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Binding of fluorophores to proteins depends on the cellular environment**

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Fluorescent small-molecules become extensively used for live-cell imaging, but mainly in the context of labeling conjugates for other protein-binding motifs, such as antibodies.[1] As most fluorescent molecules are flat and hydrophobic, it has generally been believed that these fluorophores may bind to many hydrophobic proteins in cells, without any specificity.[2] This conventional wisdom, however, has not been tested systemically due to the lack of sufficiently diverse dye sources. Recently, we developed a diversity-oriented fluorescence library approach (DOFLA) to use fluorescent dyes to distinguish directly cellular components such as GTP,[3] DNA,[4] RNA,[5] heparin,[6] and organelles.[7] In this system, the diverse structural motifs of each dye molecule in the library endowed target selectivity. From these results, we envisioned that sufficient structural modifications of fluorophore scaffold could lead us to develop probes that label specific proteins from whole proteomes.[8] In addition to our recent finding of a fluorescein derivative labeling glutathione s-transferase,[9] here we report a rosamine derivative that labels tubulin *in vitro* and a mitochondrial protein in live cells.

Previously a fluorescent small molecule capable of detecting differentiated myotubes was discovered from mitochondria-targeted rosamine library.[10, 11, 12] The hallmark of muscle

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differentiation is the fusion of mono-nucleated myoblasts to multi-nucleated myotubes.[13] During murine C2C12 myogenesis, the fluorescence intensity of one rosamine compound, E26, increased significantly. This myotube selectivity may be achieved by binding to one of the differentiation markers expressed more highly in myotubes; alternatively, the probe may detect other physiological changes after differentiation. When subjected for the further investigation, unfortunately, E26 showed photo-instability under strong and continuous light irradiation (Supplementary Fig. 1 online). The rosamine library compounds were retested under long-term light exposure and we selected two compounds (I25 and I31; Fig. 1) based on high photo-stability and fluorescence response after differentiation (I25: 2.4 ± 0.2, I31: 3.0 ± 0.5 fold increase, $N = 3$; Supplementary Fig. 2 online).

In order to identify protein binders of these compounds, affinity matrices were prepared based on careful SAR studies (Fig. 2a). Affinity pull-down assay is the most conventional method for identification of small-molecule binding proteins.[14] Affinity resins were incubated with myotube lysates and washed with buffer to get rid of non-specific binders. Then, resin-bound proteins were separated by SDS-PAGE and stained with Coomassie blue (Fig. 2b). One enriched protein band at approximately 54 kDa was observed along with several other bands. To determine the specificity of protein binders to these compounds, competition assay was followed. Myotube cell lysate was pre-incubated with 100 μM of I25, I31, rhodamine 123, or rhodamine B before affinity pull-down.

The strongest band at 54 kDa completely disappeared upon competition with unmodified I25 or I31, but not with rhodamine 123 or rhodamine B, which were included as structurally similar controls. Thus, we concluded that the 54 kDa band was the most convincing binding target protein of the compounds. The band was excised, sequenced, and identified to be tubulin (Supplementary Note 1 online).

While affinity pull-down assay identified the major binder to be tubulin, a well-known cytosolic protein, our compounds appeared to be localized to mitochondria in live cells. Affinity-based isolation greatly depends on protein abundace as well as protein binding affinity. Since tubulin is a highly abundant protein in cells (10 ~ 20 μM),[15] despite the intrinsic affinity, its isolation might be an artifact. Therefore, we further explored the endogenus binding protein in the context of live cells.

For live-cell investigation, we synthesized a cell-permeable chemical affinity derivative, which has a thiol reactive chloroacetyl group, to enable covalent binding to target proteins (Fig. 2c). The compound is named as CDy2, **C**ompound of **D**esignation **y**ellow **2**, following the biological convention of Cluster of Designation (CD) for cell-surface markers. The benefit of the chemical affinity probe is that once it forms a covalent bond with its targets in live cells. Those labeled proteins can be visualized by scanning the SDS-PAGE gel with a fluorescence scanner, even though the proteins are denatured.

