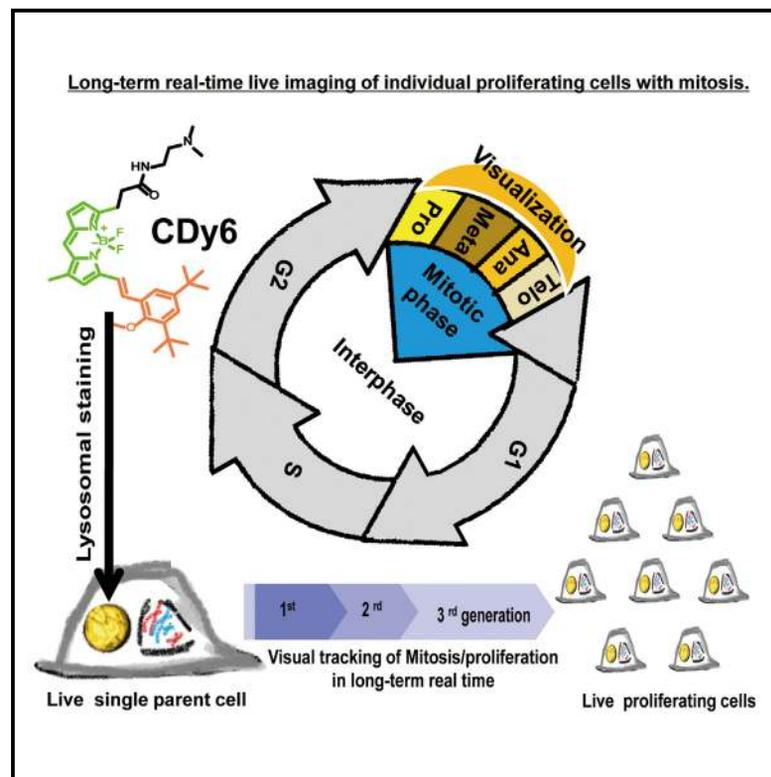


Chemistry & Biology

CDy6, a Photostable Probe for Long-Term Real-Time Visualization of Mitosis and Proliferating Cells

Graphical Abstract



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In Brief

Jeong et al. describe CDy6, a dye to mark mitotic cells. CDy6 displays numerous favorable properties including showing a sharp increase in intensity and change in localization in mitosis, improved photostability, and decreased toxicity compared with other widely used lysosomal markers in long-term real-time imaging.

Highlights

- CDy6, a BODIPY-derived compound of designation yellow 6, labels lysosome
- Fluorescence of CDy6 displays high sensitivity to acidity (pH 2–4)
- CDy6 permits visual tracking of lysosomal dynamics from mitosis to proliferation
- CDy6 exhibits excellent photostability *in vitro* and *in vivo* for long-term imaging



CDy6, a Photostable Probe for Long-Term Real-Time Visualization of Mitosis and Proliferating Cells

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SUMMARY

Long-term real-time visualization of lysosomal dynamics has been challenging at the onset of mitosis due to the lack of fluorescent probes enabling convenient imaging of dividing cells. We developed a long-term real-time photostable mitotic or proliferating marker, CDy6, a BODIPY-derived compound of designation yellow 6, which labels lysosome. In long-term real-time, CDy6 displayed a sharp increase in intensity and change in localization in mitosis, improved photostability, and decreased toxicity compared with other widely used lysosomal and DNA markers, and the ability to label cells in mouse xenograft models. Therefore, CDy6 may open new possibilities to target and trace lysosomal contents during mitosis and to monitor cell proliferation, which can further our knowledge of the basic underlying biological mechanisms in the management of cancer.

INTRODUCTION

Mitosis is one of the most remarkable events in cell biology (Santos et al., 2012; Mitchison and Salmon, 2001). In general, mitosis is broken down into five stages: prophase, metaphase, anaphase, telophase, and cytokinesis. When a mammalian cell enters mitosis, the cell condenses its chromosomes, undergoes nuclear envelope breakdown, reorganizes its microtubules into a spindle, and splits into two identical daughter cells (Webster et al., 2009). Two major challenges for the twenty-first century have been described in relation to mitosis: first, determining how the cell-cycle network operates dynamically and spatially within live cells (Mitchison and Salmon, 2001; Nurse, 2000; Tsien, 2003); second, determining how ordered inheritance of organelles responds to the changes in dividing cells (Mitchison and Salmon, 2001; Nurse, 2000; Tsien, 2003). Recently, fluorescence microscopy and time-lapse imaging have become widely used due to the increasing availability of fluorescent dyes. Modern probes are noninvasive, nonradioactive, and highly cost-effective, and offer high resolution, high sensitivity, and simple operation in comparison with conventional imaging modalities

(Méchali and Lutzmann, 2008; Wiedenmann et al., 2009; Dzyubachyk et al., 2010; Dean and Palmer, 2014). Therefore, time-lapse imaging should play an important role in addressing these questions through direct observation of living cells with specific fluorescent organelle probes.

In order to identify cells in mitosis, current approaches require the uptake of bromodeoxyuridine (BrdU) or [³H]thymidine, immunostaining of fixed cells with cell-cycle-specific antibodies, or fluorescently tagged cell-cycle reporter vectors (Hesse et al., 2012). Cell fixation is often undesirable; it does not permit monitoring of the real-time dynamic behaviors of the cell cycle. To overcome this problem, several techniques have been developed for tracking cell-cycle progression in live cells using fluorescently tagged cell-cycle reporter vectors, such as eGFP-anilin (Hesse et al., 2012), Fucci reporter (Méchali and Lutzmann, 2008; Newman and Zhang, 2008), and GFP-histone H2B (Kanda et al., 1998), but these require engineering the cell line and expression efficiency, and consistency can be a problem (Wiedenmann et al., 2009).

Lysosomes are dynamic organelles receiving membrane traffic inputs from biosynthetic, endocytic, and autophagic pathways in mammalian cells (Warren and Wickner, 1996). The dynamic lysosome changes at the onset of mitosis are not well understood, although these are of strong interest in studies of autophagy/mitophagy (Liu et al., 2009), lysosome-chromatin interactions (Ivanov et al., 2013), and the role of endosomes/lysosomes in mitosis (Bergeland et al., 2001; Pohl, 2009). In this study, we investigated the development of a photostable lysosomal-targeting fluorescent probe for long-term real-time visualization of mitotic progression and cell proliferation in live cells. Using a mitotic-cell-based high-throughput screen (HTS) with 6,000 fluorescent small molecule compounds, CDy6 was discovered and characterized with functional validation, and potential application in vitro and in vivo. Our findings suggest that CDy6 might be applied for identification of mitotic cells and long-term real-time visualization of proliferating cells both in vitro and in vivo.

