Golgi recruitment assay for visualizing small-molecule ligand–target engagement in cells

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ABSTRACT

The development of methods that allow detection of ligand–target engagement in cells is an important challenge in chemical biology and drug discovery. Here, we present a Golgi recruitment (G-REC) assay in which the ligand binding to the target protein can be visualized as Golgi-localized fluorescence signals. We show that the G-REC assay is applicable to the detection of various ligand–target interactions, ligand affinity comparison among distinct protein isoforms, and the monitoring of unmodified drug–target engagement in cells.

MAIN TEXT

Small-molecule ligands that bind to specific proteins in cells are valuable tools for biological research and can lead to new drugs for therapeutic use. In recent years, small-molecule ligands have also been used as a key component in the design of various chemical biology tools, which include protein-specific fluorescent probes,[1,2] affinity labeling reagents,[3,4] and bifunctional molecules such as chemical inducers of protein dimerization.[5,6] In the development of such small-molecule ligands and ligand-based tool compounds, evaluating and validating the binding of the ligand (or its derivative) to the target protein is a critical step. Currently, various techniques are available for measuring ligand-protein interactions *in vitro*.[7,8] However, ligands that show favorable binding properties to purified proteins *in vitro* do not necessarily lead to expected biological effects in cellular contexts. Therefore, it is critical to evaluate the direct binding of a ligand of interest to its target protein within cells. Consequently, several approaches have emerged that enable the detection of ligand–target engagement in cells.[9] Some strategies involve the use of fluorophore-labeled ligands. The direct binding of the fluorescent ligand to the target protein in cells can be detected by fluorescence resonance energy transfer (FRET),[10,11] bioluminescence resonance energy transfer (BRET),[12] or fluorescence polarization anisotropy.[13,14] Activity-based protein profiling (ABPP)[15,16] and ligand-directed protein labeling[17,18] are alternative approaches that can be used for ligand–target engagement evaluation by covalently labeling the ligand-binding protein in cellular environments. These methods require chemically labeled ligands. As a label-free approach, the cellular thermal shift assay (CETSA) was recently developed. CETSA measures cellular drug–target engagement based on the principle that the thermal stability

of proteins is affected by ligand binding.[19] Although these existing methods provide valuable information on cellular ligand–target interactions, they require complicated procedures and/or specialized instruments. Hence, there is a strong need for simple and reliable methods applicable to assessing ligand–target engagement in cells.

We herein present a new assay method for the *in situ* visualization of ligand–target engagement in mammalian cells. The principle of our assay is illustrated in **Fig. 1a**. In this assay, a small-molecule ligand of interest is attached via linker to a Golgi-targeting myristoyl-D-cysteine (m^Dc) motif recently developed by us.[20,21] The resulting m^Dc-tethered ligand can self-localize to the Golgi surface on cell entry.[22,23] Meanwhile, a target protein fused to a fluorescent protein (FP) is expressed in the cell cytoplasm. The cells are then incubated with the m^Dc-tethered ligand. When the m^Dc-tethered ligand binds to the FP-tagged target protein in the cell, Golgi-localized fluorescence is observed as a result of the ligand-induced Golgi recruitment of the protein. Notably, the Golgi apparatus has a distinct and compact perinuclear structure in cells. Therefore, the Golgi localization of the FP-tagged target protein leads to a fluorescence signal that is easily detectable by conventional confocal fluorescence microscopy.

As a proof of principle, we first attempted to demonstrate the visualization of the binding of a small-molecule ligand, trimethoprim (TMP), to *E. coli* dihydrofolate reductase (eDHFR) in cells.[24] In accordance with the strategy described above, we designed and synthesized **1** in which TMP was conjugated to the m^Dc motif via a flexible (and relatively long) linker consisting of five repeats of 8-amino-3,6-dioxaoctanoic acid (Adox) (**Fig. 1b**).[25] For the *in-cell* assay, we expressed eDHFR as a fusion with enhanced green fluorescent protein (eDHFR-EGFP) in HeLa cells. Before ligand treatment, eDHFR-EGFP was evenly distributed in the cytoplasm (**Fig. 2a**). However, after incubating the cells with **1** for 1 h, we observed EGFP fluorescence localized at a perinuclear region, which merged well with the Golgi marker (**Fig. 2a**). Subsequent treatment of the cells with free (unmodified) TMP (**Fig. S1a**) diminished the Golgi fluorescence, verifying that the Golgi localization of eDHFR-EGFP was mediated by the direct binding of eDHFR to the ligand moiety of **1**. Therefore, these results demonstrated the visualization of TMP–eDHFR binding as a Golgi-localized fluorescence signal using **1** in live-cell contexts.

