.4 Although this effect appears to be independent of fluctuations in ER ceramide or calcium levels and may potentially involve a curcumin-associated cross-linking activity (33), the underlying mechanism remains to be established. Nevertheless, we asked whether the curcumin-mediated effect on SMSr oligomerization could be resolved by single-molecule photobleaching analysis. To this end, we analyzed the photobleaching traces of 684 fluorescent spots in curcumin-treated meGFP-SMSr-expressing cells (50 μM curcumin, 6 h) and compared these with those of 921 fluo-
rescent spots in meGFP-SMSr-expressing control cells. As shown in Fig. 8A, curcumin treatment nearly doubled the fraction of fluorescent spots with three or four bleaching steps (from 19 to 36%) while causing a substantial reduction in the fraction of fluorescent spots with a single bleaching step (from 46 to 35%). We calculated that the drug increased the average number of bleaching steps from 1.74 to 2.12 steps per fluorescent spot. As curcumin absorbs blue light and acts as a redox-modifying agent (34), its impact on the average number of bleaching steps could be due to changes in meGFP photobleaching kinetics rather than a shift in the oligomeric state of SMSr. However, we did not observe any significant differences in the lifetimes of bleaching steps or photobleaching kinetics of meGFP-SMSr between control and curcumin-treated cells (Fig. 8, B and C). Together, these findings indicate that curcumin promotes formation of SMSr homo-oligomers in the ER of intact cells.
Probability Analysis of SMSr Self-assembly Detected by Single-molecule Photobleaching—Chemical cross-linking experiments revealed that SMSr proteins in cells occur as monomers, trimers, and hexamers.4 However, photobleaching analysis of hundreds of individual fluorescent spots in meGFP-SMSr-expressing cells yielded a maximum of four, occasionally five bleaching steps (Figs. 6B, 7, and 8A; see above). Although this finding was somewhat unexpected, several factors likely contribute to an underestimate of the actual number of meGFP-SMSr molecules in each spot, thus limiting the probability of directly observing higher order oligomers such as hexamers. To begin with, not all of the meGFPs may be fluorescent at the start of data acquisition due to misfolding or partial maturation of the GFP. In fact, we found that only ~72% of meGFP molecules expressed in *Escherichia coli* reach full maturation (Fig. 9A). Consistent with our analysis, a previous study involving live cell imaging of GFP-tagged channels expressed by RNA injection in *Xenopus* oocytes estimated that 77.5% of the expressed GFPs are in a fluorescent state (35). Moreover, meGFP-SMSr-expressing cells had to be located under the microscope, and even though minimal laser power was used for their detection, some bleaching may have occurred before data acquisition. To determine the photobleaching rate of meGFP under sample screening conditions, we carried out time-lapse TIRF imaging of meGFP molecules immobilized on functionalized coverslips and recorded the fluorescence intensity in a region-of-interest over time (Fig. 9B). A bleaching rate of ~0.015 s⁻¹ was obtained by mono-exponential fitting, indicating that ~36% of meGFP molecules is bleached after 30 s of sample screening, which is the average time required to select and position cells before photobleach analysis. From this we inferred that the effective labeling degree of meGFP-SMSr at the start of data acquisition is ~46%, *i.e.* the multiply of 72% maturation level and 64% unbleached meGFP molecules after sample screening. Next, we used this effective labeling degree to calculate the respective observation probabilities of fluorescent meGFP-tags for SMSr monomers, trimers, and hexamers (Fig. 9C). This indicated that a low effective labeling degree likely represents the main reason for our inability to directly observe SMSr hexamers. The calculated probability distributions were then used to decompose the experimentally obtained photobleaching step histograms (Fig. 9D), allowing us to estimate, by approximation, the actual changes in the oligomeric state of SMSr after curcumin treatment or removal of the SAM domain (see "Experimental Procedures" for details). In line with our biochemical studies,4 this numerical analysis revealed that a significant
FIGURE 9. Probability analysis of SMSr self-assembly detected by single-molecule photobleaching. A, UV-visible spectrum of 10 μM affinity-purified meGFP in PBS buffer used to assess the meGFP maturation level (~72%), as described under “Experimental Procedures.” B, time-dependent decay in fluorescence intensity of surface-anchored meGFP molecules under sample screening conditions. A bleaching rate of 0.015 s⁻¹ was obtained by mono-exponential fitting (red line). a.u., arbitrary units. C, calculated binomial distributions of bleaching steps for meGFP-SMSr monomers, trimers, and hexamers assuming a probability of 46% that the meGFP tag is fluorescent. D, the probability distributions from C were fitted to the experimentally obtained photobleaching step histograms as described under “Experimental Procedures.” Comparisons of experiment and the best fitting model are shown for meGFP-SMSrΔSAM and meGFP-SMSr in control or curcumin-treated cells. E, estimated distributions of the oligomeric states of meGFP-SMSrΔSAM and meGFP-SMSr in control or curcumin-treated cells, calculated as described under “Experimental Procedures.”
portion of ER-resident SMSr proteins forms hexamers (~15%) and that this fraction increases substantially (to ~45%) at the expense of SMSr monomers and trimers in curcumin-treated cells (Fig. 9E).

