CHAPTER

Optogenetic control of mitosis with photocaged chemical dimerizers

7

Huaiying Zhang*, David M. Chenoweth†, Michael A. Lampson*,[1]

*Department of Biology, School of Arts and Sciences, University of Pennsylvania, Philadelphia, PA, United States

†Department of Chemistry, School of Arts and Sciences, University of Pennsylvania, Philadelphia, PA, United States

[1]Corresponding author: e-mail address: lampson@sas.upenn.edu

CHAPTER OUTLINE

Abstract

Mitosis is a highly dynamic process that depends on coordination of many protein–protein interactions with temporal and spatial precision. A challenge for understanding this complex system is to manipulate it on biologically relevant temporal and spatial scales, with molecular specificity. We describe an optogenetic platform, based on photosensitive chemical inducers of dimerization, which provides control over dimerization of genetically tagged proteins with light. As examples, we drive chromosome transport and activate and silence the spindle assembly checkpoint by recruiting proteins to and releasing them from kinetochores with light.

Methods in Cell Biology, Volume 144, ISSN 0091-679X, https://doi.org/10.1016/bs.mcb.2018.03.006

1 INTRODUCTION

During mitosis the cell attaches duplicated sister chromosomes to the spindle fibers, aligns all chromosome pairs at the spindle equator, detects and corrects attachment errors, and then separates the sisters to opposite sides of the cell to ensure that exactly one copy of each chromosome is retained in each daughter cell. This highly dynamic process depends on coordination of many protein–protein interactions with temporal and spatial precision. For example, chromosome congression requires coordinated actions of plus- and minus-end directed microtubule (MT) motors to drive chromosome movements to align at the spindle equator (Auckland & McAinsh, 2015; Walczak, Cai, & Khodjakov, 2010). The spindle assembly checkpoint relies on enrichment of checkpoint signaling proteins on unattached kinetochores to generate a checkpoint-mediated cell delay and release from attached kinetochores to satisfy the checkpoint (Lara-Gonzalez, Westhorpe, & Taylor, 2012; London & Biggins, 2014). A challenge for understanding this complex system is to manipulate it on biologically relevant temporal and spatial scales, with molecular specificity. Conventional molecular approaches target specific proteins (Goshima & Vale, 2003) but lack spatial and temporal control. Small-molecule inhibitors offer temporal, but not spatial control, and their molecular specificity is variable and difficult to thoroughly characterize (Lampson & Kapoor, 2006). We developed an optogenetic platform, based on photosensitive chemical inducers of dimerization, which provides control over dimerization of genetically tagged proteins with light. We designed a set of dimerizers that offer different properties and used them to control organelle transport and kinetochore function in living cells (Ballister, Aonbangkhen, Mayo, Lampson, & Chenoweth, 2014; Ballister, Ayloo, Chenoweth, Lampson, & Holzbaur, 2015; Zhang et al., 2017).

Here, we describe two dimerizers and their application to control checkpoint signaling and molecular motor activity at kinetochores in live cells (Zhang et al., 2017). Each molecule contains a Halo ligand linked to Trimethoprim (TMP), a ligand for *Escherichia coli* dihydrofolate reductase (eDHFR), and can be used to dimerize a protein genetically fused to the Haloenzyme with another protein fused to eDHFR. The first dimerizer (*CTH*) contains a [7-(diethylamino)-coumarin-4-yl] methyl (DEACM) photocage, to prevent untargeted dimerization, TMP, and the Halo ligand that covalently binds to the Haloenzyme, a bacterial alkyldehalogenase (Fig. 1A). This molecule initially binds to the Haloenzyme, and uncaging with light exposes TMP to trigger dimerization with eDHFR. The second dimerizer (*TNH*) contains a photocleavable linker, 6-nitroveratryl oxycarbonyl (NVOC), between TMP and Haloligand (Fig. 2A). This molecule dimerizes Haloenzyme and eDHFR as soon as it is added to cells, and dimerization can be reversed by cleaving the linker with light.