RESULTS

Identification of the Lysosomal-Targeting Fluorescent Probe CDy6

HTS can lead to fast and cost-effective discovery of imaging probes (Manning et al., 2008). To develop sensitive and

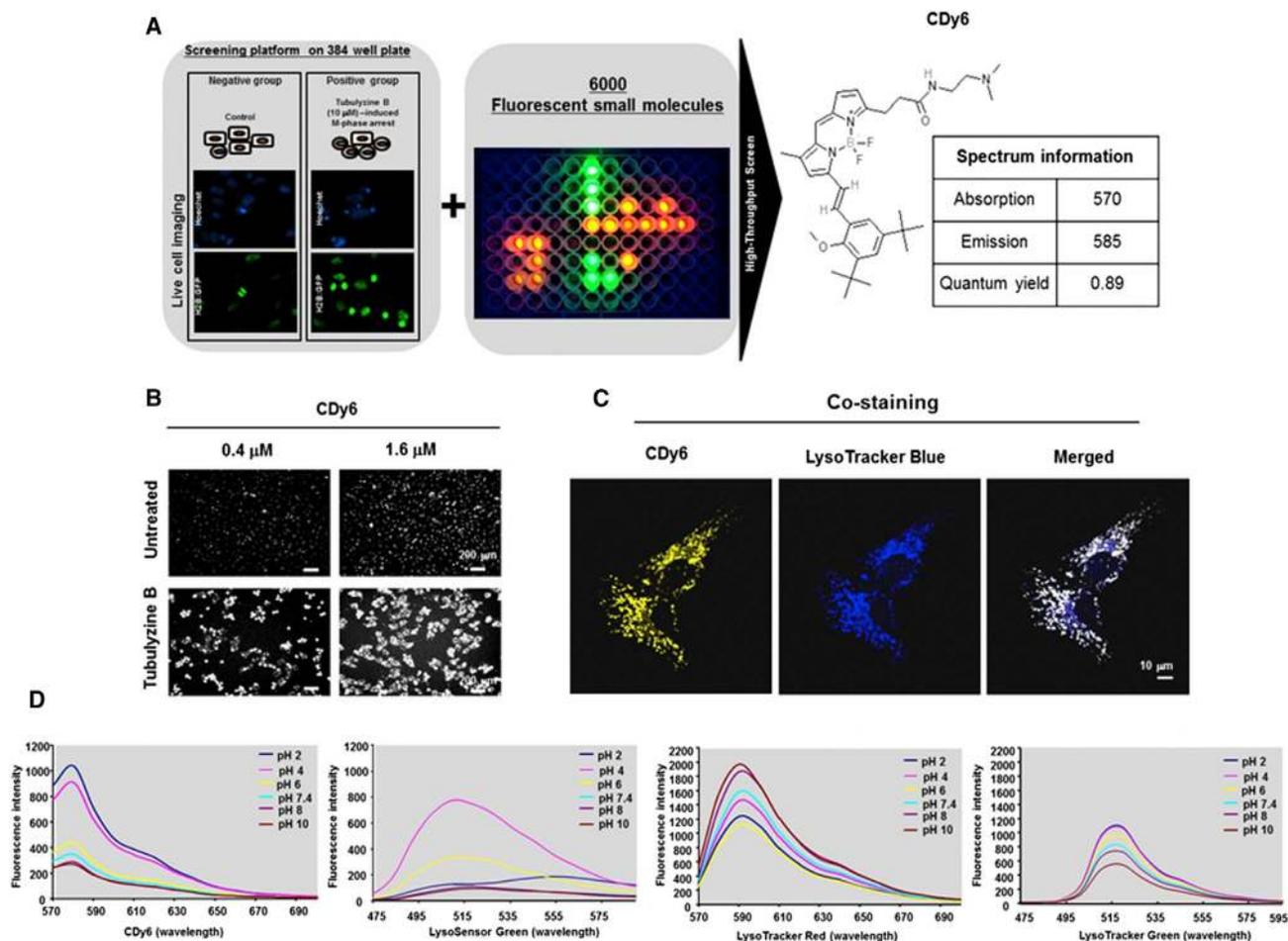


Figure 1. Discovery of CDy6 Probe

(A) Left panel: Scheme of HTS based on tubulysin B-induced M-phase arrest. Right panel: Chemical structure and normalized absorption of CDy6. (B) Live-cell imaging of CDy6-stained RPE1:H2B:GFP cells at various doses. Scale bars, 200 μ m. (C) Colocalization patterns for CDy6 (yellow) and LysoTracker Blue (blue). (D) pH sensitivity at pH 2–10 for CDy6, LysoTracker Red, LysoSensor Green, and LysoTracker Green.

easy-to-apply fluorescent probes for real-time imaging of mitosis, we established a fluorescence-based HTS assay utilizing the M-phase blocker tubulysin B (Figure 1A; Figure S1A). We used the RPE1 cell line stably expressing GFP-tagged histone 2B (H2B-GFP) as a positive marker of cell division. H2B-GFP fluorescence is located exclusively in condensed chromosomes, enabling sensitive analysis of cell-cycle dynamics in living mammalian cells (Kanda et al., 1998). This primary assay isolated 132 hits exhibiting increased staining of M-phase-arrested cells compared with asynchronous cells (data not shown), which were then further tested in RPE1:H2B:GFP cells to evaluate their reproducibility. We selected CDy6 for its high selectivity in both flow cytometry (data not shown) and imaging in M-phase-arrested conditions (Figure 1B). CDy6 is based on 4,4-difluoro-r-bora-3a,4a-diaza-s-indacene (BODIPY) with excitation/emission wavelengths of 570/585 nm (Figure 1A; Figure S2A). The extinction coefficient of CDy6 is 20,952 in aqueous condition (PBS buffer, pH 7.4) and 80,150 in organic solvent (ethanol) (Figure S2B).