Discussion

Although single-molecule photobleaching technology has been widely used to elucidate the subunit stoichiometry of receptors and channels at the plasma membrane, only a few studies have extended its application to analyze protein oligomerization in intracellular organelles. Here we report to the best of our knowledge the first successful application of this method to resolve drug-induced and domain-dependent changes in the oligomeric state of an ER-resident membrane protein, i.e. the candidate ceramide sensor SMSr.

TIRF microscopy of chemically fixed cells grown on functionalyzed substrates and expressing meGFP-tagged SMSr allowed us to quantitatively analyze photobleaching traces of hundreds of individual fluorescent spots. This analysis revealed that SMSr self-assembles into ER-resident oligomers. Formation of these oligomers proved largely independent of SMSr expression levels but critically relied on the N-terminal SAM domain of the protein. Thus, we observed that the maximum number of bleaching steps detected in fluorescent spots of meGFP-SMSr-expressing cells dropped from four to two upon removal of SAM, in line with chemical cross-linking and co-immunoprecipitation studies showing that loss of this domain causes a collapse of SMSr trimers and hexamers into dimers and monomers. Conversely, treatment of meGFP-SMSr-expressing cells with a drug promoting the recovery of SMSr trimers and hexamers in immunoprecipitation experiments, caused a significant increase in the average number of bleaching steps. Together, these data provide first insights into the actual behavior of SMSr in its native environment and establish single-molecule photobleaching as a valuable complementary method to unravel the functional implications of SAM-mediated SMSr oligomerization in intact cells.

One limitation of the method is that for complexes with five or more subunits, the distributions of bleaching steps for n and n + 1 subunits look similar, and detection of discrete steps becomes more difficult (28, 36). Indeed, although chemical cross-linking experiments indicate that SMSr partially self-assembles into trimers and hexamers, photobleaching analysis of hundreds of individual fluorescent spots in meGFP-SMSr-expressing cells yielded a maximum of four, occasionally five, bleaching steps. However, we found that this discrepancy is first and foremost due to the fact that not all meGFP-tagged proteins are fluorescent at the start of data acquisition because of partial maturation of the fluorophore and pre-photobleaching during sample screening. Experimental determination of the fraction of functional fluorophores at the start of the analysis combined with statistical modeling allowed us to reconstruct the actual assembly states of SMSr. Following this approach, we inferred that a significant portion of ER-resident SMSr proteins occurs in hexamers and that this subpopulation expands dramatically in curcumin-treated cells at the expense of monomers and trimers. Importantly, these findings are largely consistent with our biochemical analysis. For a more robust subunit counting of membrane protein complexes comprising five or more subunits, the application of improved fluorescent labeling techniques, a fast sample screening and/or a more sophisticated automatized analysis of photobleaching traces (e.g. Ref. 37) might be necessary. It deserves mention that bulk biochemical approaches like chemical cross-linking have limitations in their own right when it comes to an accurate assessment of the assembly state of membrane proteins. Combined application of single-molecule tracking and bimolecular fluorescence complementation technology recently enabled direct imaging of acutely induced dimeric protein complexes in membranes of living cells at sub-diffractional resolution (38). However, this approach is unsuited for resolving the stoichiometry of higher order protein assemblies such as those formed by SMSr. Consequently, our present work provides a valuable extension of currently available methods to decipher the oligomeric state of intracellular membrane proteins in their native environment.

