Cell Biological Analysis of DT40 Knockout Cell Lines for Cell-Cycle Genes

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ABSTRACT

DT40 is a chicken B cell line that has been widely used as a model for gene functional studies because the high level of homologous recombination in DT40 cells allows targeted disruption of a gene of interest. While our laboratory uses DT40 cells to understand kinetochore assembly and function, the approach is applicable to functional studies of other genes that are required for cell cycle progression. Protocols are presented for the creation of knockout cells and subsequent cell biological analyses for characterizing the phenotypes of these cells. *Curr. Protoc. Cell Biol.* 50:8.7.1-8.7.17. © 2011 by John Wiley & Sons, Inc.

Keywords: DT40 • transfection • gene targeting • cell biological analysis

INTRODUCTION

Gene knockout strategy is important in elucidating the precise function of a gene of interest. The chicken DT40 system is particularly powerful because DT40 cells show an unusually high ratio of targeted to random integration of knockout constructs (Buerstedde and Takeda, 1991). Other technologies have also been widely used in vertebrate cell lines to suppress expression of desired genes, e.g., RNA interference (RNAi) and analysis of knockout mice. While RNAi is a very useful technique, there are some problems, including efficiency of suppression and off-target gene regulation (Jackson et al., 2003). Therefore, it is more desirable to analyze cells in which a gene of interest is genetically disrupted. Analysis of knockout mice is also a useful and reliable method; however, compared to creating a knockout mouse, construction of knockout DT40 cells is faster and more efficient (Fukagawa, 2008).

This unit describes a method for generating DT40 cells in which a desired gene is genetically disrupted. Once the knockout construct is created, it is introduced into cells to generate the knockout cell line (Basic Protocol 1). After isolation of the knockout cells, their phenotype is characterized using cell biological analysis. These analyses include techniques for cell cycle analysis (Basic Protocol 2), immunostaining (Basic Protocols 3 and 4), and electron microscopy observation (Basic Protocol 5).

CREATION OF THE KNOCKOUT VECTOR AND CELL LINE

BASIC PROTOCOL 1

To isolate DT40 cell lines for a desired gene, the knockout vector is first constructed. After the vector is transfected into DT40 cells, it is integrated into the target locus by high-efficiency homologous recombination (Fig. 8.7.1). Positive clones are isolated on the basis of the antibiotic resistance introduced with the knockout vector, and their DNA is screened by Southern hybridization.

As our laboratory is studying centromere function, this protocol provides, as an example, the knockout of a gene required for centromere function. We have isolated the CENP-L protein, which is associated with CENP-H and CENP-I, and cDNA for *CENP-L* (Okada et al., 2006). We have identified the genomic locus of *CENP-L* by searching the NCBI

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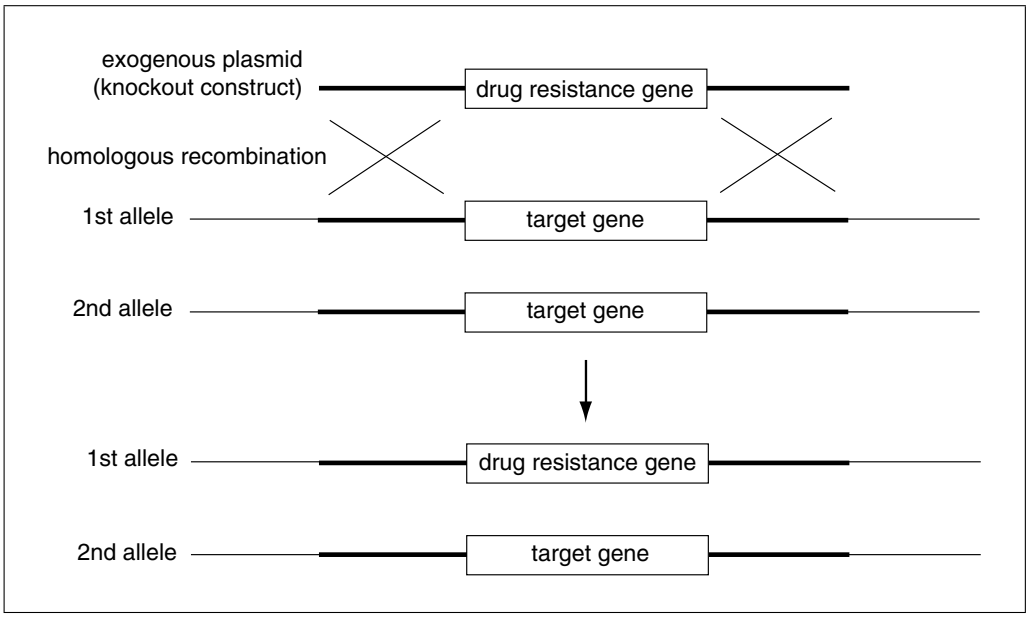