Confocal microscopy shows CDy6 as localized in the cytoplasm, in the form of bright vesicles (Figure 1C; Figure S2C). To identify the CDy6-stained vesicles in live cells, dual labeling was performed using LysoTracker Blue, MitoTracker, and ER-Tracker. The near-perfect colocalization of CDy6 and LysoTracker Blue (Figure 1C) and the lack of overlap with MitoTracker and ER-Tracker (Figures S2D and S2E) indicate that CDy6 primarily stains lysosomal vesicles. It is well known that LysoSensors are cell-permeable weak bases that selectively accumulate in acidic vesicles after being protonated. LysoSensors have higher quantum yields at lower pH ranges to allow visualization of the lysosomes (Lin et al., 2001). Based on this concept, we measured pH-dependent excitation and emission peaks of CDy6 compared with LysoTracker and LysoSensor at pH 2–10. pH sensitivity demonstrates that CDy6 has higher quantum yields under acidic conditions at pH 2–4 (Figure 1D; Figure S2B).

CDy6 Allows Complete Visual Tracking of Mitosis

To investigate whether CDy6 could be used to visualize mitosis in real time, RPE1:H2B:GFP cells were stained with CDy6

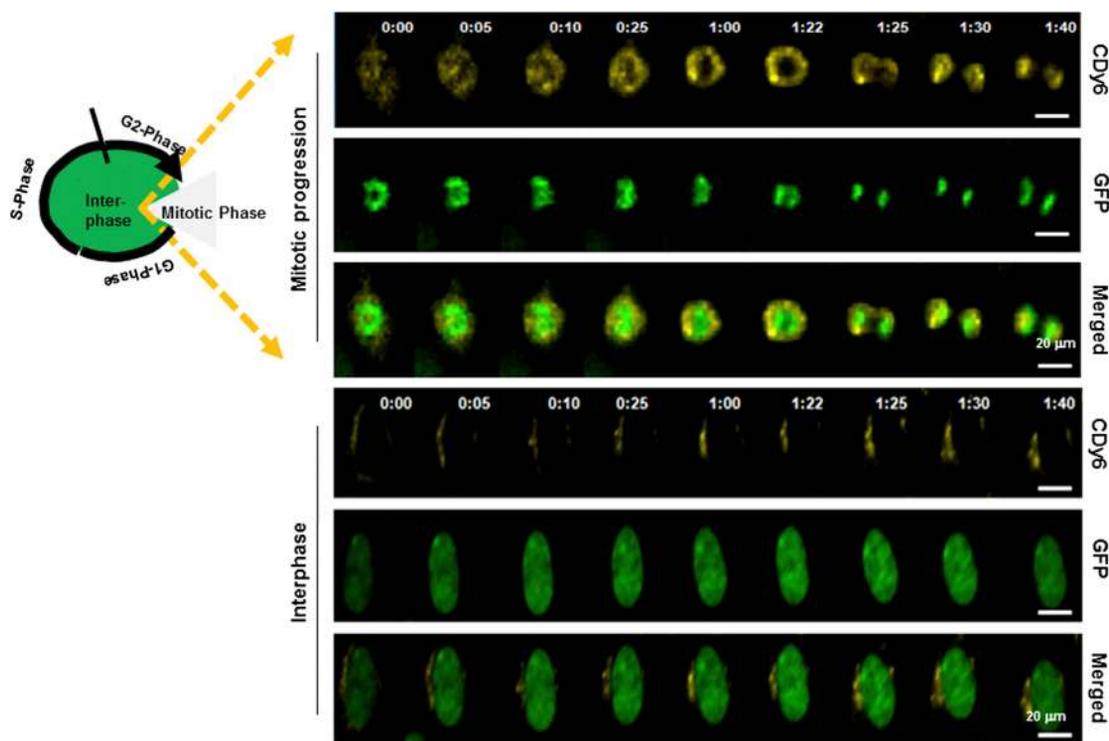


Figure 2. Lysosomal-Targeted CDy6 Labeling Effectively Visualizes Mitosis in Real-Time Live Imaging

RPE1:H2B:GFP cells were stained with CDy6 (0.5 μ M), and imaged for 8 hr. The H2B:GFP signal was used as a positive selection marker of cell division. Scale bars, 20 μ m.

and subjected to time-lapse imaging. CDy6-stained live cells showed a dramatic change in fluorescence localization and gradual increase in intensity after the beginning of mitosis (Figure 2). Similar results were seen in RPE1, U2OS, and HeLa cells (Figure S3). We next quantitatively characterized the levels and localization of CDy6 in cells, particularly during mitosis (Figure 3), by tracking the cells in time lapse and measuring the amount of CDy6 staining in the nucleus (I_{nuc}) and cytoplasm (I_{cyto}) as well as the nucleus intensity from the brightfield channel of each cell over time (see Experimental Procedures). All three nucleus signals divide approximately in half as a result of cell division a few frames after the CDy6 nuclear signal reaches a maximum (Figures 3A–3C, frame 30), confirming our qualitative observation that CDy6 marks mitosis. In addition, the ratio between these two quantities (i.e., I_{nuc}/I_{cyto}) for CDy6 exhibits a sharp and narrow peak during mitosis, increasing by a factor of >10 during mitosis (Figures 3D and 3E), which is consistent with our qualitative observation of a mostly cytoplasmic localization shifting to an accumulation near the surrounding chromosome at mitosis (Figure S2C), visualized for a sample cell in Figure 3F.

Long-Term Real-Time Live Imaging of Individual Proliferating Cells with Excellent Photostability of CDy6 In Vitro and In Vivo

Long-term time-lapse imaging demonstrated that the high photostability of CDy6 enabled the visualization of lysosomal dynamics across several cell cycles of individual proliferating cells

over a 72 hr period (Figure 4). To elucidate whether other lysosomal-targeted fluorescent probes behave similarly to CDy6 in proliferating cells, we used the commercially available LysoTracker Red, whose excitation/emission spectrum and cellular distribution is reportedly similar to CDy6 (Pierzyńska-Mach et al., 2014) (Figure S2D). In contrast to CDy6, the LysoTracker Red fluorescent signal decreased dramatically during imaging, with almost no fluorescence signal remaining after the beginning of the second mitotic event (Figure 5A). In addition, despite visualization of first mitosis using LysoSensor, the LysoSensor Green led to death after the first generation (Figure 5A). Of note, CDy6 was significantly more photostable than LysoTracker Red, LysoTracker Green, and LysoSensor Green at 72 hr (Figure 5B). Next, we tested the effects of CDy6 and Hoechst 33342 on RPE1 viability and cell proliferation. Imaging of Hoechst 33342 perturbs the cell cycle (Figure 5A). In contrast, CDy6 did not interfere with cell proliferation and subsequent progression through the cell cycle (Figures 5A, 5C, and 5D; Figure S4).