Whether SAM-mediated self-assembly of SMSr is part of the mechanism by which this protein controls ER ceramide levels and how curcumin influences this process remains to be established. Curcumin has been reported to stimulate de novo ceramide synthesis by triggering dimerization and activation of ER-resident ceramide synthases (39, 40) but also perturbs Ca2+ homeostasis (25, 41, 42) and may act as a cross-linking agent (33). Hence, future studies should reveal whether a more specific and acute manipulation of ER ceramide levels influences SMSr oligomerization. Contrary to bulk biochemical approaches, a key advantage of the current method is that it permits a systematic analysis of the oligomeric state of individual ER-resident membrane proteins in intact cells. A desirable but challenging prospect is to circumvent chemical fixation and resolve the dynamics of SMSr self-assembly in living cells. The creation of ER membrane sheets that are tethered to the plasma membrane via synthetic linkers (43) may provide a suitable starting point to achieve this goal.

Experimental Procedures

DNA Constructs—Mammalian expression vector pSEMS (Covalys Biosciences) containing meGFP is described in Wilmes et al. (32). To obtain meGFP-SMSr, a cDNA encoding human SMSr was PCR-amplified and inserted into pSEMS/meGFP via XhoI and NotI restriction sites. To obtain meGFP-SMSrΔSAM, the first 78 N-terminal amino acid residues of SMSr were removed during the PCR reaction. The addition of a C-terminal ER retention sequence KKSA yielded the constructs meGFP-SMSr–KKSA and meGFP-SMSrΔSAM–KKSA.

Cell Culture and Transfection—A HeLa SMSr-null cell line (HeLa–SMSr–/−) was created using CRISPR/Cas9 technology. Cells were cultured at 37 °C and 5% CO2 in phenol red-free DMEM (Gibco) supplemented with 1 mM sodium pyruvate (Gibco), 2 mM GlutaMAX (Gibco), and 10% FBS (PAA) and penicillin-streptomycin (Gibco). 24 h before transfection cells were seeded on regular glass coverslips or on glass coverslips coated with PLL-PEG-RGD (31) as described in Wilmes et al. (32). Cells were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer’s protocol. Where indicated, cells were treated with 50 μM curcumin (Enzo.
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Life Sciences; 50 mM stock prepared in DMSO) or with carrier only (DMSO) during the final 6 h of transfection.

**Immunofluorescence Microscopy**—24 h post-transfection, cells grown on regular glass coverslips were fixed using 3.7% paraformaldehyde, PBS (pH 7.4) for 12 min at 37 °C, washed in PBS, and then quenched in 0.3 M glycine for 15 min at room temperature. Cells were washed 5 times for 30 s, 1 min, 5 min, 10 min, and 15 min with PBS and then permeabilized using PBS containing 0.1% (w/v) saponin and 0.2% (w/v) BSA. Cells were immuno-stained with mouse anti-GM130 (BD Bioscience) and donkey anti-mouse Cy3 antibodies (Jackson ImmunolResearch) and then mounted in Prolong Gold Antifade Reagent (Thermo Fischer Scientific). Images were captured at room temperature with a Leica DM5500 B epifluorescence microscope using a 63×1.40 NA Plan Apo oil objective and a SPOT Pursuit camera. Fluorochromes used were: FITC/Alexa Fluor488, λex = 488 nm and λem = 515 nm; Texas Red/Alexa Fluor 568, λex = 568 nm and λem = 585 nm. Images were processed using ImageJ software (NIH, Bethesda, MD).

**Single-molecule Photobleaching Analysis**—HeLa-SMSr−/− cells were seeded on PLL-PEG-RGD-coated coverslips, transfected with meGFP-SMSr constructs, and then fixed, quenched, and washed in PBS as described above. Single-molecule images were taken at room temperature using an inverted TIRF microscope (Cell^TIRF, Olympus) equipped with a quad-line dichroic beam-splitter (Di01 R405/488/561/640, Chroma), a quad band emission filter (Brightline HC 446/523/600/677–25, Semrock), and an sCMOS camera (Hamamatsu, ORCAFlash 4.0, 2048 × 2048 pixels). A 100× magnification objective with a numerical aperture of 1.49 (UAPON, 100x/1.49 TIRF, Olympus) was used for time-lapse imaging during single-molecule photobleaching experiments. meGFP was excited by a 488-nm diode laser (Olympus). A laser power of ~600 micro-watts was used for sample screening (maximum 30 s). After selection of meGFP-SMSr-expressing target cells, photobleaching was performed with a power output of ~2 milliwatts at the objective, and the emission was detected through an additional single band 525/50 bandpass filter (Brightline HC 525/50, Semrock). Time-lapse image stacks were recorded at an acquisition rate of 35 ms/frame for 500–1000 frames (~18–35 s). Regions in meGFP-SMSr-expressing cells where the density of fluorescent spots ranged between 10 and 100 per 10 × 10 μm² area were selected for image analysis using ImageJ as described previously (44). For each isolated fluorescent spot, a square region of interest with 3 × 3 pixels (195 × 195 nm²) around the peak was selected to obtain the trace of fluorescence intensity versus time. Spots that were not completely immobilized or that did not show any distinct photobleaching behavior were excluded from the analysis. Intensity traces of individual spots were analyzed using a “Step Transition and State Identification” (StaSI) algorithm (45) as described previously (43). For each of the expressed meGFP-SMSr constructs, >600 individual traces were analyzed and pooled in a histogram.