When applied to myoblasts and myotubes, CDy2 showed a 2.3-fold increase in fluorescence intensity after differentiation (2.3 ± 0.4 fold increase, $N = 3$; Fig. 2d), which is comparable to the increases observed with I25 and I31. To unveil the endogenus binding protein(s), myoblasts or myotubes were incubated with CDy2 for 1 hour. Labeled lysates were

separated by SDS-PAGE and analyzed with a fluorescence scanner (Fig. 2d). Again, a unique fluorescently labeled band was observed around 54 kDa (Fig. 2e). Also, pre-treatment of myotubes with I31 reduced the intensity of CDy2-labeled protein band, indicating effective competition of CDy2 with I31 in live cells (Supplementary Fig. 3b online). To determine the identity of the labeled protein, cell lysates were separated by 2D-gel electrophoresis, and fluorescently labeled spots around 54 kDa were excised and sequenced (Fig. 2f; Supplementary Note 2 online). To our surprise, the major spots were found to be mitochondrial aldehyde dehydrogenase (ALDH2), not tubulin.

To validate the binding in live cells, firstly ALDH2 or tubulin was overexpressed in HEK cells. Each protein was tagged with green fluorescent protein (GFP) to distinguish those from the endogenous proteins. Forty-eight hours after transfection, cells were labeled with CDy2 and each lysate was subjected to SDS-PAGE analysis (Fig. 3a); the HEK293 cell line was chosen because of its relatively high transfection efficiency. The result shows that CDy2 labeled ALDH2-GFP, but not tubulin-GFP. Secondly, ALDH2 expression was suppressed by siRNA (Fig. 3b). Upon ALDH2 knock-down, the CDy2-labeled band (54 kDa) was dramatically reduced. These siRNA and overexpression experiments clearly indicate that CDy2 selectively binds to ALDH2 in living cells. However, when treated to cell lysates, CDy2 labeled tubulin instead of ALDH2 (Supplementary Fig. 4d online). Altogether, these results suggest that CDy2 binds to two distinct proteins depending on the experimental environment.

In-gel fluorescence analysis showed that CDy2 labels ALDH2 stronger in myotubes over myoblast (Fig. 2e). Interestingly, however, the total amount of ALDH2 remained unchanged before and after differentiation. Thus it was necessary to determine the mechanism of CDy2 selectivity for myotubes. For example, the mitochondrial membrane potential of skeletal muscle is quite high, possibly due to increased energy requirements for muscle contractions;[13] this elevated membrane potential may cause the myotube-selectivity of CDy2. In fact, when cells were fixed with formaldehyde, CDy2 lost its mitochondrial preference, as well as its selectivity for myotubes (Supplementary Fig. 4a online). Further, the mitochondrial membrane potential was disrupted by treating cells with the mitochondrial uncoupler CCCP (carbonyl cyanide 3-chlorophenylhydrazone).[16] Upon pre-treatment with CCCP, the amount of fluorescently labeled protein was significantly reduced (Supplementary Fig. 4c online). These results support the notion that an increase in the mitochondrial membrane potential as a result of myogenesis gives rise to the selectivity of CDy2 for myotubes.

Rosamine compounds are derivatives of rhodamine, which have long been used as mitochondrial probes. Their aromatic and cationic properties direct them to mitochondria due to the membrane potential across its bilayer.[17] The rosamine probes are sensitive to the incresed membrane potential after myogenic differentiation. Once localized in mitochondria, they labeled ALDH2 selectively. Although ALDH2 itself is not a differentiation marker, the selectivity to rosamine probes deserves careful consideration. Up until now, it has been generally believed that rhodamine dyes stain the mitochondrial membrane without showing specific interactions with mitochondrial proteins.[18]