Since CDy6 exhibited strong photostability in proliferating cells, we next tested its performance in a whole animal study. The current and widely used organic fluorophores lack photostability for long-term and continuous imaging in vitro and in vivo (Wiedenmann et al., 2009). We tested the photostability of CDy6 and LysoTracker Red using a mouse tumor xenograft model. HeLa cells were stained with and without CDy6 or LysoTracker Red and injected subcutaneously to the right and left rear flanks of the BALB/c nude mice. In vivo images demonstrated that CDy6-stained HeLa cell seeding sites of mice showed very

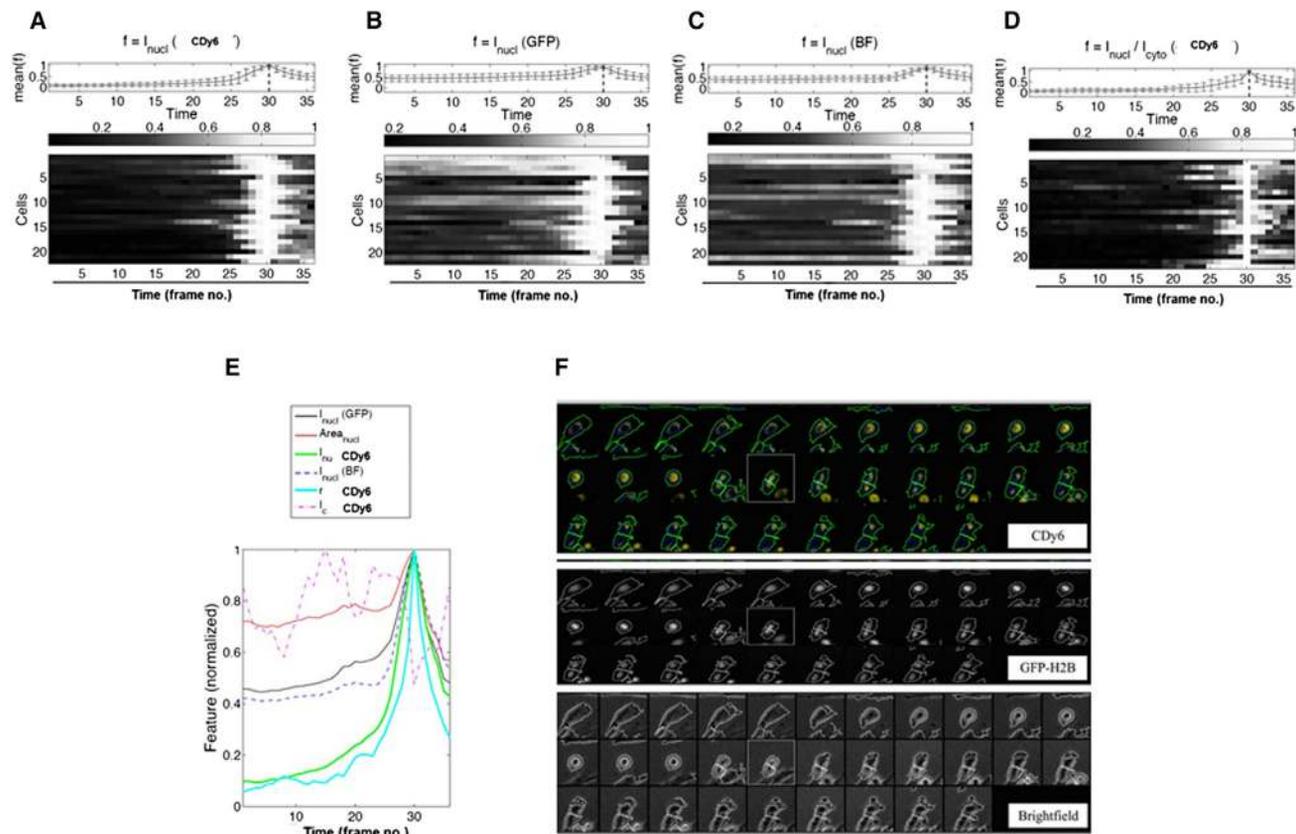


Figure 3. CDy6 Undergoes a Shift from Cytoplasmic to Nuclear Localization during Mitosis

(A–D) RPE1:H2B:GFP cells were stained with CDy6 (0.5 μM) for 72 hr. Time-lapse image series were acquired at 3 min intervals on a time-lapse imaging system Nikon Biostation IM (Nikon), as described in Experimental Procedures. Ten experiments were performed. The integrated intensity I_{nucl} is shown for (A) CDy6, (B) H2B:GFP, and (C) brightfield, and the ratio of CDy6 staining in the nucleus to the cytoplasm (i.e., $I_{\text{nucl}}/I_{\text{cyto}}$) for selected and synchronized mitotic cells (D); see Experimental Procedures. Upper panel: Plot of the average measurement from the selected cells across time over the selected cells; error bars indicate standard deviation. Cells were synchronized to $t = 30$ as indicated by the vertical dashed line.

(E) Comparison of the averaged intensities over the synchronized mitotic cells from (A)–(D).

(F) Image montages from a representative mitotic event (cell no. 4 in (A)–(C)). Image is pseudocolored in yellow for CDy6 (top), and in grayscale for H2B:GFP (middle) and brightfield (bottom). Blue and green outlines indicate the detected nuclei and cell boundaries, respectively.

stable fluorescence signals (Figure 6). We detected a clear CDy6 fluorescent sign in xenograft-bearing nude mice up to 21 days after injection of CDy6-stained HeLa cells (Figure 6B). On the other hand, the LysoTracker Red fluorescent signal disappeared in xenograft-bearing nude mice up to 12 hr after injection of LysoTracker Red-stained HeLa cells (Figure S5). This finding indicates that CDy6 can be used for the long-term tumor xenograft-targeted imaging and cell-tracking experiments in vivo.

DISCUSSION

Although mitosis plays a central role in the operation of all eukaryotes, to the best of our knowledge no photostable fluorescent probes were available that were capable of identifying live mitotic cells without significant cytotoxicity in long-term real-time live-cell imaging. In the present study, we describe a lysosomal-targeting fluorescent probe, CDy6. This probe provides several benefits for real-time and time-lapse imaging of mitosis. First, lysosomal-targeting CDy6 enables identification of mitotic

progression without nucleus staining in live cells via an easy and rapid protocol with 1 hr of incubation, without the need to engineer the cell line. Second, lysosomal dynamics of CDy6 labeling permit real-time visualization of proliferating cells without any cytotoxicity or inhibitory effect on the cell cycle for 72 hr. Lastly, CDy6 displays excellent photostability in vitro and in vivo for long-term imaging.

