Chapter 20

Probing Mitosis by Manipulating the Interactions of Mitotic Regulator Proteins Using Rapamycin-Inducible Dimerization

Edward R. Ballister and Michael A. Lampson

Abstract

Inducible dimerization is a general approach to experimentally manipulate protein–protein interactions with temporal control. This chapter describes the use of rapamycin-inducible dimerization to manipulate mitotic regulatory proteins, for example to control kinetochore localization. A significant feature of this method relative to previously described protocols is the depletion of endogenous FKBP12 protein, which markedly improves dimerization efficiency.

Key words Rapamycin, Kinetochore, Dimerization

1 Introduction

Protein–protein interactions underlie the regulation of essentially every process in biology, and mitosis is no exception. These interactions can be experimentally controlled through inducible dimerization, a family of techniques that has been applied to diverse biological processes. One of the most widely used tools for inducible dimerization is the small molecule rapamycin, which simultaneously binds two different proteins, FKBP12 (hereafter FKBP) and mTOR, with very high affinity (~2 nM) [1]. Rapamycin is used as a general tool for heterodimerization by genetically fusing proteins of interest (POIs) to FKBP12 and a minimal rapamycin-binding fragment of mTOR (FRB) [2, 3]. In this way, rapamycin can be applied to any pair of POIs (POI1 and POI2) amenable to genetic manipulation. An advantage of rapamycin over other chemical dimerizers is that FRB binds only to the rapamycin-FKBP complex, which prevents formation of unproductive rapamycin-FRB complexes.

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Inducible dimerization has been used to probe mitosis in different ways. In the example described in this method, the Spindle Assembly Checkpoint (SAC) protein Mad1 is recruited to kinetochores during metaphase, a time when SAC proteins are normally removed from kinetochores [4, 5]. Returning Mad1 to kinetochores at metaphase reactivates the SAC. Inducible dimerization has also been used to control cohesin ring closure [6], manipulate SUMO isopeptidase activity at kinetochores [7], and rapidly remove TACC3-ch-TOG-clathrin complexes from kinetochore-microtubule fibers by sequestering the complexes to mitochondria, where they cannot perform their normal functions [8].

One factor which limits the effectiveness of rapamycin-induced dimerization is the presence of endogenous FKBP, a highly abundant cytosolic protein in yeast and animal cells which competes against exogenous POI1-FKBP for ternary complex formation with POI2-FRB upon addition of rapamycin [4, 9]. FKBP is not essential, at least in tissue culture cells [10–13], so this problem can be overcome by depleting endogenous FKBP using RNAi, using either siRNA transfection or expression of an engineered shRNA/miRNA as described here [4]. FKBP null mice develop fatal cardiac defects [14], but tissue-specific FKBP knockout later in development can be well tolerated [11].

The overall method involves construction and expression of a pair of fusion proteins (POI1-FKBP and POI2-FRB), RNAi depletion of endogenous FKBP, and addition of rapamycin as the experimental perturbation (Fig. 1). We use Recombinase Mediated Cassette Exchange (RMCE) [15] to generate cell lines stably expressing POI1-FKBP and POI2-FRB fusion proteins plus shRNA against endogenous FKBP. The specific protocol used here (HiLo RMCE) allows rapid generation of stable cell lines constitutively and/or inducibly expressing a number of cDNAs and miRNA-based shRNAs [16]. The reagents are generally available for use in other academic or nonprofit environments at no cost.

2 Materials

2.1 Generation and Culture of Stable Cell Lines

1. HiLo acceptor cell line (*see* **Note 1**).
2. RMCE donor plasmid capable of expressing FKBP- and FRB-fusion proteins as well as shRNA against endogenous FKBP (in this case, Mis12-GFP-FKBPx3 and FRB-mCherry-Mad1) (*see* **Notes 2** and **3**).
3. Cre expression plasmid, preferably with nuclear localization sequence (*see* **Note 4**).