To control molecular motor activity at kinetochores, we fused Haloenzyme to the kinetochore protein SPC25 and eDHFR to K560, the active motor domain of kinesin-1 (Fig. 1B). We then used light to recruit K560 to kinetochores by uncaging CTH, driving chromosome movement (Fig. 1C). To control checkpoint signaling, we fused Haloenzyme to the kinetochore protein CENP-T and eDHFR to the checkpoint protein Mad1 (Fig. 2B). We recruited Mad1 to kinetochores with TNH to trigger a

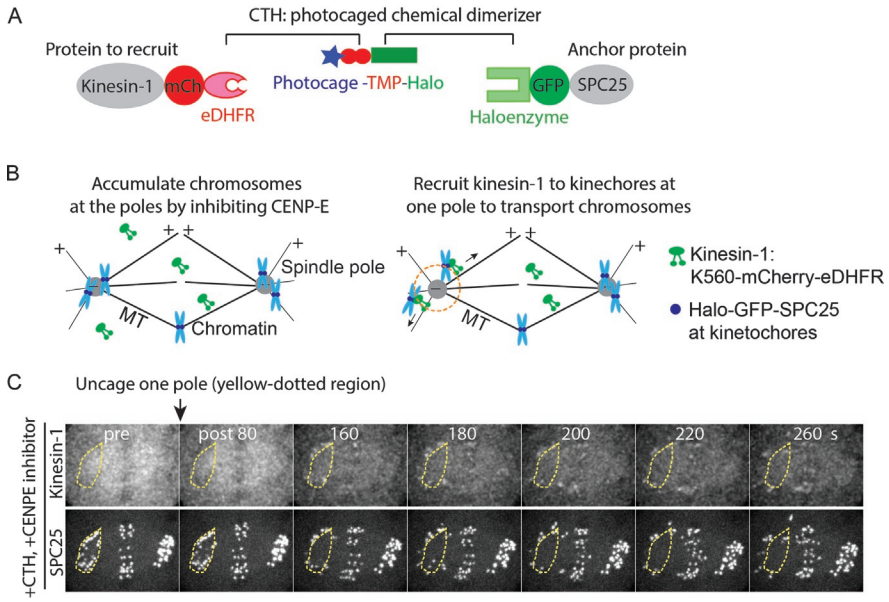

FIG. 1

Optogenetic control of molecular motor activity at kinetochores. (A) Schematic of the photocaged dimerizer CTH and fusion proteins used in the experiment. (B) Schematic of the experiment: A CENP-E inhibitor is used to accumulate some chromosomes at the spindle poles. Uncaging CTH at one pole (*orange-dashed region*) recruits kinesin-1 to kinetochores to transport chromosomes toward microtubule plus-ends. (C) Cells expressing the two fusion proteins shown in the schematic were incubated with CTH and treated with CENP-E inhibitor. Uncaging CTH (*yellow-dashed region*) recruits kinesin-1 to kinetochores at one pole, which leads to chromosome movement away from the pole.

These data are reproduced from Zhang, H., Aonbangkhen, C., Tarasovetc, E. V, Ballister, E.R., Chenoweth, D.M., & Lampson, M. A. (2017). Optogenetic control of kinetochore function. Nature Chemical Biology, 13, *1096–1101.*

checkpoint-mediated response and arrest cells in metaphase, and then released Mad1 from kinetochores by cleaving TNH with light, leading to checkpoint satisfaction and anaphase onset (Fig. 2C).

2 MATERIALS

2.1 GENERATION AND CULTURE OF STABLE CELL LINES

1. HiLo acceptor cell line (Khandelia, Yap, & Makeyev, 2011) (see Note 1).
2. RMCE donor plasmid capable of expressing Halo and eDHFR fusion proteins (in this case, mCherry-eDHFR-K560, Halo-GFP-SPC25, Halo-GFP-CENP-T, mCherry-eDHFR-Mad1) (see Note 2).
3. Cre expression plasmid (see Note 3).