CDy6 is a BODIPY-derived compound of designation yellow 6, developed through a mitotic cell-based HTS of 6,000 small fluorescent molecules, based on changes in fluorescence intensity. CDy6-labeled mitotic arrested cells showed a significantly stronger fluorescent signal than asynchronous cells, and the signal colocalizes with LysoTracker Blue, which stains lysosomal vesicle-like compartments. In particular, CDy6 contains a weakly basic N,N-ethylenediamine bridging unit like that of LysoTracker and LysoSensor. Of note, by comparison of pH changes with LysoTracker and LysoSensor, the fluorescence increase of CDy6 was more highly selective than LysoSensor and LysoTracker at acidic pH 2 and pH 4 (Figure 1D). Thus, CDy6 is notable for its ability to track lysosomal dynamics arising from mitosis.

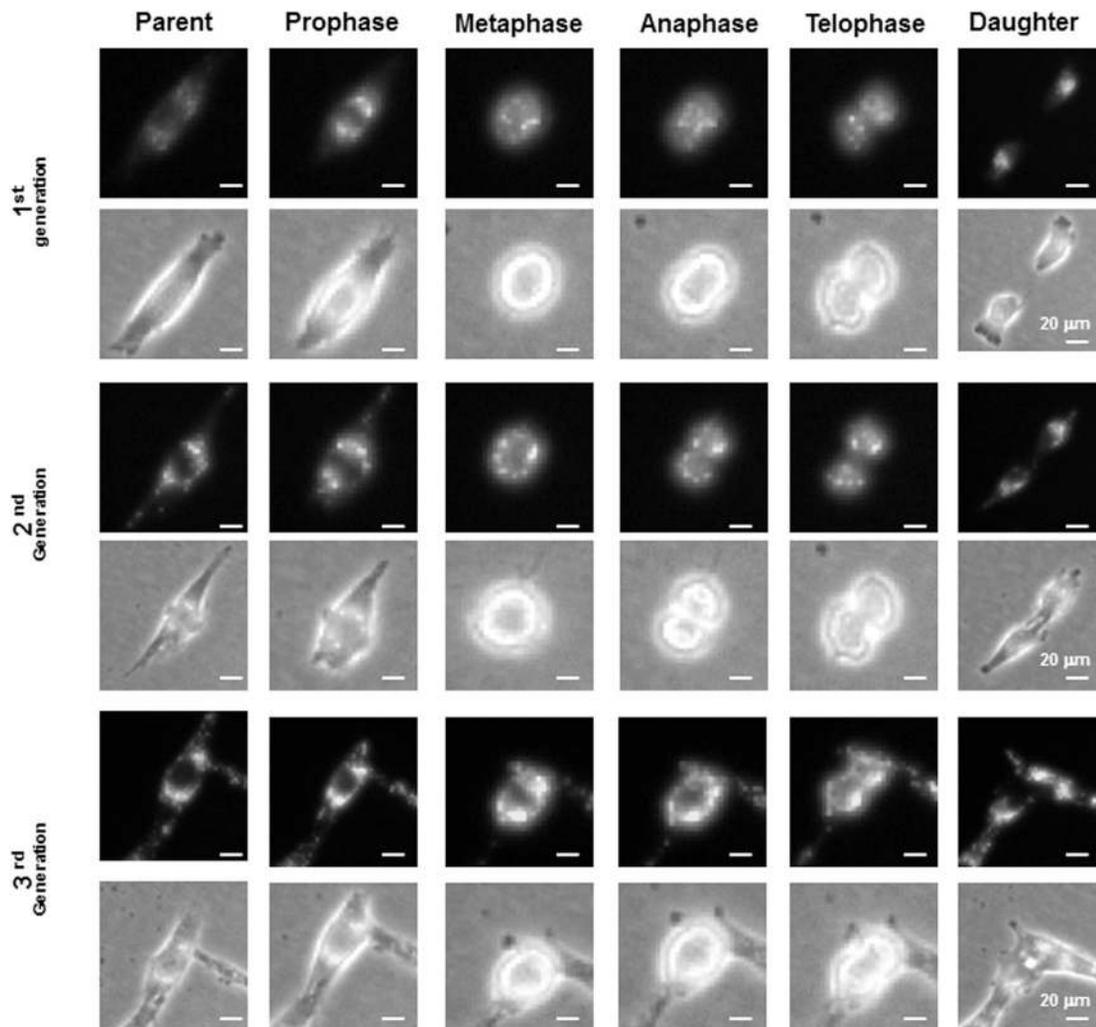


Figure 4. Real-Time Visualization of CDy6-Labeled Mitosis during Cell Proliferation

RPE1:H2B:GFP cells were stained with CDy6 (0.5 μ M), and the frames acquired at 3 min intervals over a period of 72 hr, as described in Experimental Procedures. Scale bars, 20 μ m.

Although mitotic progression can be visually distinguished from the G₁, S, and G₂ phases of the cell cycle and the redistribution of the chromosomes and organelles are well characterized (Mitchison and Salmon, 2001; Wareen and Wickner, 1996), the lysosomal dynamics at the onset of mitosis are still unclear. A prior study has suggested that the endosomes and lysosomes do not disintegrate during mitosis but are partitioned as separate, intact vesicles, with the endosome/lysosome copy number approximately halved after cell division (Bergeland et al., 2001). We observe that CDy6-labeled lysosomal vesicles are strongly localized near the DNA in mitotic cells, compared with a dim, cytoplasmic localization in interphase cells (Figures S2C–S2E). In live cells using time-lapse imaging, the fluorescent signal of CDy6 showed a remarkable change in localization and increase in intensity at the beginning of mitosis. These results suggest that CDy6 would be useful to study how the processing of lysosomal-related vesicles is involved in mitotic events. In addition, CDy6 could be used to detect mitotic events because the cytoplasm/nucleus ratio for CDy6 increases by more than a

factor of ten during mitosis. The long-standing enthusiasm for in vitro and in vivo microscopy derives from the ability to observe biological phenomena evolve in real time under physiological conditions (Tsien, 2003; Méchali and Lutzmann, 2008; Wiedenmann et al., 2009; Dzyubachyk et al., 2010; Dean and Palmer, 2014). In particular, time-lapse imaging techniques are essential for understanding how the cell cycle network operates dynamically during proliferation and how organelles are inherited during cell division (Dzyubachyk et al., 2010; Dean and Palmer, 2014). CDy6 offers advantages over previous markers for these studies. For example, tracing cell proliferation using BrdU requires fixation and does not permit the real-time visualization of individual dividing cells. eGFP-anillin (Hesse et al., 2012), H2B:GFP (Kanda et al., 1998), and Fucci reporters (Newman and Zhang, 2008) require engineering a cell line which can be time consuming and disruptive to cells' functions, and expression levels can diminish during long-term culture (Wiedenmann et al., 2009). Several fluorescent probes designed to interact with inorganic ions, metals, thiols, and sulfides have been developed to allow

