COMMUNICATION

Spatiotemporal Regulation of CENP-E-guided Chromosomes Using A Fast-relaxing Arylazopyrazole Photoswitch

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We developed a centromere-associated protein E (CENP-E) inhibitor employing *trans* to *cis* photoisomerization with 405 nm visible light illumination and fast thermal relaxation. This photoswitching characteristic of the inhibitor enabled selective blockage or release of the motion of particular chromosomes within a single mitotic cell. Using this technique, we successfully demonstrated targeted chromosome gain and loss in daughter cells by introducing asymmetric chromosome segregation.

Chromosome engineering, when integrated with cell reprogramming technology, holds significant promise as a strategy for advancing cell therapy and drug discovery. The predominant techniques in chromosome engineering currently rely on the use of artificial chromosome vectors, genome editing technologies, or cell-autonomous chromosome correction[1b]. The precise control of chromosome segregation during mitosis opens up novel avenues for the effective manipulation of chromosome numbers. Consequently, there is a pressing need for the creation of a molecular instrument capable of precisely and adjustably modulating chromosome segregation at a subcellular level, which would be a valuable asset from the standpoint of chromosome engineering.

Centromere-associated protein E (CENP-E) is a kinesin-like motor protein that associates with and transports the mitotic chromosomes to the cellular equatorial plane during prometaphase in mitosis[2]. CENP-E inhibition causes chromosome misalignment during prometaphase, which results in prolonged mitosis, and often leads to an unequal distribution of replicated chromosome pairs in daughter cells (i.e., chromosome mis-segregation)[3]. The direct and continuous involvement of CENP-E in prometaphase chromosome transportation makes it an ideal molecular target for the artificial control of chromosome segregation. We previously reported a photoswitchable CENP-E inhibitor **1**[4] bearing an arylazopyrazole photoswitch[5] as a potent photopharmacological tool[6] (Fig. 1a) based on the authentic CENP-E inhibitor of **GSK923295** (Fig. 1b). The thermally stable *trans* isomer of **1** exhibited the strong inhibition of CENP-E, whereas the inhibitory effect was significantly much less for the metastable *cis* isomer induced by 365 nm light illumination. The drastic change in CENP-E inhibition upon photoisomerization enabled the dynamic photocontrol of chromosomal movement and mitotic progression in live cells under alternating 365 nm and 510 nm light illumination. However, there is a need to overcome several critical limitations in achieving versatile engineering of chromosome segregation through CENP-E photocontrol. Some of these limitations are 1) the usage of ultraviolet light at 365 nm to liberate CENP-E inhibition by **1** is

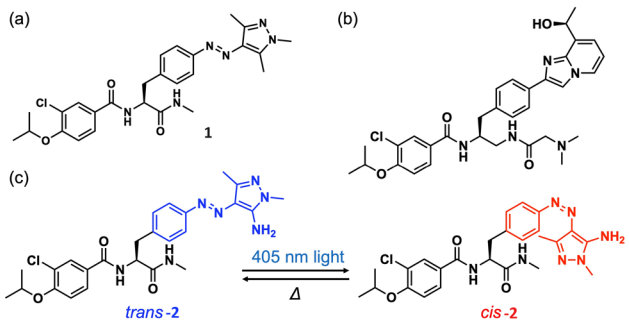

Fig. 1 Design of the photoswitchable CENP-E inhibitor controlled with single visible light. (a) Previously reported arylazopyrazole type of inhibitor **1** (b) Authentic CENP-E inhibitor, **GSK923295**. (c) Photoisomerization and thermal relaxation reaction of **2**.

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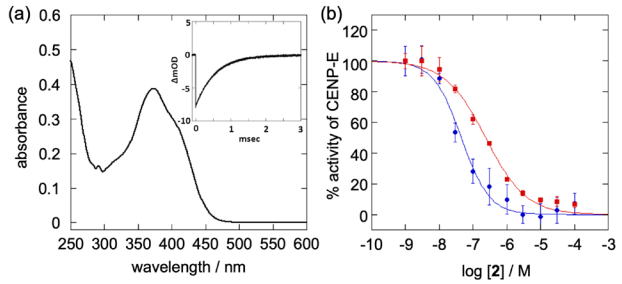

Fig. 2 Photophysical properties and *in vitro* CENP-E inhibition properties of **2**. (a) Absorption spectrum of **2** (20 µM) in BRB80 buffer. Transient absorbance measurements at 405 nm of **2** (20 µM) in BRB80 buffer (inset). (b) *In vitro* ATPase assay depending on the concentration of **2** with/without 405 nm LED light illumination (10 mW/cm^2). Blue circle: without light illumination. Red circle: with 405 nm light illumination. Error bars show the standard errors from triplicate experiments.