This Golgi recruitment (G-REC) assay was also applicable to monitor other ligand–target interactions in cells. Small-molecule SLF* is a synthetic analog of FK506, which

binds to the F36V mutant of FKBP12 ($FKBP_{36V}$).[26] When m^Dc-tethered SLF* (**2**; **Fig. 1b**) was used, $FKBP_{36V}$ fused to mCherry was recruited to the Golgi surface (**Fig. S1b**), demonstrating the detection of intracellular $FKBP_{36V}$–SLF* binding. We also synthesized and tested **3** bearing benzenesulfonamide (BS), a small-molecule inhibitor for carbonic anhydrase (CA) (**Fig. 1b**).[27] Likewise, **3** allowed the monitoring of the BS binding to CA isoform 2 (CA2) in cells (**Fig. S1c**). More significantly, when we performed the assay with all combinations of the m^Dc-tethered ligands (**1**–**3**) and proteins (eDHFR, $FKBP_{36V}$, and CA2), the ligands induced Golgi-localized fluorescence signals only in cells expressing their corresponding target protein (**Fig. 2b** and **Table S1**), demonstrating the reliability of this G-REC assay platform to detect specific ligand–target engagement in cells.

Various proteins have isoforms with different affinities to a ligand. Thus, the ability to evaluate the relative affinity of a ligand to distinct protein isoforms in cellular contexts would be useful. Here, we sought to perform an affinity comparison assay using **3** by targeting CA isoform 1 (CA1) and CA2. For this purpose, we used cells expressing EGFP-fused CA1 (CA1-EGFP) or CA2 (CA2-EGFP) at similar levels, and quantified the ratio of the Golgi to the cytosolic fluorescence intensity (G/C ratio) of the proteins after adding **3**. In the G-REC assay for CA2, a minimal detectable level of Golgi-recruitment of CA2-EGFP (with the G/C ratio of >1.2) was observed at 5 μM after increasing the concentration of **3** (**Fig. 3**). From the dose-dependent curve of the G/C ratio, a half maximal effective concentration (EC_{50}) was estimated to be 6.8 μM (**Fig. 3b**). In contrast, the same assay targeting CA1 required at least 50 μM of **3** to induce a noticeable level of Golgi-recruitment of CA1-EGFP with an EC_{50} value of 65 μM. This one-order magnitude difference in the EC_{50} values was in good agreement with the difference in reported affinities (K_i values) of CA1 (3.4 μM) and CA2 (0.26 μM) toward 4-sulfamoylbenzoic acid (SBA).[27] These data demonstrate that the dose-dependent G-REC assay can be used to compare the affinity between a ligand and its target protein isoforms in cells.

Methods that allow *in situ* monitoring of target engagement of "unmodified" small-molecule drugs in cells would be highly valuable in drug screening. We thus finally applied the G-REC assay to quantifying target occupancy of unmodified drugs based on competitive binding. In our approach, a target protein previously recruited to the Golgi surface by the corresponding m^Dc-tethered ligand is released to the cytoplasm by an unmodified drug that competes for the binding to the target protein (**Fig. S2a**). To test

this, CA2-EGFP-expressing cells were first treated with **3** to recruit the protein to the Golgi surface, which was followed by the addition of an increasing concentration of unmodified drugs. As shown in **Fig. 4**, non-CA2-binder TMP had little to no effects on the G/C ratio. In contrast, a significant dose-dependent decrease in the G/C ratio was observed by the addition of CA2-binding drugs, ethoxzolamide (EZA), benzenesulfonamide (BS), and SBA with EC_{50} values of 1.8 nM, 1.0 μM, and 11 μM, respectively (**Fig. 4** and **Fig. S2b–f**). K_i values of EZA, BS, and SBA for CA2 determined *in vitro* were reported to be 8 nM, 1.5 μM, and 0.26 μM, respectively.[27,28] Notably, EZA showed a higher binding (competition) ability to CA2-EGFP than BS as expected from the reported K_i values. However, whereas SBA has the higher K_i value toward CA2 than BS *in vitro* (see above), the binding ability of SBA to CA2-EGFP was lower than that of BS in cells (**Fig. 4b**). This is likely attributed to the lower cell membrane permeability of anionic SBA compared with neutral BS.[29] Overall, this proof-of-principle experiment illustrates that the G-REC assay combined with ligand displacement is applicable to the monitoring of unmodified drug–target engagement inside cells, which may provide a new useful tool for cell-based drug screening.