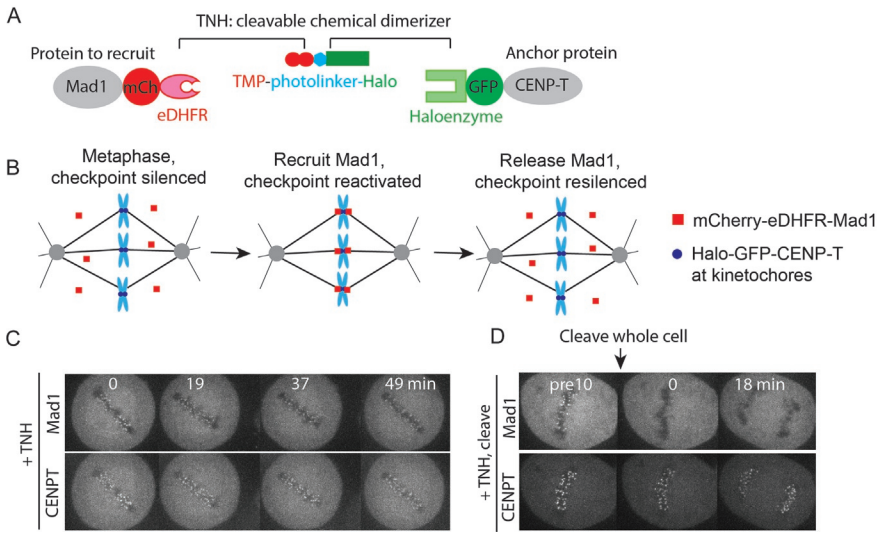

FIG. 2

Optogenetic control of checkpoint signaling. (A) Schematic of the photocleavable dimerizer TNH and fusion proteins used in the experiment. (B) Schematic showing control of checkpoint signaling by manipulating Mad1 localization to kinetochores. (C) Cells expressing the two fusion proteins shown in the schematic were incubated with TNH to recruit Mad1 to kinetochores, leading to metaphase arrest (*bottom left*). Cleaving TNH with light releases Mad1 from kinetochores, resulting in anaphase onset (*bottom right*).

4. Puromycin, 1 μg/mL (see Note 4).
5. Growth medium: Dulbecco's-modified Eagle's medium (DMEM) supplemented with 1% PenStrep and 10% fetal bovine serum (FBS).
6. Transfection medium: DMEM supplemented with 10% FBS.
7. DMSO.
8. Cryopreservation medium: 50% FBS, 40% DMEM, 10% DMSO.
9. Selection medium: growth medium supplemented with 1 μg/mL puromycin (see Note 4).
10. Lipofectamine 2000 (Invitrogen) (see Note 5).
11. Serum-free media such as Opti-MEM (Life Technologies).

2.2 LIVE-CELL IMAGING

1. Microscope suitable for live-cell fluorescent imaging (see Note 6).
2. Polylysine coated #1.5 coverslips.
3. Live-cell imaging chamber.
4. Imaging medium: Leibovitz-15 (L15) CO_2-independent media, supplemented with 1% PenStrep and 10% FBS.

5. 10 μM CTH in growth medium and 15 μM TNH in imaging medium.
6. The CENP-E inhibitor GSK923295 (Cayman Chemical Company) dissolved in DMSO.

3 METHODS

3.1 PRODUCTION AND MAINTENANCE OF STABLE CELL LINE

1. Culture acceptor cells in one well of a 6-well plate in growth medium until they reach ~60% confluency.
2. Replace growth medium with transfection medium prior to transfection.
3. Cotransfect cells with RMCE donor plasmid (Halo-GFP-SPC25 or Halo-GFP-CENP-T in this case) and Cre expression plasmid at a 100:1 mass ratio of donor plasmid and Cre plasmid (1 μg of donor plasmid + 10 ng of Cre plasmid).
4. After 24 h, replace transfection medium with growth medium.
5. 48 h after transfection, replace growth medium with selection medium. Continue to cultivate cells in selection medium from now on.
6. After 24 h of puromycin selection, many cells should die. Change media to remove dead cells.
7. After another 24 h, the vast majority of cells should have died, but some microcolonies of resistant cells should be apparent. Change media again to remove dead cells.
8. Monitor culture until colonies merge and become confluent, or until the cell density in the interior of the colonies becomes too high (usually ~7–10 days after transfection).
9. Passage 80% of cells to a 10-cm dish. Seed the remaining 20% on a coverslip to check for appropriate expression and localization of fluorescently tagged fusion proteins.
10. Culture cells in selection medium until they reach 80% confluence.
11. Harvest cells and freeze several aliquots in cryopreservation medium (see Note 2).