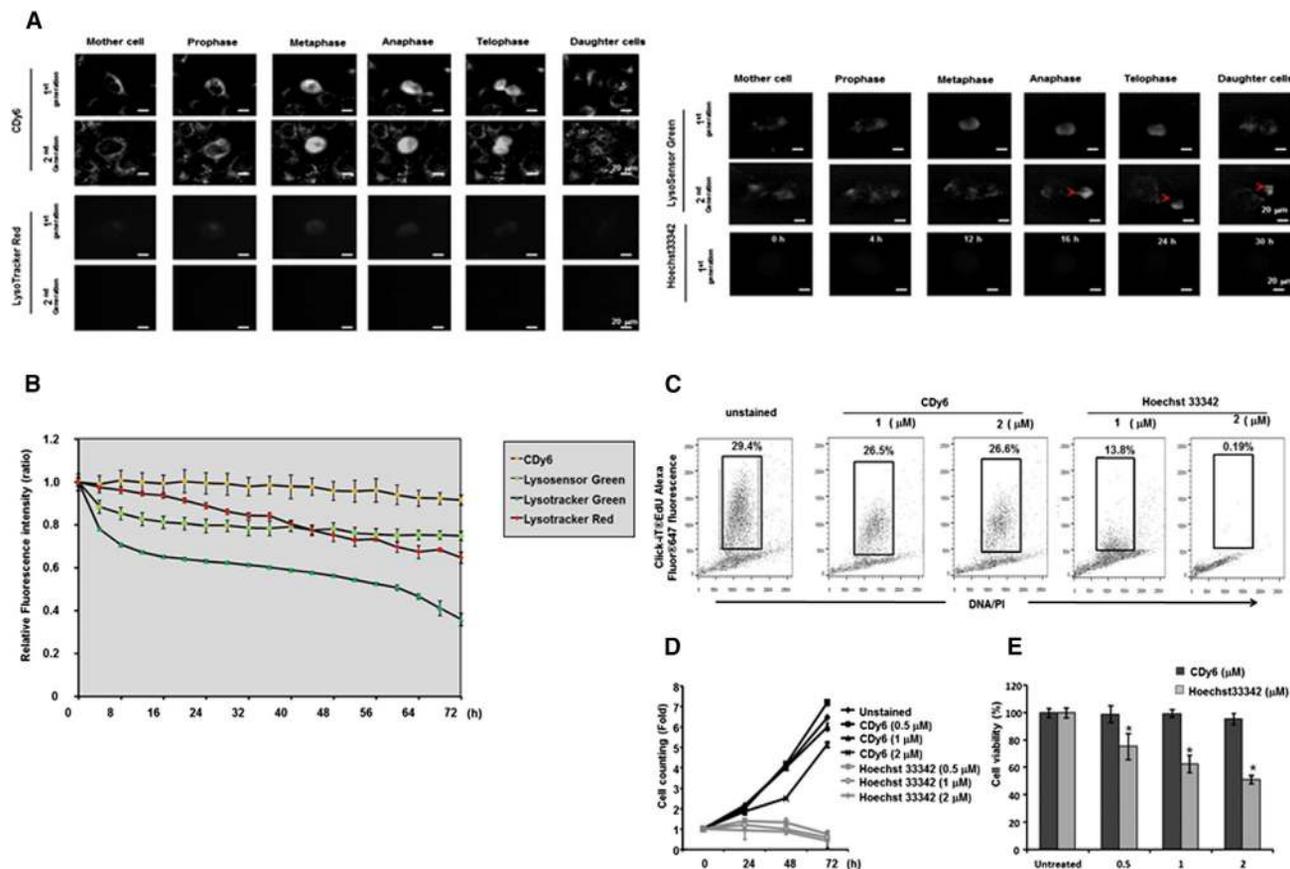


Figure 5. Long-Term Time-Lapse Live-Cell Imaging of CDy6 Shows More Stable Photostability Than in LysoSensor Green, LysoTracker Red, and Hoechst33342 during Cell Proliferation

(A) U2OS cells were stained with CDy6 (0.5 μM), LysoTracker Red (1 μM), LysoSensor Green (1 μM), or Hoechst 33342 (1 μM), and images were acquired at 3 min intervals over a period of 50 hr (scale bars, 20 μm). Only CDy6 displayed brightness after the second generation. LysoSensor showed the first mitosis, then the cells finally died prior to the beginning of mitosis in the second generation. Red arrowhead indicates a dead cell.

(B) Photostability of CDy6, compared with LysoTracker Red, LysoSensor Green, and LysoTracker Green. CDy6, LysoTracker Green, LysoSensor Green, and LysoTracker Red at 5 μM in phenol red complete media were placed in a 96-well black opaque plate. Photostability of fluorescence was recorded every 4 hr for 72 hr (two different excitation/emission spectra: 480–520 nm and 560–590 nm) using a spectraMaxM2 microplate reader. 0.5% DMSO was the control. The representative graph was normalized to control signal at time = 0.

(C and D) Effect of CDy6 on cell proliferation. RPE1 cells were stained with the indicated doses of CDy6 for indicated time points, and the cell proliferation rate was measured by manual counting of cells under microscopic observation and EdU assay; see Experimental Procedures.

(E) The effect of CDy6 or Hoechst 33342 on cell viability. p Values (*) of less than 0.01 were considered significant.

rapid, effective, and bright live-cell imaging; they tend to have higher cytotoxicity or low photostability for long-term and continuous time-lapse imaging in vitro and in vivo (Pierzyńska-Mach et al., 2014; Purschke et al., 2010). For example, Hoechst 33342 is the most popular dye used to visualize chromatin in live cells by fluorescence microscopy, but is phototoxic (Purschke et al., 2010). LysoTracker Red is less phototoxic, but its rapid photobleaching limits the range of useful applications considerably for long-term imaging (Pierzyńska-Mach et al., 2014). The present study demonstrates the characteristics and potential application of CDy6 in vitro and in vivo. Time-lapse imaging of CDy6 labeling offers a unique opportunity for real-time visualization of lysosomal dynamics after the onset of mitosis during cell proliferation. CDy6 labeling is also straightforward; this is precisely why it is interesting as a step forward in visual tracking of the cell-cycle progression of living dividing cells

in automatic long-term real-time imaging. We suggest that our results may have a powerful impact on future studies relating to the monitoring of lysosomal contents during cell proliferation, creating an attractive avenue for future therapeutic approaches to cancer in general.