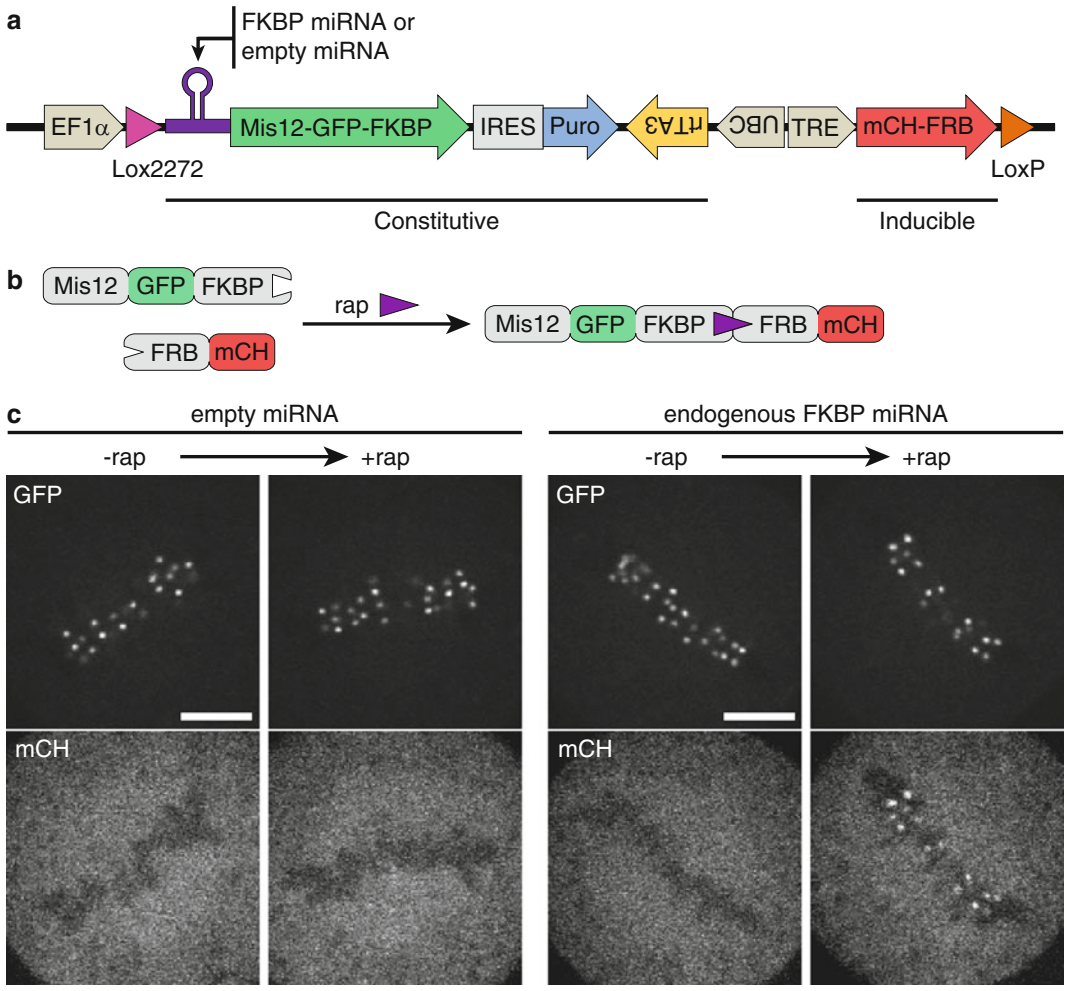

Fig. 1 Rapamycin-mediated recruitment to kinetochores. (**a**) Diagram of a DNA cassette used to constitutively express Mis12-GFP-FKBP and miRNA, and inducibly express mCherry-FRB. The cassette is integrated between Lox acceptor sites downstream of the EF1a promoter [16]. (**b**) Schematic representation of rapamycin-mediated recruitment of mCherry-FRB to kinetochore-localized Mis12-GFP-FKBP. (**c**) HeLa cells expressing Mis12-GFP-FKBP, mCherry-FRB and either an empty miRNA backbone or miRNA against the 3′ UTR of endogenous FKBP were imaged before and ~1 min after the addition of 500 nM rapamycin (rap). This figure was originally published in The Journal of Cell Biology (Ballister et al. [4] Fig. 1)

4. Puromycin ready-made solution 10 mg/mL.
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. FuGENE 6 (Promega) (*see* **Note 6**).
11. Serum-free media such as Opti-MEM (Life Technologies).
12. Doxycycline hydrochloride.

2.2 Live Cell Imaging

1. Microscope suitable for live-cell fluorescent imaging (*see* **Note 7**).
2. Poly-lysine 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. Rapamycin.