Figure 8.7.1 Scheme for gene disruption using homologous recombination. The knockout construct with a drug resistance gene and the homologous regions of the genome bracketing the gene of interest (indicated by the darker lines) is introduced into cells. Homologous recombination occurs, and the target gene is replaced with the knockout construct.

database, and it contains 4 exons and spans a 4-kb region (see Fig. 8.7.2). We typically replace a 3- to 4-kb region, corresponding to several exons, with an appropriate drug resistance gene. A total of >10 kb of the upstream and downstream sequences surrounding the region that we want to knock out are amplified by PCR and sequentially cloned into an appropriate vector such as pBluescript KS or SK(Stratagene), using standard molecular biology techniques (e.g., see Ausubel et al., 2011). In the case of the *CENP-L* knockout vector, we inserted the puromycin resistance gene between the upstream and downstream regions (Fig. 8.7.2; also see Okada et al., 2006). It is important to delete a critical region of a target protein. Alternatively, if you target the first exon, it is possible to repress whole region of mRNA of the desired gene.

Materials

30 to 40 μg of knockout construct plasmid for the gene of interest/transfection, e.g., *CENP-L* construct (Fig. 8.7.2)
Restriction enzymes (e.g., *Not*I, *Sal*I, and *Nco*I for the *CENP-L* construct in Fig. 8.7.2)
0.7% (w/v) agarose gels
1:1 (v/v) phenol/chloroform
100% and 70% (v/v) ethanol
Phosphate-buffered saline (PBS, *APPENDIX 2A*)
$>10^6$ DT40 cells (ATCC)/ml in suspension: culture from frozen stock in 30 ml Dulbecco's modified medium (DMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; see *APPENDIX 2A*), 1% (v/v) chicken serum, 1% (v/v) penicillin/streptomycin (P/S, Gibco) in a 75-cm^2 culture flask for 3 to 4 days
DT40 medium: DMEM supplemented with 10% (v/v) FBS, 1% (v/v) chicken serum
2× antibiotic selection medium: e.g., DT40 medium containing 1 μg/ml puromycin for knockout of *CENP-L*
1:1 (v:v) phenol/chloroform
Lysis buffer without sodium dodecyl sulfate (SDS) and proteinase K: 100 mM Tris (pH 8.0; see *APPENDIX 2A*)/200 mM NaCl/5 mM EDTA (see *APPENDIX 2A*)

Lysis buffer with SDS and proteinase K: 100 mM Tris (pH 8.0)/200 mM NaCl/5 mM EDTA/0.6% (w/v) SDS/0.6 mg/ml proteinase K

Isopropanol

Tris/EDTA buffer, pH8 (TE; *APPENDIX 2A*)

Repressible promoter system (e.g., Tet-Off, Clontech) with cDNA for the gene of interest

1.5-ml microcentrifuge tubes

50-ml polypropylene tubes (e.g., Corning)

4-mm electropolation cuvettes (Bio-Rad)

Electropolator (e.g., Gene Pulser, Bio-Rad)

96-well flat-bottom microtiter plates (e.g., Corning cat. no. 3596)

Multichannel pipettor and sterile troughs

24-well flat-bottom microtiter plates (e.g., Corning cat. no. 3526)

25-cm^2 tissue culture flasks

Additional reagents and equipment for performing agarose gel electrophoresis (Voytas, 2000) and Southern hybridization (Brown, 2004), and for determining cell numbers (e.g., see *UNIT 1.1*)

Prepare construct plasmid DNA

1. Linearize the knockout construct plasmid DNA (30 to 40 μg per each transfection), by incubating for 4 hr with an appropriate restriction enzyme in a 200-μl reaction, according to the supplier's directions, and examine the quality of the construct DNA by electrophoresis of a 1-μl sample on a 0.7% agarose gel (e.g., see Voytas, 2000).

   In the case of CENP-L, the construct was created in pBluescript. We used 100 U NotI in a 200-μl reaction in a 1.5-ml tube. The electrophoresis will confirm whether the digestion is complete.

2. Extract the DNA by adding 200 μl of 1:1 phenol/chloroform to the reaction mixture and mix gently.

3. Centrifuge 1 min at 15,000 × *g*, room temperature.

4. Move the upper layer to a fresh 1.5-ml microcentrifuge tube, and add 500 μl of 100% ethanol.

5. Centrifuge the DNA 10 min at 15,000 × *g*, 4°C.

6. Remove the supernatant, add 200 μl of 70% ethanol to the pellet, and mix.

7. Centrifuge the DNA 5 min at 15,000 × *g*, 4°C.

8. Remove the supernatant, and dry the pellet for 10 min at room temperature in a flow hood.

9. Resuspend the DNA in 200 μl phosphate-buffered saline (PBS).

Prepare DT40 cells

10. Centrifuge 10 to 30 ml of DT40 cell culture in 50-ml polypropylene tube for 5 min at 250 × *g*, room temperature.

11. Remove the supernatant by aspiration, and wash the pellet by gently mixing the cells with 30 ml PBS in.

12. Centrifuge 5 min at 250 × *g*, room temperature.

13. Remove the supernatant and resuspend the cells in PBS (~600 μl) to a final concentration of 1.66×10^7/ml. See, e.g., *UNIT 1.1* for cell-counting methods.