SIGNIFICANCE

In recent years, fluorescence-based probes have been widely used for real-time analysis of various biological events within cell cycles in fluorescence microscopy, fluorescence immunology, and flow cytometry. Although the numbers of available fluorescent probes for live-cell imaging have increased rapidly, photostable fluorescent probes that are able to detect mitotic phase and cell proliferation in real time have not been reported. Hence, we have developed a

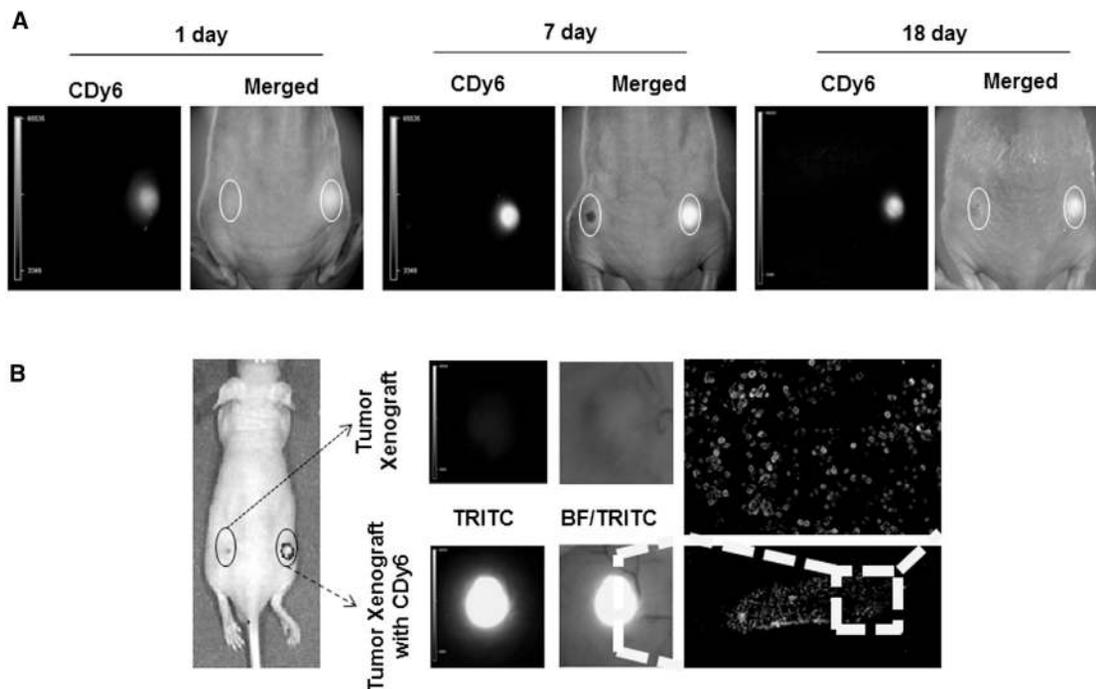


Figure 6. The Survival Signal of CDy6-Stained HeLa Cells in Tumor Xenograft

(A) HeLa cells were stained with and without CDy6 (1 μ M) for 1 hr and injected subcutaneously to the right and left rear flanks of the BALB/c nude mice. Time-dependent fluorescence images were obtained with a Leica M205 FA fluorescent stereo microscope using a 3.95 \times objective lens with TRITC filter.

(B) Twenty-one days after injection of the cells, in vivo images were obtained using an IVIS spectrum imaging system (Caliper Life Sciences) in the Biological Resource Center.

photostable lysosomal-targeting cytoplasmic fluorescent probe for long-term real-time visualization of mitosis and proliferation in living cells, named CDy6. By utilizing a weak base such as an N,N-ethylenediamine bridging unit, CDy6 specifically recognizes lysosomal vesicles. Using CDy6, we demonstrated that lysosomal vesicles are retained in mitotic cell division during cell proliferation. By comparison with LysoTracker and LysoSenser, CDy6 probe was also demonstrated to be useful in validating a time-lapse real-time visualization of proliferating cells that modulates the lysosomal dynamics between parent and daughter cell. Therefore, CDy6 is a tool not only for investigating the volume and activity of lysosomal compartment at the onset of mitotic progression but also for evaluating temporal analysis of dramatic lysosomal changes in various biological behaviors. Recent papers demonstrated the importance of lysosome-mediated cell death as a target for an anticancer drug discovery (Pohl, 2009; Ivanov et al., 2013; Liu et al., 2009). We suggest that CDy6 can be used as a reliable tool for gaining deeper insights into the biological pathway of lysosomes, and promises to be an attractive avenue for future therapeutic approaches to cancer.

EXPERIMENTAL PROCEDURES

Chemicals and Cell Culture

The reagents Hoechst 33342 (1:5000, H1399), LysoTracker Red DND-99 (referred to as LysoTracker Red; 1 μ M), LysoSensor Geen DND-189 (referred to as LysoSensor Green; 1 μ M), LysoTracker Geen DND-26 (referred to as

LysoTracker Green; 1 μ M), LysoTracker Blue DND-22 (referred to as LysoTracker Blue; 1 μ M), MitoTracker Deep Red FM (referred to as MitoTracker; 1 μ M), and ER-Tracker Blue-White DPX (referred to as ER-Tracker; 1 μ M) were purchased from Life Technologies (Carlsbad, CA, USA). Tubulysin B (Kim et al., 2006) and CDy6 (Figure 1A; Figure S2A) were contributed by the Department of Chemistry at the National University of Singapore. A stock solution of CDy6, LysoTracker Red, LysoSensor Green, and LysoTracker Green were first prepared in PBS buffer at a concentration of 0.1 mM, then diluted with pH buffers to a final concentration of 20 μ M. The fluorescence excitation and emission spectra were measured using a Spectra Max M2 plate reader (Molecular Devices Corp, USA). The human osteosarcoma U2OS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 8% fetal bovine serum (FBS)/1% penicillin and streptomycin (PS) at 37 $^{\circ}$ C with 5% CO₂. Human retinal pigmented epithelial cells stably expressing histone H2B fused to GFP (RPE1:H2B:GFP), were prepared as previously described (Kanda et al., 1998), and were cultured in DMEM supplemented with 10% FBS/1% PS at 37 $^{\circ}$ C with 5% CO₂. RPE1 cells (hTERT retinal pigmented epithelial cell line, American Type Culture Collection [ATCC] strain CRL-4000) were maintained in ATCC-formulated DMEM F12 1:1 supplemented with 10% FBS with 0.01 mg/ml hygromycin B (Life Technologies). HeLa cells were purchased from ATCC and grown in DMEM media supplemented with 10% FBS/1% PS at 37 $^{\circ}$ C with 5% CO₂.