3 Methods

3.1 Production and Maintenance of Stable Cell Line

1. Culture acceptor cells in a 35 mm dish (or 1 well of a 6-well plate) in growth medium until they reach ~60 % confluency (seeding a 35 mm plate with ~125,000 HeLa acceptor cells, followed by overnight culture is typically sufficient).
2. Replace growth medium with transfection medium for >30 min prior to transfection.
3. Cotransfect cells with RMCE donor plasmid 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. Optionally, passage transfected cells to a larger dish if desired.
5. 48 h after transfection, replace growth medium with selection medium. Cells may be quite dense at this stage.
6. After 24 h of puromycin selection, many cells should die. Remove these by rinsing the plate vigorously 2× with selection medium, continue to culture in selection medium.
7. After an additional 24 h, the vast majority of cells should have died, but ~100–1000 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). At this point, passage 80 % of cells to a 10 cm dish, and seed the remaining 20 % on a coverslip to check for appropriate expression and localization of fluorescently tagged fusion proteins.
9. Culture cells in selection medium until they reach 80 % confluence.
10. Harvest cells and freeze several aliquots in cryopreservation medium. At this point, generating the stable cell line is complete.

3.2 Inducible Dimerization Live Cell Imaging Experiment

1. Seed a sufficient number of cells on coverslips so that they will reach the desired density after 48 h of culture.
2. If using dox-inducible expression: 48–30 h prior to experiment, add doxycycline to a final concentration of 200 ng/mL.
3. Set up microscope and environmental control apparatus, if the experiment requires their use.
4. Warm imaging medium to 37 °C.
5. Prepare a stock of imaging medium + 750 μM rapamycin, hold at 37 °C.
6. Replace growth medium with pre-warmed imaging medium.
7. Transfer coverslip to imaging chamber and quickly add 500 μL pre-warmed imaging medium.
8. Mount chamber on microscope and locate mitotic cells.
9. Image cells to document "pre-dimerization" state. In this example, kinetochores should be clearly identifiable at the metaphase plate in the GFP channel, and FRB-mCherry-Mad1 should be diffuse in the cytosol (and concentrated at any unattached or misaligned kinetochores which may be present).
10. Add 1 mL of imaging medium + rapamycin to the chamber, mixing quickly but gently to achieve a final concentration of 500 μM rapamycin.
11. Resume imaging to document effects of rapamycin on FRB-POI fusion localization and cellular phenotype, relative to controls. In this example, FRB-mCherry-Mad1 signal accumulates at kinetochores within 1 min, and most cells treated in this manner remain arrested in metaphase [4].

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. 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). 11 monoclonal acceptor cell lines are available, including human (HeLa, HeLa-S3, A549, HT1080, HEK293T, and U2OS) and mouse (NIH3T3, CAD, L929, N2a, and P19) cell lines.

2. Depending on the particular proteins being dimerized, it may be preferable for both to be expressed constitutively, or one or both to be expressed inducibly. In this example, Mis12-GFP-FKBPx3 is expressed constitutively and FRB-mCherry-Mad1 is expressed inducibly.
3. In the plasmids described in this Method, an shRNA against endogenous FKBP is transcribed and processed as part of an engineered miRNA nested within an intron in the same RNA PolII transcript as Mis12-GFP-FKBPx3. This obviates the need for a distinct RNA PolIII promoter for shRNA transcription. The shRNA sequence used here is 5′-AUAUGGAU UCAUGUGCACAUGGUUUUGGCCACUGACUGA CCAUGUGCAUGAAUCCAUAU-3′ which targets a region (5′-CAUGUGCACAUGAAUCCAUAU-3′) within the human FKBP 3′ untranslated region (3′ UTR). Because the endogenous FKBP 3′ UTR is not included in the exogenous FKBP transgene transcript, it is not necessary to add silent mutations to the exogenous FKBP transgene to protect it from targeting by the shRNA. siRNA transfection has also proved effective in our hands for FKBP knockdown, targeting the endogenous 3′ UTR with the following pair of annealed siRNA oligos: 5′-GCACAAGUGGUAGGUUAACdTdT-3′ + 5′-GUUAACCUACCACUUGUGCdTdT-3′ [10].
4. 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.
5. 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.
6. This protocol uses Fugene 6 for transfection, but other DNA transfection reagents or electroporation may be used instead.
7. 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).