    The total number of cells should be ~1×10^7 in 600 μl of PBS.

Transfect DT 40 cells

14. Mix 600 µl of the cell suspension with 200 µl of DNA solution (from step 9) very well by gently inverting the tube, and transfer to a 4-mm electroporation cuvette.
15. Incubate the cells and DNA mixture on ice for 5 min.
16. Perform electroporation for ~0.7 msec at 550 V, 25 µF.

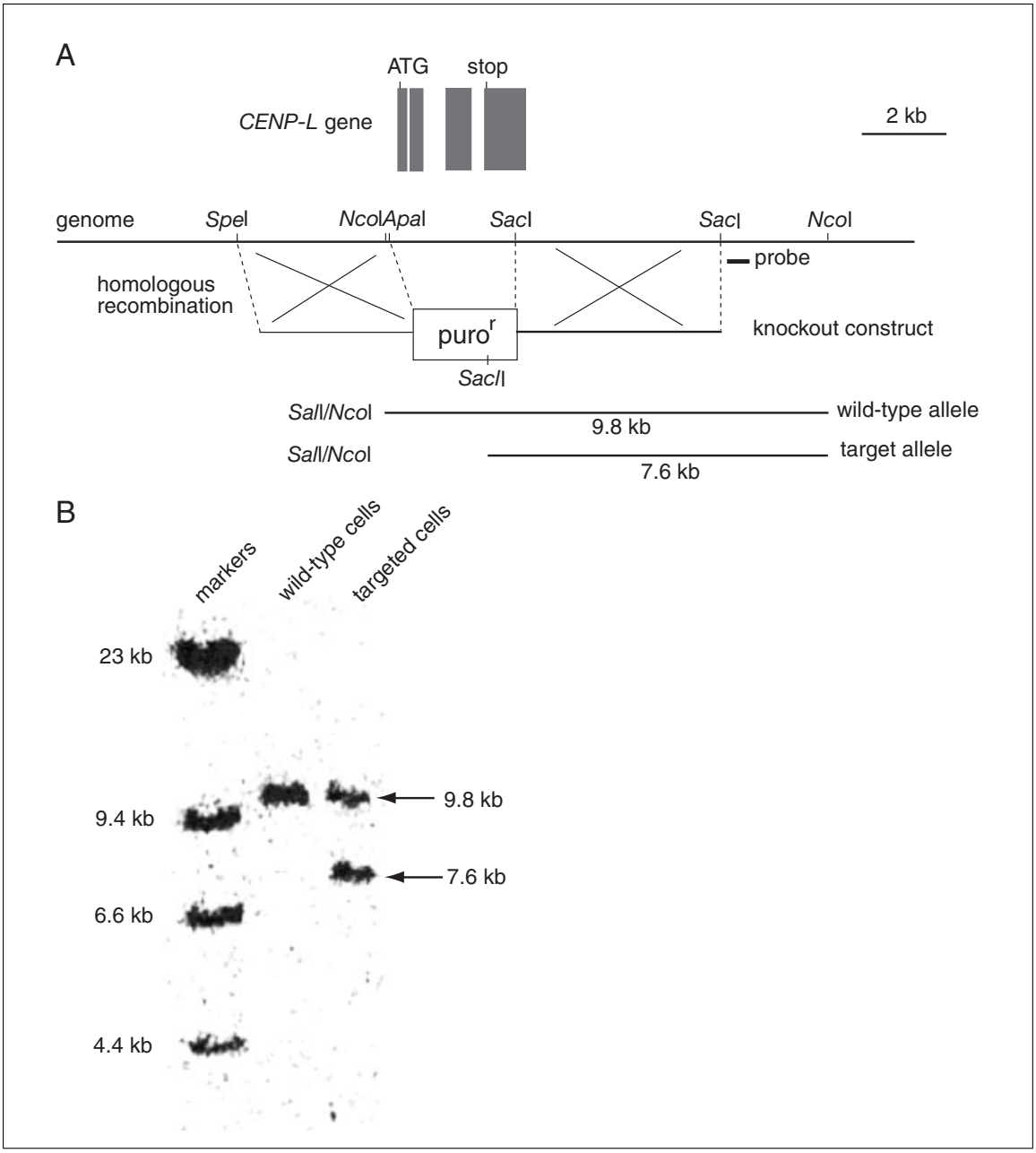

Figure 8.7.2 Example of gene disruption. (**A**) Restriction maps of the *CENP-L* locus, the gene knockout construct, with a drug resistance gene (puro^r, in this example), and targeted loci. Black boxes indicate the positions of the four exons to be disrupted. *Sal*I and *Nco*I restriction sites are shown, and the position of the probe used for Southern hybridization (which should be >0.5 kb) is indicated by the dark line. The novel 7.6-kb fragment digested with both *Sal*I and *Nco*I hybridizes to the probe if targeted integration of the construct has occurred. (**B**) Restriction analysis of the targeted integration of the *CENP-L* disruption construct. Genomic DNA from wild-type DT40 cells and a clone after first-round targeting are analyzed by Southern hybridization with the probe indicated in (A). The wild-type allele is detected at 9.8 kb, and the target allele is detected at 7.6 kb. The molecular size markers are a *Hind*III digest of λ DNA.