Live-Cell Protocol and Time-Lapse Imaging

Live-cell imaging experiments were performed on a Nikon ECLIPSE Ti microscope using a tetramethylrhodamine isothiocyanate (TRITC)/fluorescein isothiocyanate filter or an inverted Nikon A1R+ confocal laser microscope system using 562/672/405 nm lasers with Plan Apo TIRF 100 \times DiC oil H H2 objectives (Nikon Instruments, Japan). Nuclei were stained with Hoechst 33342 (5 mg/ml stock, 1:5000 dilution to 1 μ g/ml). Time-lapse imaging of CDy6 was performed with a Nikon Biostation IM (10 \times objective/TRITC filter) or Nikon ECLIPSE Ti microscope (10 \times objective/TRITC filter). Cells were seeded onto a 35 mm dish

(Nunc, Fisher Scientific, Tustin, CA, USA), then monitored using microscopy at the indicated time points. Image processing and overlay analysis were performed using NIS-Elements 3.10 software (Nikon Instruments) as previously described (Brito and Rieder, 2006; Crasta et al., 2012; Ye et al., 2011). In particular, long-term time-lapse image series were acquired such that cells were seeded onto a 35 mm dish, stained with CDy6 (0.5 μ M), LysoTracker Red (1 μ M), LysoSensor Green (1 μ M), and Hoechst 33342 (1 μ M) in complete medium, placed in a Nikon Biostation IM system (epifluorescence lamp, 6%; exposure time, 1/10 s; interval time, every 3 min for 50–72 hr at 37°C with 5% CO₂). Ten experiments were performed. A minimum of 200 cells was monitored per experiment. Comparison of relative fluorescence intensity in CDy6 and other positive dyes was accomplished using NIS-Elements 3.10 software. Time-lapse images of cell pairs ($n = 30$ pairs) in prophase, metaphase, anaphase, and telophase were collected, then the threshold level of analysis was adjusted to specifically highlight fluorescent-positive vesicles in metaphase cell pairs.

Quantitative Time-Lapse Characterization of CDy6

We quantitatively characterized the levels and localization of CDy6 in cells, particularly during mitosis. We used automated image analysis (CellProfiler [Kamentsky et al., 2011], www.cellprofiler.org) of time-lapse live-cell movies to measure the amount of CDy6 staining in the nucleus (I_{nuc}) and cytoplasm (I_{cyto}) of each cell over time. The GFP-histone channel was used to identify the nucleus for frame-to-frame tracking. All collected measurements were then normalized to a maximum value of 1, and a subset of cells from the recording were selected based on tracking fidelity for a sufficient length of time and the strength of the maximal I_{nuc} (CDy6) or maximal relative intensity $r = I_{nuc}/I_{cyto}$. These cells were then synchronized with respect to the mitotic phase for further analysis and visualization.

Cell Proliferation Analysis

RPE1 cells were stained with CDy6 (0.5, 1, and 2 μ M) or Hoechst 33342 (0.5, 1, and 2 μ M) at the indicated time points. After incubation the cells were trypsinized, and the RPE1 cell proliferation rate was measured by manually counting cells under the microscope at the indicated time points. The effect of CDy6 on cell-cycle distribution was confirmed by staining RPE1 cells with ethynyldeoxyuridine (EdU) (10 μ M) following CDy6 (1 and 2 μ M) or Hoechst 33342 (1 and 2 μ M) labeling. S-Phase was detected using the Click-iT EdU Alexa Fluor 647 flow cytometry kit (Life Technologies) following the manufacturer's protocol. The cell proliferation rate with EdU assay was analyzed by the BD LSRiI flow cytometer system (BD Biosciences, Franklin Lakes, NJ, USA).

Cell Viability

Cell viability was assessed using the crystal violet staining assay (Jeong et al., 2011). RPE1 cells (1×10^5) were seeded on 12 mm culture dishes; cells were stained with or without CDy6 (0–2 μ M) for 24 hr. After 24 hr, the culture medium was removed. Cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature and then rinsed four times. The crystal violet retained by the adherent cells was extracted with 95% ethanol and absorbance was determined at 590 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

Time-Dependent In Vivo Imaging

HeLa cells (1×10^7) were seeded on 150 mm culture dishes; cells were stained with or without CDy6 (1 μ M) or LysoTracker Red (1 μ M) for 1 hr. After labeling, cells were washed in PBS, trypsinized, spun down at 1500 rpm for 3 min, and the pellet resuspended in PBS (150 μ l). BALB/c nude mice (18–20 g) were obtained from the Biological Resource Centre (Biomedical Sciences Institutes) and anesthetized by intraperitoneal injection of ketamine (150 mg/kg)/xylazine (10 mg/kg) at 6 weeks of age. CDy6-labeled and control HeLa cells were then injected subcutaneously into the left and right rear flank, respectively, of three separate mice (injection volume: $5 \times 10^6/100 \mu$ l). Time-dependent fluorescence images were acquired with a Leica M205 FA fluorescent stereo microscope using a 3.95 \times objective lens with a TRITC filter, measured and processed with NIS-Elements 3.10.

Whole-body in vivo images were acquired using an IVIS Spectrum imaging system (Caliper Life Sciences) at the Biological Resource Center. The images were obtained 21 days after injection of HeLa cells stained with CDy6 (1 μ M).

The mice were euthanized 21 days after injection of HeLa cells, with the tumor tissue isolated from the mouse and stored in Tissue Freezing Medium (Triangle Biomedical Science, Durham, NC, USA) after removing the water on the surface. The frozen tissues were sectioned at 10 μ M thickness and tissue imaging performed using a Nikon NIE fluorescence microscope system. All animal experimental procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee.

Statistics

The statistical significance of the differences between groups was assessed by ANOVA, followed by Student's t-test. p Values less than 0.01 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two movies and can be found with this article online at <http://dx.doi.org/10.1016/j.chembiol.2014.11.018>.

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