A cell is a highly ordered structure,[19] where small-molecule localization is precisely controlled based on chemical properties. Once a small molecule is sequestered in an organelle such as mitochondria, its interaction with proteins will be greatly limited within the cellular compartment. In this study, CDy2 showed an apparent binding affinity to tubulin *in vitro*, but resulted in binding to ALDH2 in live cells. This implies that CDy2 molecules are sequestered in mitochondria rapidly before they have a chance to react with tubulin in the cytoplasm. Elucidating small molecule's binding protein is the most challenging part of chemical genetics work. Our observations cast important warning that the binding partner should be carefully evaluated in the context of the environmental factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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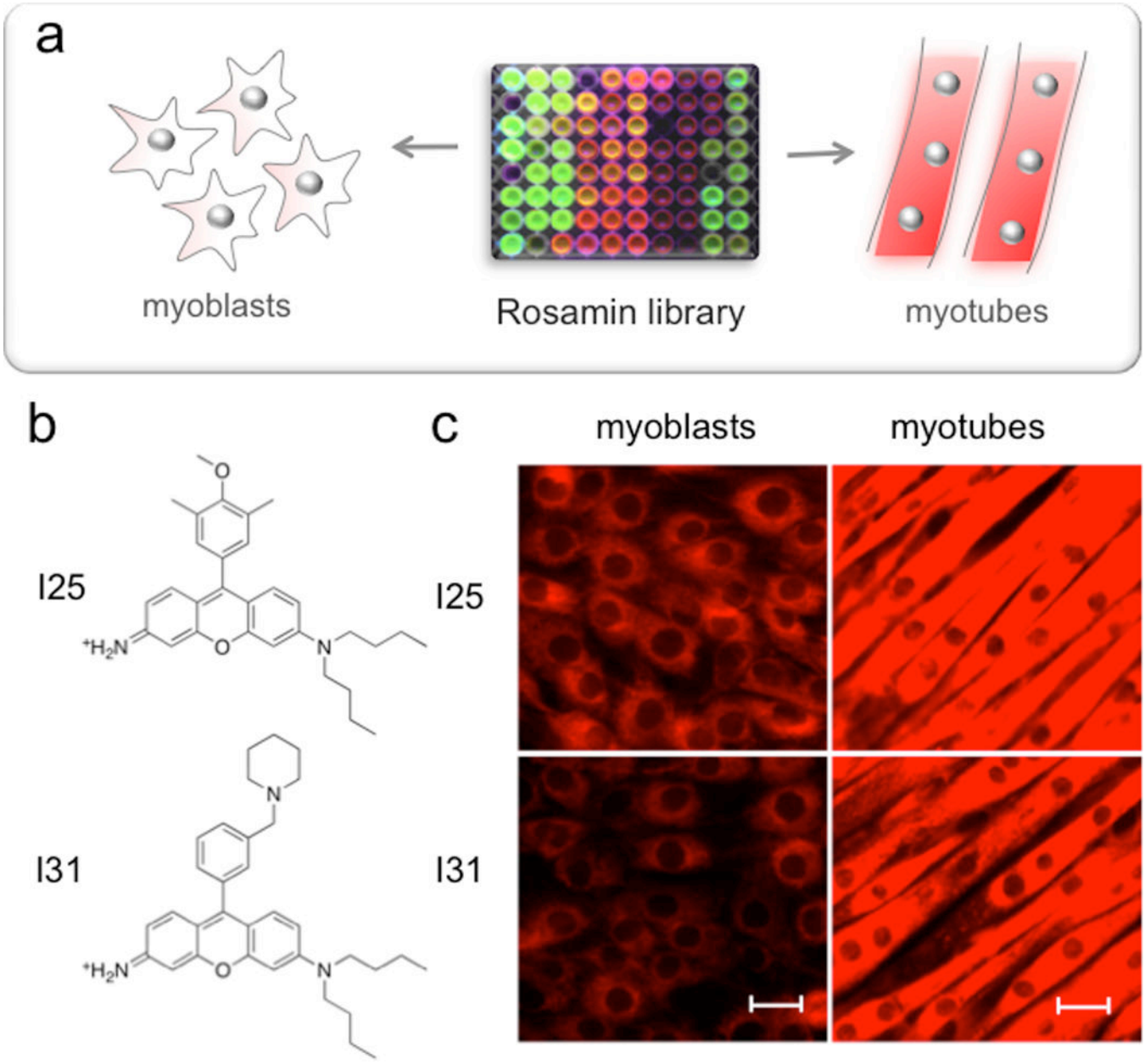

Figure 1. Probes for myogenic differentiation

(**a**) Screen of rosamine library. Myoblasts or myotubes were incubated with 500 nM of library compounds for 2 hours before imaged (**b**) Chemical structures of selected probes, I25 and I31. (**c**) Fluorescent images of I25 and I31 before (right) and after (left) muscle differentiation. Scale bar = 20 μm.