17. Incubate the cells on ice for 5 min.
18. Transfer the cells to 50 ml of DT40 medium, and distribute into five 96-well flat-bottom microtiter plates, adding 100 μl into each well using a multichannel micropipettor.
19. Incubate the cells for 24 hr at 5% CO_2, 38°C.
20. Add 100 μl of 2× antibiotic selection medium to each well using a multichannel pipettor.

    In the case the CENP-L construct, puromycin (0.5 μg/ml final concentration) was used. Other antibiotics that may be used, depending on the construct plasmid, include 2 mg/ml G418, 1 mg/ml histidinol, 2.5 mg/ml hygromycin, 1 mg/ml zeocin, or 25 μg/ml blasticidin (final concentration).
21. Incubate the cells for 7 to 10 days at 5% CO_2, 38°C, without changing the medium.

    Approximately 100 drug-resistant colonies per five 96-well plates should be visible after 7 to 10 days culture.

Isolate genomic DNA for Southern hybridization

22. Transfer the entire 200 μl of cell suspension from each positive well (containing a colony of >3 mm) in the 96-well plates into a well of a 24-well flat-bottom microtiter plate. Add 1 ml of DT40 medium to each well.
23. Incubate the cells in the 24-well plate for 3 days.

    DT40 cells grow in suspension culture.
24. Transfer 1 ml of the cell suspensions into a 1.5-ml microcentrifuge tubes and centrifuge 5 min at 250 × *g*, room temperature. Hold the remainder of the cell suspensions for expansion of clones identified as positive.
25. Remove the supernatant, add 0.2 ml PBS, and centrifuge 5 min at 250 × *g*, room temperature.
26. Remove the supernatant and resuspend the cells in 200 μl lysis buffer without SDS and proteinase K and mix well.
27. Add 100 μl lysis buffer with SDS and proteinase K, and incubate overnight at 55°C.
28. Add 300 μl isopropanol, and mix well.

    DNA precipitate should be visible.
29. Pick up the DNA precipitate using a micropipet tip (as a stick), and soak in 1 ml of 70% ethanol for ~10 sec.

    It is sufficient to just soak the DNA in 70% ethanol; it is not necessary to centrifuge.
30. Place the precipitate into 50 μl TE.

    There should be ~10 mg genomic DNA in the TE solution.
31. Add the appropriate restriction enzymes (*Sal*I, and *Nco*I for the *CENP-L* construct in Fig. 8.7.2), and incubate 1 hr at 55°C
32. Electrophorese ~3 μg DNA on a 0.7% agarose gel (see Voytas, 2000), and perform Southern hybridization with an appropriate probe (see Brown, 2004)

    See Figure 8.7.2B for an example of Southern hybridization results.