In conclusion, we presented a G-REC assay that visualizes ligand–target engagement in live-cell environments using fluorescent protein-tagged proteins and m^Dc-tethered small-molecule ligands. In contrast to existing methods[9–19] that require complicated protocols and/or specialized instruments, the G-REC assay is simple to perform, allowing researchers to detect ligand–target engagement as Golgi-localized fluorescence signals using conventional confocal or epi-fluorescence microscopy. However, there are two major limitations. First, the G-REC assay requires the conjugation of the m^Dc motif to ligands of interest. Derivatization of ligands without the loss of target binding activity is often difficult. Second, because the G-REC assay relies on the anchoring of a target protein to the Golgi surface membrane via a m^Dc-conjugated ligand, an optimization of the linker length between the m^Dc motif and the ligand may be required when the target protein has a deep ligand-binding pocket or forms a large protein complex.[30,31] Nevertheless, we showed that the G-REC assay is applicable to the detection of various cellular ligand–target interactions and for ligand affinity comparison among distinct protein isoforms in cells. We further demonstrated the feasibility of the G-REC assay combined with ligand displacement as a tool for monitoring target engagement of unmodified drugs in cells. These features make the G-REC assay a new attractive

platform for assessing ligand–target engagement in chemical biology and drug development research.

SUPPLEMENTARY INFORMATION

Fig. S1–S4, Table S1, and Supplementary Methods.

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Conflict of Interest

S.S., M.I., T.Y., A.N., and S.T. are co-inventors on a patent application related to this work. K.K. declares no competing interests.

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NOTES and REFERENCES

1. M. A. Miller, E. Kim, M. F. Cuccarese, A. L. Plotkin, M. Prytyskach, R. H. Kohler, M. J. Pittet and R. Weissleder, *Chem. Commun.*, 2018, **54**, 42–45.

2. P. D. Koch, M. S. Ahmad, R. H. Kohler, R. Li and R.Weissleder, *ACS Chem. Biol.*, 2020, **15**, 151–157.
3. T. Hayashi and I. Hamachi, *Acc. Chem. Res.*, 2012, **45**, 1460–1469.
4. T. Tamura and I. Hamachi, *J. Am. Chem. Soc.*, 2019, **141**, 2782–2799.
5. T. W. Corson, N. Aberle and C. M. Crews, *ACS Chem. Biol.*, 2008, **3**, 677–692.
6. A. Rutkowska and C. Schultz, *Angew. Chem. Int. Ed.*, 2012, **51**, 8166–8176.
7. J.-P. Renaud, C. Chung, U. H. Danielson, U. Egner, M. Hennig, R. E. Hubbard and H. Nar, *Nat. Rev. Drug Discov.*, 2016, **15**, 679–698.
8. C. M. Santiveri, B. López-Méndez, S. Huecas, C. Alfonso, J. R. Luque-Ortega and R. Campos-Olivas, *eLS*, John Wiley & Sons, Ltd., Chichester, 2017, DOI: 10.1002/9780470015902.a0027015.
9. M. Schürmann, P. Janning, S. Ziegler and H. Waldmann, *Cell Chem. Biol.*, 2016, **23**, 435–441.
10. F. J. Dekker, O. Rocks, N. Vartak, S. Menninger, C. Hedberg, R. Balamurugan, S. Wetzel, S. Renner, M. Gerauer, B. Schölermann, M. Rusch, J. W. Kramer, D. Rauh, G. W. Coates, L. Brunsveld, P. I. H. Bastiaens and H. Waldmann, *Nat. Chem. Biol.*, 2010, **6**, 449–456.
11. H. Dückert, V. Pries, V. Khedkar, S. Menninger, H. Bruss, A. W. Bird, Z. Maliga, A. Brockmeyer, P. Janning, A. Hyman, S. Grimme, M. Schürmann, H. Preut, K. Hübel, S. Ziegler, K. Kumar and H. Waldmann, *Nat. Chem. Biol.*, 2012, **8**, 179–184.
12. M. B. Roberts, M. L. Dart, C. C. Woodroofe, C. A. Zimprich, T. A. Kirkland, T. Machleidt, K. R. Kupcho, S. Levin, J. R. Hartnett, K. Zimmerman, A. L. Niles, R. F. Ohana, D. L. Daniels, M. Slater, M. G. Wood, M. Cong, Y.-Q. Cheng and K. V. Wood, *Nat. Commun.*, 2015, **6**, 10091.
13. J. M. Dubach, C. Vinegoni, R. Mazitschek, P. Fumene Feruglio, L. A. Cameron and R. Weissleder, *Nat. Commun.*, 2014, **5**, 3946.
14. J. M. Dubach, E. Kim, K. Yang, M. Cuccarese, R. J. Giedt, L. G. Meimetis, C. Vinegoni and R. Weissleder, *Nat. Chem. Biol.*, 2017, **13**, 168–173.
15. B. F. Cravatt, A. T. Wright and J. W. Kozarich, *Annu. Rev. Biochem.*, 2008, **77**, 383–414.
16. G. M. Simon, M. J. Niphakis and B. F. Cravatt, *Nat. Chem. Biol.*, 2013, **9**, 200–205.
17. S. Tsukiji and I. Hamachi, *Curr. Opin. Chem. Biol.,* 2014, **21**, 136–143.
18. T. Ueda, T. Tamura and I. Hamachi, *Biochemistry*, 2020, **59**, 179–182.