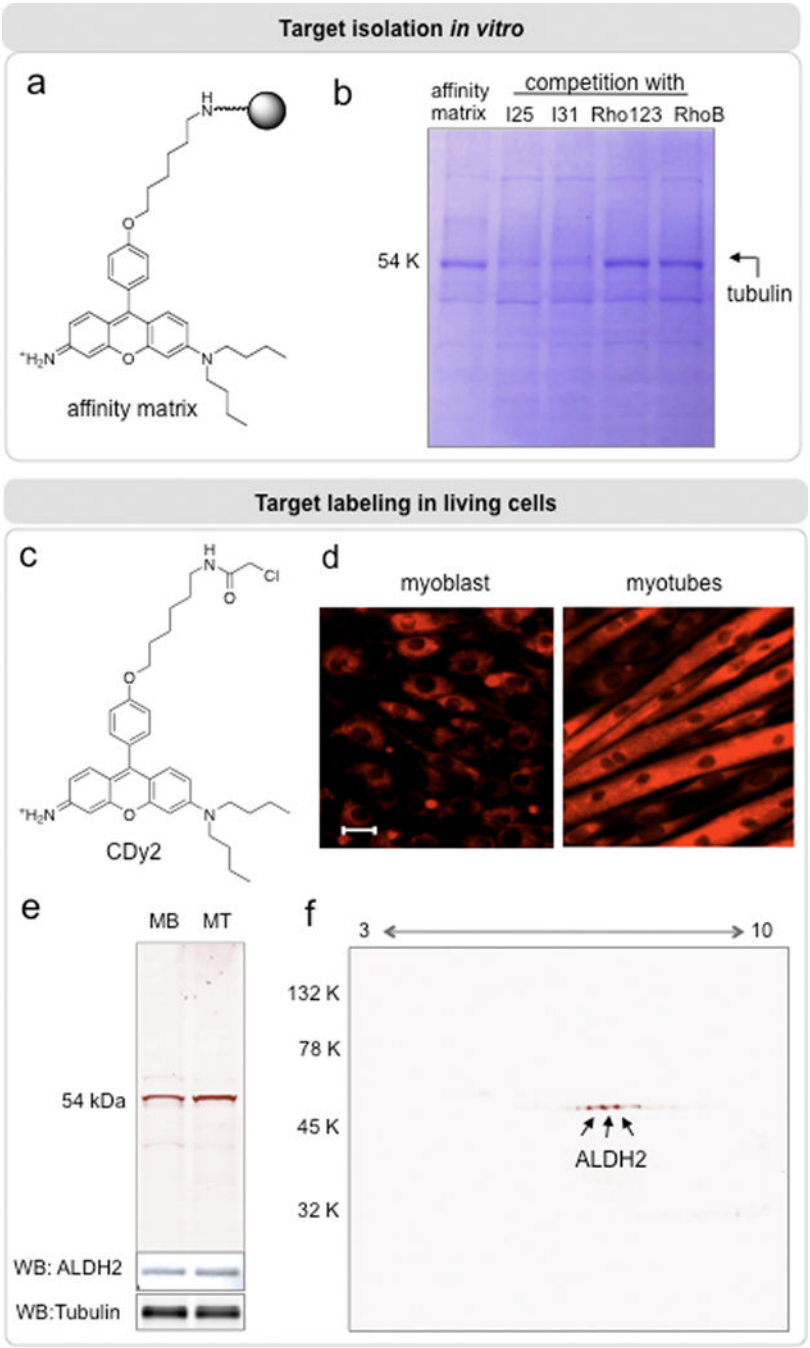

Figure 2. Identification of protein-binders *in vitro* vs. in living cells

(a) Chemical structure of the affinity matrix used to isolate cellular proteins. (**b**) SDS-PAGE analysis of bead-bound proteins from C2C12 myotubes. In a competition assay, myotube lysates were pre-incubated with 100 μM of I25, I31, or control compounds (rhodamine 123 and rhodamine B) before the affinity pull-down experiment. (**c**) Chemical structure of CDy2 for labeling target protein in living cells. (**d**) Myoblasts (MB) or myotubes (MT) were incubated with CDy2 (500 nM) for 30 min and imaged with a fluorescent microscope. Then,