**Probability Analysis of SMSr Self-assembly Detected by Single-molecule Photobleaching**—Chemical cross-linking experiments revealed that SMSr partially self-assembles into trimers and hexamers, whereas SMSrΔSAM can only form homodimers. Hence, the photobleaching step histograms measured here represent the collective contributions of photobleaching from monomers, trimers, and hexamers in the case of meGFP-SMSr and monomers and dimers in the case of meGFP-SMSrΔSAM. To decipher the actual changes in the oligomeric state of SMSr upon removal of the SAM domain or drug treatment, we used mixture models of monomers, trimers, and hexamers for meGFP-SMSr and of monomers and dimers for meGFP-SMSrΔSAM. Due to incomplete maturation of the meGFP fluorophore and pre-photobleaching during sample screening, only a certain fraction of meGFP-tagged proteins can be directly observed. Thus, the observation probabilities are governed by binomial distributions Equation 1,

\[
p(k|N,DOL) = \frac{N!}{k!(N-k)!}DOL^k(1-DOL)^{N-k} \quad (\text{Eq. 1})
\]

where \( p(k|N,DOL) \) is the probability to observe \( k \) photobleaching steps in a protein complex with stoichiometry of \( N \) (e.g. \( n = 1 \) for monomer, \( n = 3 \) for trimer, etc.) and \( DOL \) is the effective degree of labeling, reflecting the fraction of fluorescent meGFP-tagged proteins (35, 36, 46). The effective labeling degree was determined as the multiply of the meGFP maturation level and the non-bleached fraction after sample screening. To estimate the meGFP maturation level, a UV-visible absorption spectrum of \( 10 \mu M \) meGFP in PBS buffer was recorded by a spectrophotometer (JASCO V-650). To this end meGFP was expressed in *E. coli* and purified through gel filtration as described (31). Using the concentrations derived from 488 nm (\( C_{280} = A_{280}/56000 \) cm⁻¹ M⁻¹) and 280 nm (\( C_{280} = A_{280}/22000 \) cm⁻¹ M⁻¹), extinction coefficient predicted by ExPaSy, a maturation percentage of 72% was obtained as the ratio of \( C_{488}/C_{280} \) (Fig. 9A). Absorption of the fluorophore at \( A_{280} \) was omitted in this estimation, as the correction factor for meGFP is not available. To determine the photobleaching rate of meGFP under sample screening conditions, time-lapse TIRF imaging of HaloTag-fused meGFP immobilized on PLL-PEG-HaloTag²-O₂-amine ligand (HTL)-functionalized coverslips was carried out as described (44). The recorded fluorescence intensity in a region-of-interest was plotted versus time. A photobleaching rate of ~0.015 s⁻¹ was obtained by fitting the curve with a monoeponential decay function (Fig. 9B). Thus, an effective labeling degree of 46% was obtained as the multiply of 72% maturation level and 64% unbleached meGFP molecules after 30 s of sample screening before data acquisition. This effective labeling degree was used to calculate the binomial observation probabilities of fluorescent meGFP tags for monomers, dimers, trimers, and hexamers. The binomial distributions were subsequently used for fitting of the measured photobleaching step histogram by minimizing the error between the data and mixture model using Equation 2,

\[
SSR = \left\| \frac{B(k)}{N} - \sum_{N} a(N)p(k|N,DOL) \right\|_2^2 \quad (\text{Eq. 2})
\]

where SSR is the sum of squared residuals and \( B(K) \) denotes the experimentally obtained photobleaching step histogram. \( a(N) \) is the calculated percentage of each subpopulation.

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