19. L. Dai, N. Prabhu, L. Y. Yu, S. Bacanu, A. D. Ramos and P. Nordlund, *Annu. Rev. Biochem.*, 2019, **88**, 383–408.
20. A. Nakamura, C. Oki, S. Sawada, T. Yoshii, K. Kuwata, A. K. Rudd, N. K. Devaraj, K. Noma and S. Tsukiji, *ACS Chem. Biol.*, 2020, **15**, 837–843.
21. In our previous work, we showed that the $m^{D}c$ motif localized to the Golgi surface as well as the inner leaflet of the plasma membrane.[20] However, as shown in this work, when a relatively long linker is used, the $m^{D}c$-tethered ligand predominantly localizes to the Golgi surface.
22. M. Ishida, H. Watanabe, K. Takigawa, Y. Kurishita, C. Oki, A. Nakamura, I. Hamachi and S. Tsukiji, *J. Am. Chem. Soc.*, 2013, **135**, 12684–12689.
23. A. Nakamura, R. Katahira, S. Sawada, E. Shinoda, K. Kuwata, T. Yoshii and S. Tsukiji, *Biochemistry*, 2020, **59**, 205–211.
24. D. P. Baccanari, S. Daluge and R. W. King, *Biochemistry*, 1982, **21**, 5068–5075.
25. It should be noted that although we have previously reported an $m^{D}c$-TMP conjugate containing a linker consisting of three repeats of Adox,[20] **1** is a novel compound we synthesized and tested in this work for the Golgi recruitment assay.
26. T. Clackson, W. Yang, L. W. Rozamus, M. Hatada, J. F. Amara, C. T. Rollins, L. F. Stevenson, S. R. Magari, S. A. Wood, N. L. Courage, X. Lu, F. Cerasoli Jr., M. Gilman and D. A. Holt, *Proc. Natl. Acad. Sci. U.S.A.*, 1998, **95**, 10437–10442.
27. J.-Y. Winum, J.-M. Dogné, A. Casini, X. de Leval, J.-L. Montero, A. Scozzafava, D. Vullo, A. Innocenti and C. T. Supuran, *J. Med. Chem.*, 2005, **48**, 2121–2125.
28. D. K. Srivastava, K. M. Jude, A. L. Banerjee, M. Haldar, S. Manokaran, J. Kooren, S. Mallik and D. W. Christianson, *J. Am. Chem. Soc.*, 2007, **129**, 5528–5537.
29. S. Tsukiji, M. Miyagawa, Y. Takaoka, T. Tamura and I. Hamachi, *Nat. Chem. Biol.*, 2009, **5**, 341–343.
30. A. Nakamura, C. Oki, K. Kato, S. Fujinuma, G. Maryu, K. Kuwata, T. Yoshii, M. Matsuda, K. Aoki and S. Tsukiji, *ACS Chem. Biol.*, 2020, **15**, 1004–1015.
31. W. Hatanaka, H. Takeuchi, M. Koga, T. Ryujin, A. Kishimura, Y. Katayama, S. Tsukiji and T. Mori, *Chem. Lett.*, 2019, **48**, 433–436.