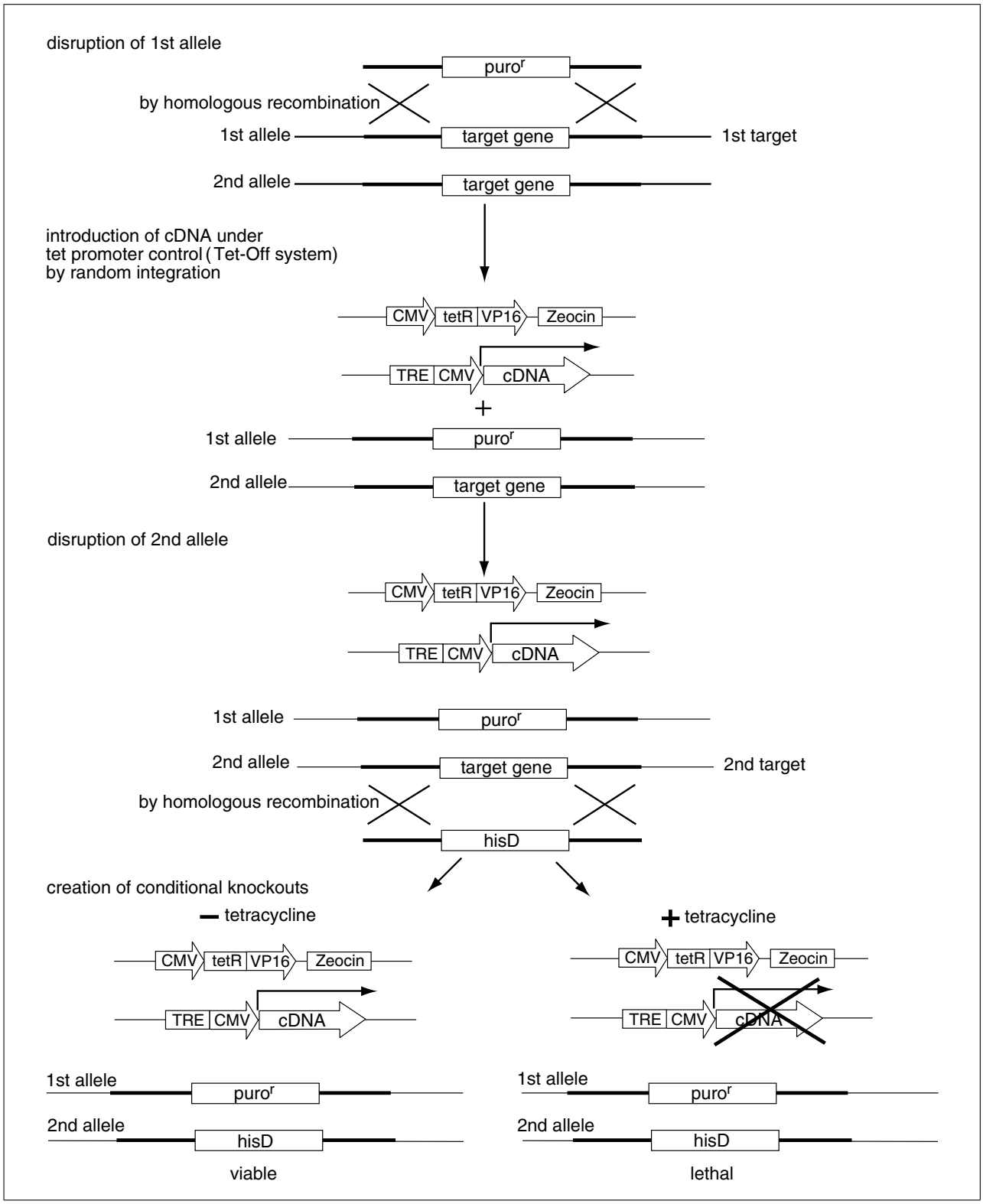

Figure 8.7.3 Strategy for creation of a conditional DT40 knockout cell line. The 1st-round target construct containing the puromycin resistance gene puro^r is transfected into DT40 cells. After 1st-round targeting, one clone is cotransfected with a cDNA construct under the control of a tet-repressible promoter and a tet-repressible transactivator (Tet-Off system) to provide conditional gene activity when the 2nd essential target allele is disrupted. Then the clone with the DNA transgene is transfected with the 2nd-round target construct containing an appropriate drug resistance gene (in this case histindinol resistance, *hisD*) to disrupt the remaining allele. Conditional knockout cells die after addition of tetracycline.

Create the knockout cell line

33a. *If the gene product is not essential for cell growth*: Expand the clones from step 23 that were identified in step 32 as having one allele knocked out, insert a different drug resistance gene into the knockout construct plasmid, and carry out a 2nd round of transfection to knock out the 2nd allele (steps 1 to 32).

This will result in the isolation of a homozygous mutant cell, knocked out for both alleles of the gene. Using the mutant cells, it is possible to perform further cell cycle analysis such as FACS and immunofluorescence to understand the phenotype of the mutant cells.

33b. *If the gene product is essential for cell growth:* Create a conditional knockout cell line by expanding the clones from step 23 that were identified in step 32 as having one allele knocked out, and first incorporating into each a repressible promoter system (e.g., the Tet-Off system) to control expression of the cDNA for the gene of interest. Then insert a different drug resistance gene into the knockout construct plasmid, and carry out a 2nd round of transfection (steps 1 to 32) to knock out the 2nd allele (see Fig.8.7.3).

We usually use the Tet system (Clontech) to create a conditional knockout cell line. After 1st-round targeting, a cDNA construct under control of tetracycline-repressive promoter and a tet-repressible transactivator (tTA) are cotransfected into a clone in which 1st-round targeting occurred (Fig. 8.7.3). The cDNA construct can be generated by cloning cDNA for the gene of interest into the Clontech Tet-Off vector. The tTA construct with several drug resistance markers can be purchased from Clontech. Then, 2nd-round targeting should be performed to create a conditional knockout cell line (Fig. 8.7.3).

34. Maintain the knockout cell lines in 10 ml of DT40 medium in 25-cm^2 tissue culture flasks, and passage daily by splitting 1:5 to 1:8 to generate cell cultures of 10^5 to 10^6 cells/ml for the analyses in Basic Protocols 2 to 5.

Once a cell line is established, the phenotype can be analyzed after addition of tetracycline. For example, as CENP-H is an essential gene, we took this conditional knockout strategy (Fukagawa et al., 2001). After addition of tet to the CENP-H knockout cells, we perform cell cycle analysis studies, e.g., FACS analysis (Basic Protocol 2).