inherently cellular toxic, which limits its biocompatibility[7]; 2) the concomitant use of two different light sources complicates the optical system; and 3) the bistability of the photostationary states of **1** allows diffusive propagation of either *cis* or *trans* isomers throughout the cytoplasm in a cell during the photoswitching, which restricts the controllability of local chromosome motion at a subcellular scale. In this study, to overcome these challenges, we developed a new photoswitchable CENP-E inhibitor **2** (Fig. 1c), which can be controlled using a single visible wavelength (405 nm) light. This photoswitching characteristic enables precise subcellular control of chromosomal segregation using a simple experimental setup.

To achieve CENP-E photocontrol using a single visible wavelength light, we introduced an amino group to the pyrazole unit in **1**. The electron-donating properties of the amino group decreases the energy barrier for thermal isomerization[8], which theoretically shortens the lifetime of the *cis* isomer. Some experiments of photoswitchable ligands and inhibitors for other proteins have proved these expected properties of the aminated azobenzene derivatives.[9] Therefore, we expected that the 1,3-dimethyl-5-aminopyrazole type of inhibitor **2** would photoisomerize from the *trans* to *cis* isomer under visible light illumination. The resulting *cis* isomer would immediately isomerize back to the *trans* isomer owing to the fast thermal relaxation in an aqueous solution. Based on this expectation, we hypothesized that this unique property of **2** would allow CENP-E inhibition without light illumination, and local illumination with visible light would locally release CENP-E molecules from the inhibition.

We chemically synthesized **2** via the azo-coupling reaction of the corresponding diazonium salt with 1,3-dimethyl-5-aminopyrazole, which was characterized by ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, and mass spectroscopy (Scheme S1, Fig. S1, and Fig. S2).

To study the photophysical properties of **2**, we measured its absorption spectrum in an aqueous solution (Fig. 2a). Fig. 2 shows the visible light region of absorption (over 400 nm)

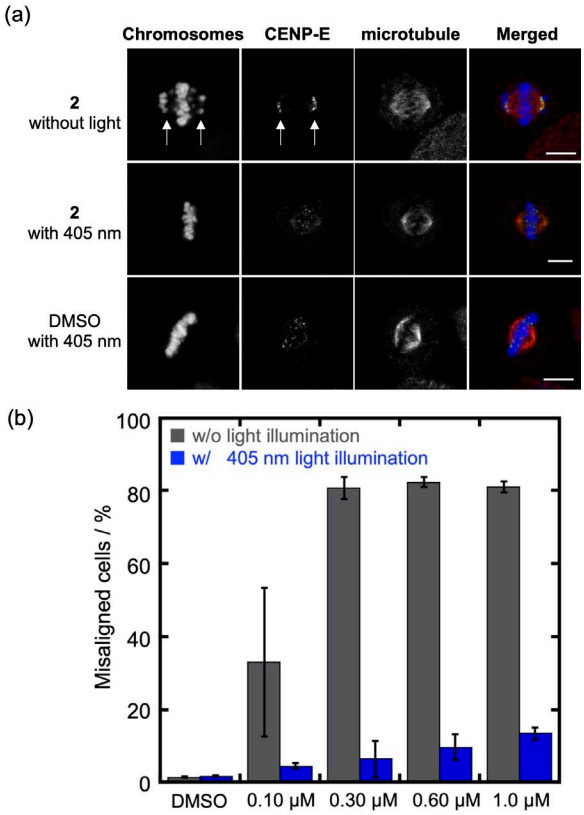

Fig. 3 Cell-based assay for CENP-E inhibition using **2**. (a) Immunofluorescence imaging of HeLa Kyoto cells was performed using 4',6-diamidino-2-phenylindole (DAPI) and antibodies for CENP-E and microtubules. Compound **2** (0.30 µM) or DMSO was incubated for 1 hour with or without 405 nm light illumination at 5 mW/cm^2, a condition that does not affect cell viability (Figure S4). The representative images are shown from triplicate experiments (> 10 cells in total for each condition). Arrows in chromosomes and CENP-E panels indicate the misaligned chromosomes and CENP-E on their kinetochores. Scale bars: 10 µm. (b) Population of misaligned chromosomes because of CENP-E inhibition. Error bars show the standard errors from triplicate experiments (> 100 cells for each experiment).

without light illumination with no obvious separation of π-π* and n-π* transition bands because of the introduction of the amino group on the pyrazole ring. After light irradiation, no absorbance change was detected using a standard UV–Vis absorbance spectrometer, implying fast thermal relaxation. Consistent with this finding, the flash laser photolysis experiment[10] revealed that the lifetime of the *cis* isomer of **2** in the BRB80 buffer was 567 µsec (Fig. 2a, inset), which was approximately 5.9×10^8-fold less than that of **1** (τ = 92.5 h)[4].