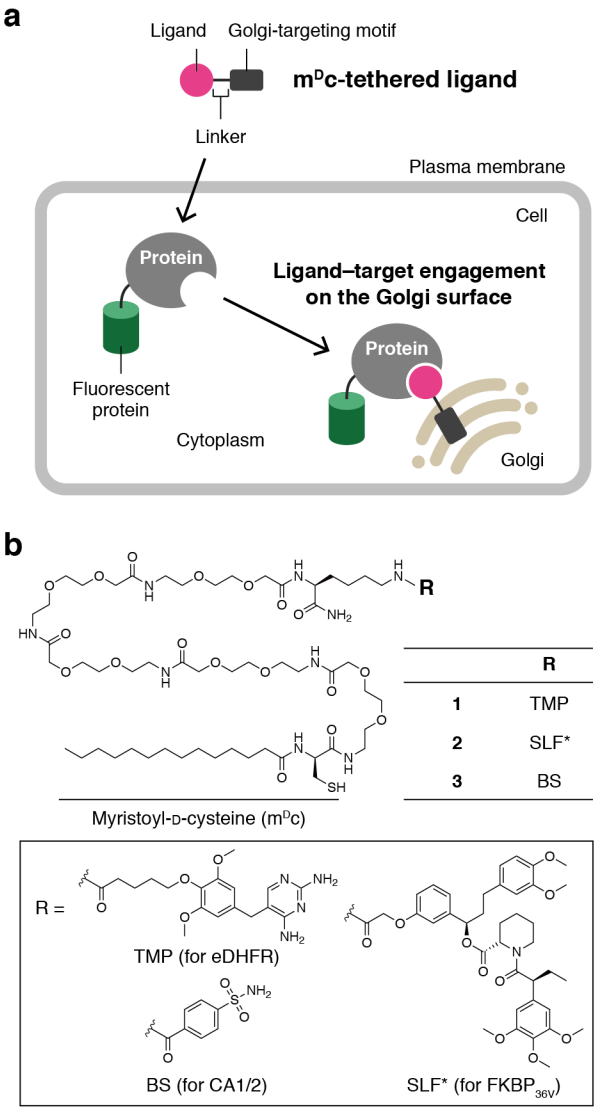

Fig. 1. The Golgi recruitment (G-REC) assay for visualizing ligand–target engagement in cells. (**a**) Schematic illustration of the system. In this system, the direct binding of a myristoyl-D-cysteine (m^{D}c)-tethered ligand to its target protein tagged with a fluorescent protein can be visualized as Golgi-localized fluorescence signals. (**b**) Chemical structures of m^{D}c-tethered ligands used in this study.