ANALYSIS OF CELL CYCLE PROFILE BY FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

BASIC PROTOCOL 2

Once a knockout clone is isolated, the phenotype of the knockout cells can be examined. A cell cycle profile in these cells is helpful in understanding the phenotype (Fig. 8.7.4). If the candidate gene is essential for mitotic progression, the main population of the knockout cells will be in the G2/M portion of the cell cycle (Fukagawa et al., 2001; Nishihashi et al., 2002; Okada et al., 2006; Hori et al., 2008). This protocol describes preparation of knockout and control DT40 cells for cell cycle analysis by FACS.

Materials

10 mM bromodeoxyuridine (BrdU)
5–10 × 10^6 knockout (Basic Protocol 1) and control DT40 cells in 10 ml of DT40 medium in 25-cm^2 tissue culture flasks
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
70% (v/v) ethanol, ice-cold
1% (v/v) bovine serum albumin (BSA)/PBS (*APPENDIX 2A*)
2 M HCl/0.5% (v:v) Triton X-100
Anti-BrdU antibody (BD Biosciences, cat. no. 347580)
Anti-mouse IgG, FITC conjugated (Jackson Laboratory, cat. no. 115-095-003)
Propidium iodide (PI)

15-ml polypropylene tubes (Falcon or Corning)
Polystyrene FACS tubes (Becton-Dickinson)
Flow cytometer (e.g., FACS scan, Becton-Dickinson)

Treat cells with BrdU

1. Add 20 µl of 10 mM BrdU to the 5–10 × 10^6 knockout and control DT40 cells and incubate the cells 10 min at 38 °C. Do not expose the cells to strong light.
2. Centrifuge the cells in a 15-ml polypropylene tube 5 min at 250 × *g*, room temperature.
3. Remove the supernatant, and add 10 ml PBS. Centrifuge the cells 5 min at 250 × *g*, room temperature.
4. Remove the supernatant, and resuspend the cells in 50 µl PBS. Add 2 ml ice cold 70% ethanol, and mix well. Leave the cells overnight at −20°C.

   Cells can also be kept at −20°C for up to 1 month.

5. Centrifuge the cells 5 min at 250 × *g*, room temperature.
6. Remove the supernatant, and add 10 ml of 1% BSA/PBS. Centrifuge the cells 5 min at 250 × *g*, room temperature.
7. Remove the supernatant, and resuspend the cells by vortexing in 10 ml of 2 M HCl/0.5% Triton X-100. Incubate 30 min at room temperature.
8. Centrifuge the cells 5 min at 250 × *g*, room temperature.
9. Remove the supernatant, and wash the cell pellet with 10 ml 1% BSA/PBS. Centrifuge the cells 5 min at 250 × *g*, room temperature. Repeat this step twice to neutralize.

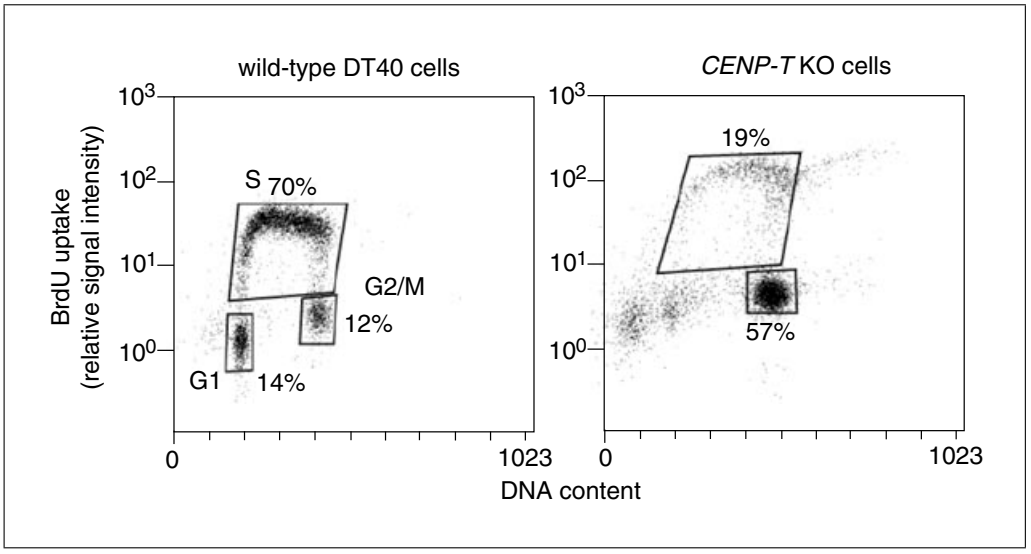

Figure 8.7.4 Cell cycle profile of wild-type DT40 cells and *CENP-T* knockout (KO) cells. Cells are stained with FITC-anti-BrdU (*y* axis, log scale) to detect BrdU incorporation (DNA replication) and with propidium iodide to detect total DNA (*x* axis, linear scale). The lower-left box represents G1-phase cells, the upper box represents S-phase cells, and the lower-right box represents G2/M-phase cells. The numbers given in the boxes indicate the percentage of gated events. Experimental data are derived from Hori et al. (2008).