We conducted a chemiluminescent ATPase assay with the purified CENP-E motor domain using the ADP-Glo system. In this assay, **GSK923295** inhibited CENP-E at a half maximal inhibitory concentration (IC_{50}) of 24 nM (Fig. S3). Without light illumination (dark conditions), **2** exhibited an inhibitory efficacy comparable to that of **GSK923295**[11] (IC_{50} = 44 nM; Fig. 2b).

Under continuous illumination with 405 nm light (to maintain *cis* isomer; light condition in Fig. 2b), the IC_{50} of **2** was 233 nM, which was 5.3-fold less potent than that under dark conditions. The affinity differences with or without 405 nm light illumination indicated that the ratio of *cis* isomer under 405 nm light was roughly estimated to be 80%. These results suggested that **2** was a photoswitchable CENP-E inhibitor that was controlled by a single visible light illumination.

Next, we addressed the feasibility of photoswitching of CENP-E function using **2** in cells. For this experiment, HeLa cells were treated with **2** in the presence of the proteasome inhibitor MG132 to block the anaphase onset[12] with or without 405 nm light illumination for 1 h before fixation. Subsequently, the cells were subjected to immunofluorescence to analyse the intracellular localization of chromosomes and CENP-E protein (Fig. 3a). In control dimethyl sulfoxide (DMSO; vehicle) -treated cells, all chromosomes were regularly aligned in the cell equatorial plane (metaphase plate), with CENP-E localized at the kinetochores on the chromosomes. Under dark conditions in the presence of **2**, substantial portions of chromosomes and CENP-E accumulated around the mitotic spindle poles (arrows in Fig. 3a), which is a typical defect caused by CENP-E inhibition[3,11]. In contrast, the regular intracellular distribution of chromosomes and CENP-E was restored under light illumination, even in the presence of **2**. Quantification of the cell population with misaligned chromosomes in the cells treated with different concentrations of **2** revealed that over 80% of mitotic cells possessed misaligned chromosomes when treated with 0.30 μM of **2** or higher under dark conditions (Fig. 3b). However, < 10% of the mitotic cells possessed misaligned chromosomes under light conditions, even when treated with 1.0 μM of **2**. These results confirmed the cellular application of photoswitchable CENP-E inhibitor **2** and enabled the precise control of mitotic chromosomes movement. Moreover, these results demonstrate the photoswitching of CENP-E function and mitotic chromosome positioning with **2** in cells using a single visible wavelength light.

To evaluate the temporal controllability of chromosomal movement using **2**, we performed live cell imaging using confocal laser scanning microscopy (CLSM). The chromosomes were visualized using a silicon rhodamine-based far-red probe, SiR-DNA[13]. Several chromosomes were misaligned in the mitotic HeLa cells under dark conditions in the presence of **2** (0.30 μM). However, upon illumination with 2% intensity[14] of a 405 nm laser (equipped with CLSM) over the entire field of view, these misaligned polar chromosomes started to move towards the metaphase plate (Fig. 4a). All these chromosomes eventually reached the metaphase plate within 20 min. However, chromosome alignment was not restored within the same time range in **GSK923295**-treated cells with 405 nm laser illumination or in **2**-treated cells without light illumination (Fig. S5 and S6). These results demonstrate the feasibility of temporal photoswitching of mitotic chromosome movement and the establishment of a metaphase plate by a single wavelength light control using **2** in live cells.

Next, we addressed the feasibility of cellular- or subcellular-scale photocontrol of chromosomal movements. Using local laser illumination in the designated area under CLSM, we used a 405 nm laser to illuminate only one of the two lined up mitotic cells. Before illumination, both cell types possessed misaligned polar chromosomes. However, upon the targeted 405 nm illumination, only the illuminated cells underwent chromosome re-alignment (Fig. S7). This demonstrates the cellular-scale spatial resolution of the photocontrol using **2**.