3.2 CONTROL OF DIMERIZATION AND LIVE-CELL IMAGING

1. Seed a sufficient number of cells on coverslips so that they will reach 70%–80% confluency after 24 h of culture.
2. Transfect another plasmid (mCherry-eDHFR-K560 or mCherry-eDHFR-Mad1 in this case, 1 μg) and wait 24–48 h before imaging.
3. Set up microscope and environmental control apparatus.
4. Warm imaging medium to 37°C.
5. For the molecular motor experiment with CTH, incubate cells with 10 μM CTH and 50 nM CENP-E inhibitor for 1 h, wash three times with fresh growth medium, then incubate with fresh growth medium containing 50 nM CENP-E inhibitor for 30 min.

6. Replace growth medium with prewarmed imaging medium, transfer coverslip to imaging chamber, and quickly add 1 mL prewarmed imaging medium. For the molecular motor experiment with CTH, add CENP-E inhibitor to final concentration of 50 nM.
7. Mount chamber on microscope and locate mitotic cells with bright GFP signal on kinetochores (some will be at the poles with CENP-E inhibitor) and diffusive mCherry signal in the cytosol.
8. For the molecular motor experiment with CTH, use the ROI tool in the imaging software to draw a region covering the kinetochores at one of the poles. Setup an imaging sequence to take one image, then activate the region of interest with the 405 nm laser at 7% power for 20 repetitions, and then take images every 20 s for 10 min.
9. For the checkpoint experiment with TNH, take an image as the "predimerization" state. Make 0.5 mL imaging media containing 10 μL of 15 μM TNH and add it to the imaging chamber on the stage so that the final TNH concentration is 100 nM. Manually take images every several minutes for 30 min to check for cell cycle progression. Cells should arrest in metaphase. To cleave TNH, take an image as "after-dimerization" and "precleavage." Use the ROI tool in the imaging software to draw a region covering the whole cell and activate this region with the 405 nm laser at 8% power for 40 repetitions. Take one image immediately and then manually every several minutes for 30 min or until cells proceed to anaphase.

4 NOTES

1. RMCE involves recombination between a donor plasmid and a specially designed acceptor locus in the genome of the cells being genetically modified (Khandelia et al., 2011). Typically, this acceptor locus is present at single copy at a unique site in the genome of the acceptor cells. In the HiLo system, the donor plasmid contains two nonidentical Lox sites (LoxP and Lox2272) which flank the "cassette" of DNA which is to be exchanged. The acceptor locus contains the same pair of Lox sites, with an EF1α promoter immediately upstream of the Lox2272 site. This promoter is used to drive transcription of a selection marker immediately downstream of the Lox2272 element (within the acceptor cassette). Multiple monoclonal acceptor cell lines are available from the Makeyev lab, including human (HeLa, HeLa-S3, A549, HT1080, HEK293T, and U2OS) and mouse (NIH3T3, CAD, L929, N2a, and P19) cell lines. Here, we use the HeLa cell line.
2. We make stable cell lines that express an anchor protein fused to the Haloenzyme (e.g., Halo-GFP-SPC25 or Halo-GFP-CENP-T) and transiently express eDHFR fusion proteins (mCherry-eDHFR-K560 or mCherry-eDHFR-Mad1). During imaging, we select cells with bright GFP signals at kinetochores and

intermediate mCherry signal in the cytosol, which give the best recruitment. To obtain cells with more Haloenzyme at kinetochores, stable cell lines can be further sorted with flow cytometry for bright GFP signal (top 5%) before transfecting with eDHFR plasmids.

3. The HiLo system uses Cre recombinase to catalyze recombination between donor and acceptor Lox sites. Plasmids for transient Cre expression (including a Cre construct containing a nuclear import sequence) are available from the Makeyev lab and repositories such as Addgene.
4. An appropriate concentration for the selection agent must be determined empirically from a kill-curve experiment. 1 μg/mL puromycin works well for HeLa cells.
5. This protocol uses Lipofectamine 2000; other methods of transfection can also be used.
6. In this example: a microscope (DM4000; Leica) with a 100×1.4 NA objective, an XY Piezo-Z stage (Applied Scientific Instrumentation), a spinning disk (Yokogawa), an electron multiplier charge-coupled device camera (ImageEM; Hamamatsu Photonics), and a laser merge module equipped with 488- and 593-nm lasers (LMM5; Spectral Applied Research) controlled by MetaMorph software (Molecular Devices) equipped with an environmental chamber for temperature control (Incubator BL; PeCon GmbH). Targeted uncaging or cleavage was performed with an iLas2 illuminator system (Roper Scientific), equipped with a 405 nm laser (output of 27 mW after fiber coupling) controlled using the iLas2 software module within Metamorph.