Stain cells for DNA

10. Remove the supernatant, and resuspend the cells in 50 µl anti-BrdU antibody diluted 1:1 to 1:10 with 1% BSA/PBS. Incubate 1 hr at room temperature.

    The best antibody dilution to use is determined by the signal intensity in the FACS analysis, and can be adjusted in subsequent experiments.

11. Add 1 ml of 1% BSA/PBS, and centrifuge the cells 5 min at 250 × *g*, room temperature.

12. Remove the supernatant, and add 10 ml of 1% BSA/PBS. Centrifuge the cells 5 min at 250 × *g*, room temperature. Repeat the wash.

13. Remove the supernatant, and resuspend the cells in 50 µl FITC-conjugated anti-mouse antibody diluted 1:10 to 1:100 with 1% BSA/PBS. Incubate 30 min at room temperature.

14. Add 1 ml 1% BSA in PBS, and centrifuge 5 min at 250 × *g*, room temperature.

15. Remove the supernatant, and add 10 ml of 1% BSA/PBS. Centrifuge the cells 5 min at 250 × *g*, room temperature.

16. Remove the supernatant, and resuspend the cells in 500 µl PBS containing 5 µg/ml propidium iodide (PI). Incubate 1 hr at room temperature.

17. Analyze the cells by flow cytometry.

    A typical cell cycle profile is shown in Figure 8.7.4. In the case of knockout cells for kinetochore protein, G2/M accumulation was observed, due to failure of chromosome segregation.

INDIRECT IMMUNOFLUORESCENCE ANALYSIS IN DT40 CELLS

BASIC PROTOCOL 3

When using DT40 knockout cells, it is essential to examine the localization of proteins related to the disrupted gene (Okada et al., 2006; Hori et al., 2008). This protocol describes a standard method for examining the localization of intracellular proteins. If a kinetochore protein is disrupted, it is very important to examine the localization of the other kinetochore proteins in order to understand their assembly pathway.

Materials

5–10 × 10^6 knockout (Basic Protocol 1) and control DT40 cells in 10 ml of DT40 medium in 25-cm^2 tissue culture flasks
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
3% (w/v) paraformaldehyde (PFA)/250 mM HEPES (pH7.4)
0.5% w/v NP-40 (Sigma)/PBS
Primary antibodies for proteins of interest (e.g., anti-CENP-T; generated in-house)
0.5% (v/v) bovine serum albumin (BSA)/PBS
Appropriate secondary dye-conjugated antibody (e.g., FITC- or Cy3-conjugated anti-rabbit IgG, Jackson Laboratory, cat. no. 111-097-003 or 111-167-003)
0.2 µg/ml 4,6-diamidino-2-phenylindole (DAPI)/Vectashield Antifade (Vector Laboratories)

Glass microscope slides
Cytospin centrifuge (e.g., Cytospin 3, Shandon)
Coplin jars
Parafilm
Fluorescence light microscope (e.g., Olympus IX71) with CCD camera

Additional reagents and equipment for determining cell numbers (e.g., see *UNIT 1.1*)

Prepare cells for staining

1. Centrifuge ~5 × 10^6 (10 ml) DT40 knockout cells and control DT40 cells 5 min at 250 × *g*, room temperature.
2. Remove the supernatant, and resuspend the cells in 10 ml PBS. Centrifuge 5 min at 250 × *g*, room temperature.
3. Remove the supernatant, and resuspend the cells in PBS, adjusting the cell concentration to 1–10 × 10^5/ml. See, e.g., *UNIT 1.1* for cell-counting methods.
4. Disperse 300 µl cell suspension onto a glass microscope slide using a Cytospin device by centrifuging 5 min at 250 × *g*, room temperature.
5. Insert the slide into a Coplin jar with 3% PFA /250 mM HEPES, and incubate 15 min at room temperature to fix the cells.

   This procedure should be performed in a Coplin jar, where appropriate.