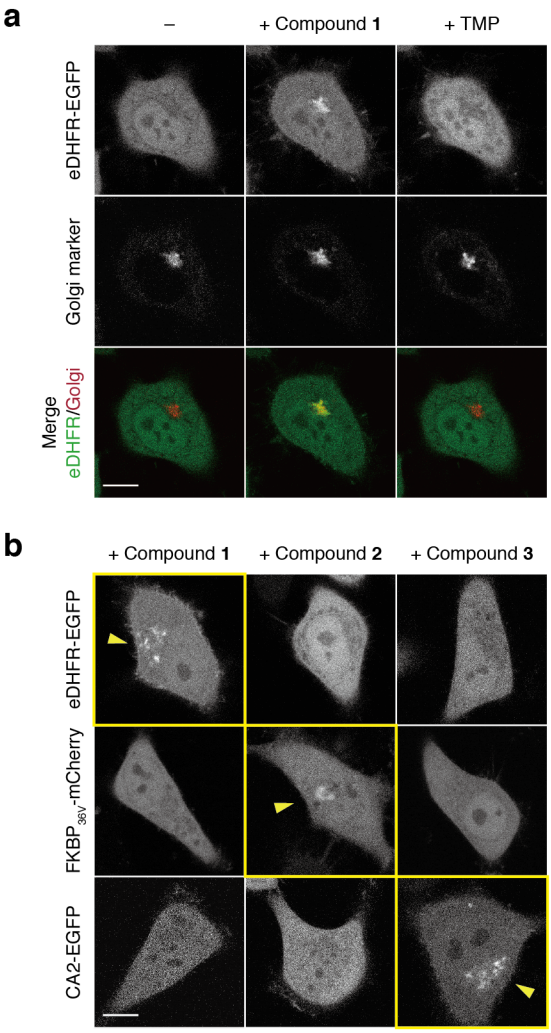

Fig. 2. Evaluation of the G-REC assay. (**a**) Detection of TMP–eDHFR interaction in living cells. Confocal fluorescence images of HeLa cells coexpressing eDHFR-EGFP and mCherry-giantin (Golgi marker) were taken before (left), 60 min after incubation with 5 μM **1** (center), and 30 min after subsequent incubation with 50 μM TMP (right). (**b**) Specific ligand–target engagement detection by the G-REC assay. Confocal fluorescence images of HeLa cells expressing eDHFR-EGFP (top), $FKBP_{36V}$-mCherry (middle), or CA2-EGFP (bottom) were taken 60 min after incubation with 5 μM **1** (left), 5 μM **2** (center), or 10 μM **3** (right). Arrowheads in the yellow-framed images indicate Golgi-localized fluorescence signals. Quantitative analysis also indicates that only cells with specific ligand-protein pairs showed significant Golgi-localized fluorescence signals (**Table S1**). Scale bars, 10 μm.

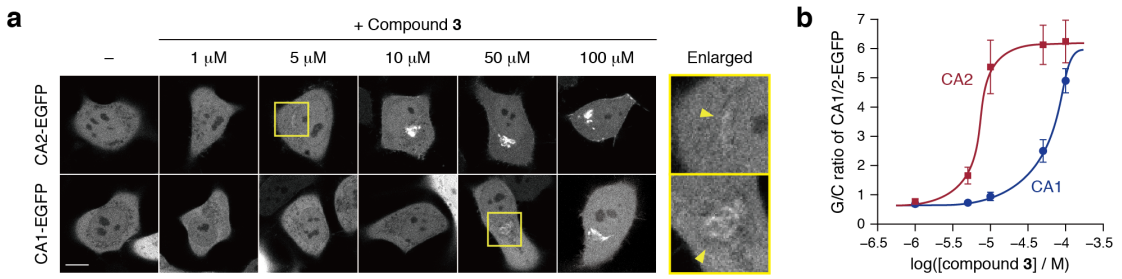

Fig. 3. Affinity comparison assay for CA isoforms. (**a**) Confocal fluorescence images of HeLa cells expressing CA2-EGFP (top) or CA1-EGFP (bottom) were taken 60 min after incubation with **3** at the indicated concentration. Cells with similar expression levels were used. Enlarged views of the yellow line frames are shown in the right-hand column of the panel. Arrowheads in the enlarged images indicate Golgi-localized fluorescence signals. Scale bar, 10 μm. (**b**) Quantification of the dose-dependent Golgi localization of CA1/2-EGFP. The ratios of the Golgi complex to the cytosolic fluorescence intensity (G/C ratios) of CA1-EGFP- (circle, blue) and CA2-EGFP-expressing cells (square, red) were quantified after treatment with **3** at the indicated concentration for 60 min. Data are represented as the mean ± SD (n = 6 cells).

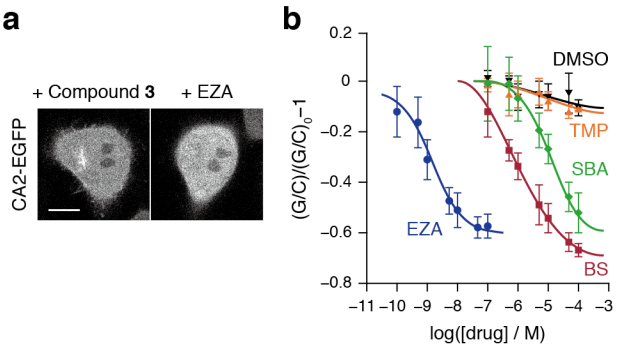

Fig. 4. Unmodified drug–target engagement assay for CA2 inhibitors. (**a**) Competition by EZA. CA2-EGFP-expressing HeLa cells were pre-treated with 10 μM **3** for 30 min to localize the protein to the Golgi surface. After washing, confocal fluorescence images of the cells were taken before (left) and 15 min after incubation with 100 nM EZA (right). Scale bar, 10 μm. (**b**) Titration profile of the change in Golgi-localized fluorescence signals after adding EZA (circle, blue), BS (square, red), SBA (diamond, green), TMP (triangle, orange), and DMSO (inverse triangle, black). The *y* axis is given as $(G/C)/(G/C)_0–1$, where (G/C) and $(G/C)_0$ are the G/C ratios after and before inhibitor treatment, respectively, and is plotted against the inhibitor concentration. Data are represented as the mean ± SD. ($n = 4$ cells). Representative confocal fluorescence images of the titration assay are shown in **Fig. S2**.

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