5 OUTLOOK

This method highlights the advantages of a hybrid chemical and genetic approach for controlling kinetochore function with light. We can induce both gain and loss of activities, using checkpoint signaling and motor proteins as examples, with flexibility provided by the choice of proteins to tag and the choice of chemical probes. We envision that this approach can be readily adapted to other processes, such as regulation by kinetochore kinases and phosphatases, tension sensing and MT capture (Funabiki & Wynne, 2013; Heald & Khodjakov, 2015; Lampson & Grishchuk, 2017), and to manipulate other structures in dividing cells, such as spindle poles (Akera et al., 2017). Furthermore, the modular design of our dimerizers supports the development of new probes, for example, to allow sequential recruitment and release with different wavelengths of light. The continued development of such optogenetic tools will challenge cell biologists to design creative experiments that take advantage of the opportunity to probe dynamic cellular processes with spatial and temporal precision.

REFERENCES

Akera, T., Chmátal, L., Trimm, E., Yang, K., Aonbangkhen, C., Chenoweth, D. M., et al. (2017). Spindle asymmetry drives non-Mendelian chromosome segregation. *Science*, *358*, 668–672.

Auckland, P., & McAinsh, A. D. (2015). Building an integrated model of chromosome congression. *Journal of Cell Science*, *128*, 3363–3374.

Ballister, E. R., Aonbangkhen, C., Mayo, A. M., Lampson, M. A., & Chenoweth, D. M. (2014). Localized light-induced protein dimerization in living cells using a photocaged dimerizer. *Nature Communications*, *5*, 5475.

Ballister, E. R., Ayloo, S., Chenoweth, D. M., Lampson, M. A., & Holzbaur, E. L. F. (2015). Optogenetic control of organelle transport using a photocaged chemical inducer of dimerization. *Current Biology*, *25*, R407–R408.

Funabiki, H., & Wynne, D. J. (2013). Making an effective switch at the kinetochore by phosphorylation and dephosphorylation. *Chromosoma*, *122*, 135–158.

Goshima, G., & Vale, R. D. (2003). The roles of microtubule-based motor proteins in mitosis: Comprehensive RNAi analysis in the Drosophila S2 cell line. *The Journal of Cell Biology*, *162*, 1003–1016.

Heald, R., & Khodjakov, A. (2015). Thirty years of search and capture: The complex simplicity of mitotic spindle assembly. *The Journal of Cell Biology*, *211*, 1103–1111.

Khandelia, P., Yap, K., & Makeyev, E. V. (2011). Streamlined platform for short hairpin RNA interference and transgenesis in cultured mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, *108*, 12799–12804.

Lampson, M., & Grishchuk, E. (2017). Mechanisms to avoid and correct erroneous kinetochore-microtubule attachments. *Biology (Basel).*, *6*, 1.

Lampson, M. A., & Kapoor, T. M. (2006). Unraveling cell division mechanisms with small-molecule inhibitors. *Nature Chemical Biology*, *2*, 19–27.

Lara-Gonzalez, P., Westhorpe, F. G., & Taylor, S. S. (2012). The spindle assembly checkpoint. *Current Biology*, *22*, R966–R980.

London, N., & Biggins, S. (2014). Signalling dynamics in the spindle checkpoint response. *Nature Reviews. Molecular Cell Biology*, *15*, 736–748.

Walczak, C. E., Cai, S., & Khodjakov, A. (2010). Mechanisms of chromosome behaviour during mitosis. *Nature Reviews. Molecular Cell Biology*, *11*, 91–102.

Zhang, H., Aonbangkhen, C., Tarasovetc, E. V., Ballister, E. R., Chenoweth, D. M., & Lampson, M. A. (2017). Optogenetic control of kinetochore function. *Nature Chemical Biology*, *13*, 1096–1101.