References

1. Banaszynski LA, Liu CW, Wandless TJ (2005) Characterization of the FKBP.rapamycin.FRB ternary complex. J Am Chem Soc 127:4715–4721. doi:10.1021/ja043277y
2. Rivera VM, Clackson T, Natesan S, Pollock R, Amara JF, Keenan T et al (1996) A humanized system for pharmacologic control of gene expression. Nat Med 2:1028–1032
3. Putyrski M, Schultz C (2012) Protein translocation as a tool: the current rapamycin story. FEBS Lett 586:2097–2105. doi:10.1016/j.febslet.2012.04.061
4. Ballister ER, Riegman M, Lampson MA (2014) Recruitment of Mad1 to metaphase kinetochores is sufficient to reactivate the mitotic checkpoint. J Cell Biol 204:901–908. doi:10.1083/jcb.201311113
5. Kuijt TEF, Omerzu M, Saurin AT, Kops GJPL (2014) Conditional targeting of MAD1 to kinetochores is sufficient to reactivate the spindle assembly checkpoint in metaphase. Chromosoma. doi:10.1007/s00412-014-0458-9
6. Gruber S, Arumugam P, Katou Y, Kuglitsch D, Helmhart W, Shirahige K et al (2006) Evidence that loading of cohesin onto chromosomes involves opening of its SMC hinge. Cell 127:523–537. doi:10.1016/j.cell.2006.08.048
7. Cubeñas-potts C, Goeres JD, Matunis MJ, Bloom KS (2013) SENP1 and SENP2 affect spatial and temporal control of sumoylation in mitosis. Mol Biol Cell 24:3483–3495. doi:10.1091/mbc.E13-05-0230
8. Cheeseman LP, Harry EF, McAinsh AD, Prior IA, Royle SJ (2013) Specific removal of TACC3-ch-TOG-clathrin at metaphase deregulates kinetochore fiber tension. J Cell Sci 126:2102–2113. doi:10.1242/jcs.124834
9. Coutinho-Budd JC, Snider SB, Fitzpatrick BJ, Rittiner JE, Zylka MJ (2013) Biological constraints limit the use of rapamycin-inducible FKBP12-Inp54p for depleting PIP2 in dorsal root ganglia neurons. J Negat Results Biomed 12:13. doi:10.1186/1477-5751-12-13
10. Weiwad M, Edlich F, Kilka S, Erdmann F, Jarczowski F, Dorn M et al (2006) Comparative analysis of calcineurin inhibition by complexes of immunosuppressive drugs with human FK506 binding proteins. Biochemistry 45:15776–15784. doi:10.1021/bi061616p
11. Hoeffer CA, Tang W, Wong H, Santillan A, Patterson RJ, Martinez LA et al (2008) Removal of FKBP12 enhances mTOR-Raptor interactions, LTP, memory, and perseverative/repetitive behavior. Neuron 60:832–845. doi:10.1016/j.neuron.2008.09.037
12. De Angelis B, Dotti G, Quintarelli C, Huye LE, Zhang L, Zhang M et al (2009) Generation of Epstein-Barr virus-specific cytotoxic T lymphocytes resistant to the immunosuppressive drug tacrolimus (FK506). Blood 114:4784–4791. doi:10.1182/blood-2009-07-230482
13. Gerard M, Deleersnijder A, Daniëls V, Schreurs S, Munck S, Reumers V et al (2010) Inhibition of FK506 binding proteins reduces alpha-synuclein aggregation and Parkinson's disease-like pathology. J Neurosci 30:2454–2463. doi:10.1523/JNEUROSCI.5983-09.2010
14. Shou W, Aghdasi B, Armstrong DL, Guo Q, Bao S, Charng MJ et al (1998) Cardiac defects and altered ryanodine receptor function in mice lacking FKBP12. Nature 391:489–492. doi:10.1038/35146
15. Turan S, Galla M, Ernst E, Qiao J, Voelkel C, Schiedlmeier B et al (2011) Recombinase-mediated cassette exchange (RMCE): traditional concepts and current challenges. J Mol Biol 407:193–221. doi:10.1016/j.jmb.2011.01.004
16. Khandelia P, Yap K, Makeyev EV (2011) Streamlined platform for short hairpin RNA interference and transgenesis in cultured mammalian cells. PNAS 108:12799–12804. doi:10.1073/pnas.1103532108