cells were lysed for in-gel fluorescence analysis (λ_{ex} = 530 nm, λ_{ex} = 580 nm) (**e**). (**f**) 2D-gel analysis for the identification of labeled-protein.

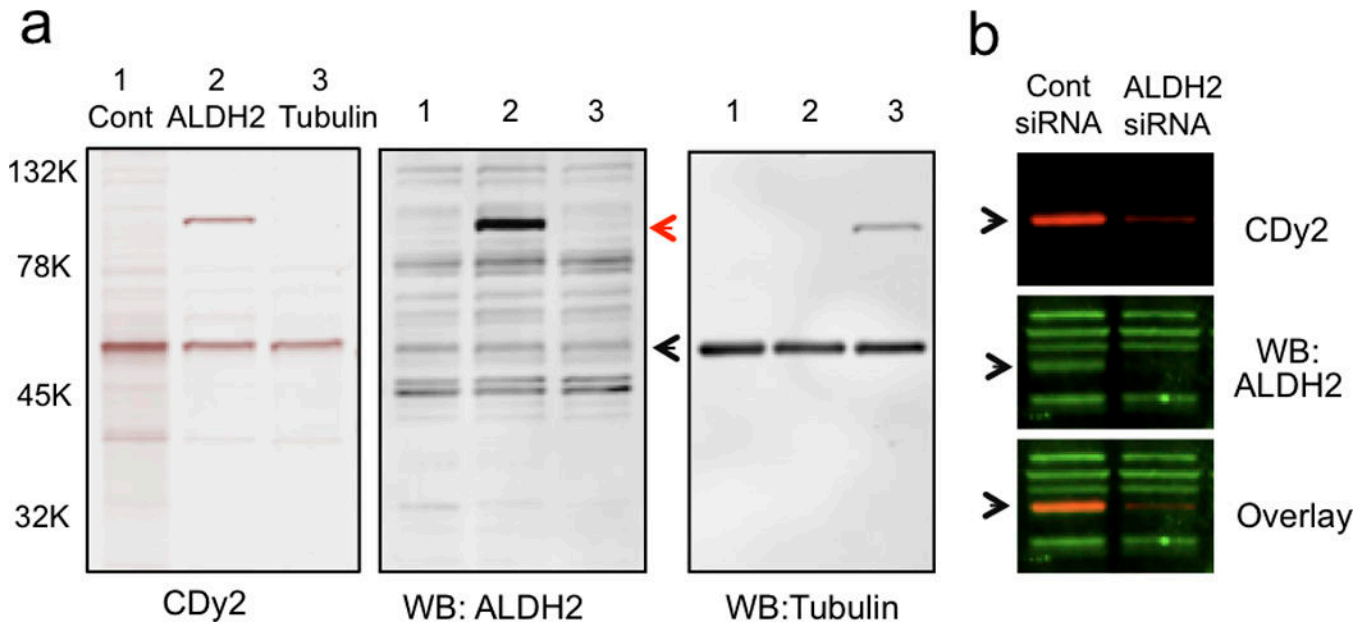

Figure 3. Validation of labeled protein identity in living cells

(**a**) GFP-tagged ALDH2 or tubulin constructs were transfected into HEK293 cells. After 48 hours, cells were labeled with CDy2 and subjected to in-gel fluorescence imaging. Immunoblot analysis shows the endogenous (black arrow) and over-expressed (red arrow) proteins. (**b**) C2C12 myoblasts were transfected with siRNA against ALDH2. After 72 hours of transfection, cells were labeled with CDy2. Fluorescence labeling patterns of CDy2 were directly compared to ALDH2 immunofluororescence (green).