To further narrow down the target scale and test the feasibility of selective photocontrol of a small number of chromosomes within a cell, we used laser illumination around only one pole of the mitotic spindle in **2**-treated mitotic cells (Fig. 4b). Before 405 nm illumination, misaligned polar chromosomes were observed at both poles of the **2**-treated cell. However, upon 405 nm

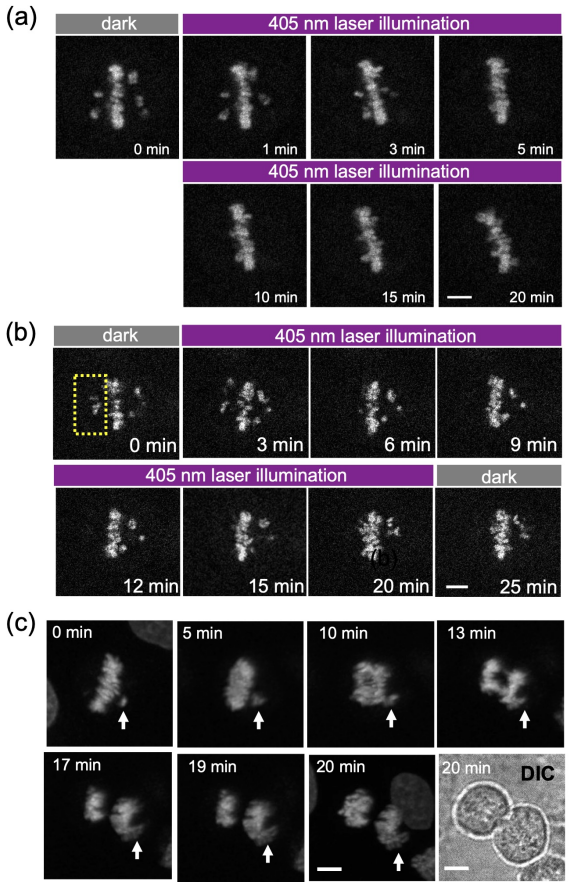

Fig. 4 Live cell experiments for the photocontrol of mitotic chromosomes. (a) Time lapse imaging of photocontrol of chromosome movement caused by **2** in single cell using 405 nm laser illumination (2% intensity) under a confocal laser scanning microscope. (b) Time lapse imaging of the pin-point control of chromosome positioning and their asymmetric distribution at the subcellular level. Yellow dotted square indicates the area of 405 nm laser illumination. The line profiles of the fluorescence intensities were also described in Fig. S8. (c) Time lapse imaging of asymmetric cell division by the spindle assembly checkpoint suppression using the Mps1 inhibitor. White arrows show the misaligned chromosomes. The representative examples are depicted from triplicate experiments. All scale bars in Fig. 4 are 5 μm.

illumination at the side of the spindle, the polar chromosomes on the illuminated side started to move towards and, subsequently, reached the metaphase plate. Notably, the polar chromosomes on the non-illuminated side remained misaligned, resulting in a prominent asymmetric chromosomal distribution. Line profiles of chromosomal fluorescence intensities along the spindle axis revealed spatially selective induction of chromosome movement on a single mitotic spindle structure (Fig. S8). Therefore, the high spatiotemporal resolution and the monostability of the photoswitching of **2** enabled the selective control of the targeted chromosome subset, with no effect on the untargeted chromosomes in the shared cytoplasm.

The prominently asymmetric (one-sided) distribution of misaligned polar chromosomes upon the partial laser illumination has rarely been observed under preexisting experimental conditions, providing an opportunity for unique manipulation of chromosome number. Therefore, we finally demonstrated chromosome number manipulation using the subcellular-scale photocontrol of chromosome segregation in dividing cells. In the presence of misaligned chromosomes following chemical inhibition of CENP-E, mitotic progression is precluded by a cell-intrinsic surveillance mechanism known as the spindle assembly checkpoint (SAC)[15]. Thus, we used **AZ3146**, a chemical inhibitor of Mps1[16], which is a protein essential for SAC, to override SAC after the photoinduction of one-sided misaligned polar chromosomes using **2**. Under these conditions, the cells underwent chromosome separation and cytokinesis in the presence of one-sided polar chromosomes. This resulted in the formation of daughter cells that either gained or lost the targeted chromosome sets (Fig. 4c). We successfully constructed a simple system to selectively modulate chromosome segregation and chromosome number using single wavelength light with a high spatiotemporal resolution.

In conclusion, we developed the CENP-E inhibitor **2** based on the *cis-trans* photoisomerization of the azophotoswitch and fast thermal relaxation of the aminated arylazopyrazole unit, enabling photocontrol of CENP-E function both *in vitro* and *in vivo* using a single visible wavelength (405 nm) light with superior spatiotemporal accuracy. Combined with highly controllable laser illumination under a CLSM, we achieved subcellular-scale manipulation of mitotic chromosome segregation within a single cell. In the future, the technique developed in this study can be applied as a photocontrol method for a single chromosome. Our laboratory is currently working on the detailed characterization of the daughter cells produced through asymmetric chromosome segregation.

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Conflicts of interest

There are no conflicts to declare.

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