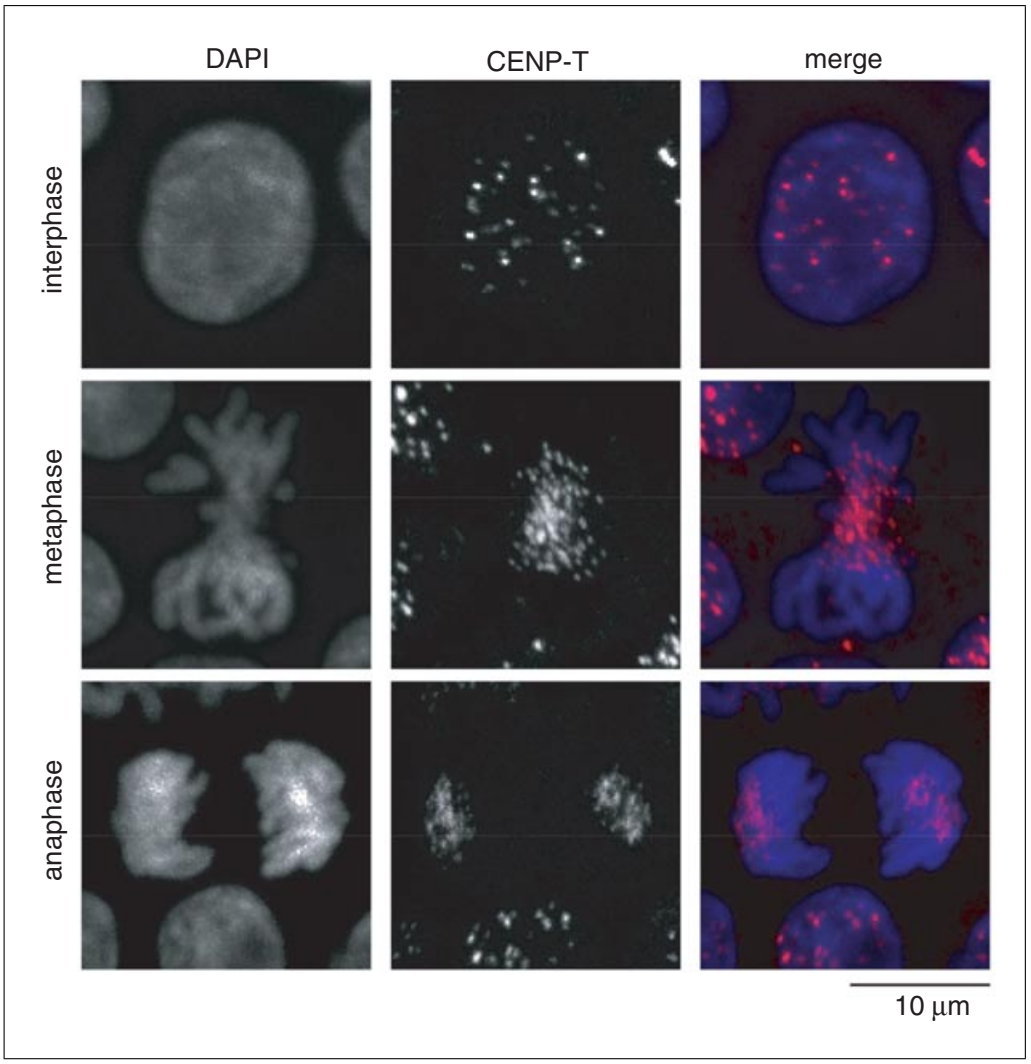

Figure 8.7.5 Localization of CENP-T at progressive stages of the cell cycle in DT40 cells. Cells are fixed and stained with anti-CENP-T antibody (red). Nuclei and chromosomes are visualized by counterstaining with DAPI (blue). The right column shows the merged images for the DAPI and antibody-stained cells. The scale bar corresponds to 10 µm. For the color version of this figure go to *http://www.currentprotocols.com*.

6. Wash the slide briefly in 40 ml PBS, and incubate 15 min at room temperature in 40 ml of 0.5% NP40/PBS.

7. Wash the slide briefly in 40 ml PBS, and incubate 40 min in 0.5% NP-40/PBS

Stain cells with primary antibody

8. Remove the slide from the Coplin jar, and add 100 μl of a primary antibody diluted 1:1,000 to 1:10,000 in 0.5% BSA/PBS.

   Various antibodies against kinetochore and cell-cycle protein are available from several companies (e.g., Santa Cruz, Abcam, or MBL).

   The optimal antibody dilution to use is determined by the final result, and can be adjusted in subsequent experiments. If background signals are high, you can use a high dilution.

9. Cover the slide with Parafilm, and incubate 1 hr at 37°C.

10. Wash the slide three times, 5 min each, in 40 ml of 0.5% BSA/PBS.

Stain cells with secondary antibody

11. Add to the slide 100 μl of a secondary antibody diluted 1:1,000 to 1:10,00 in 0.5% BSA/PBS.

    If back ground signals are high, you can use a high dilution.

12. Cover the antibody solution with a Parafilm and incubate 45 min at 37°C.

13. Wash the slide three times, 5 min each, in 40 ml of 0.5% BSA/PBS.

Stain cells with DAPI and analyze

14. Carefully remove all of the liquid from the slide using a soft tissue. Add 30 μl DAPI in Vectashield Antifade, and cover with a cover slip.

15. Analyze the cells with a fluorescence microscope.

    Typical centromere staining with an anti-CENP-T antibody is shown in Figure 8.7.5.

INDIRECT IMMUNOFLUORESCENCE ANALYSIS OF METAPHASE CHROMOSOME SPREADS

BASIC PROTOCOL 4

Kinetochore proteins localize into the centromere region of mitotic chromosomes. To examine localization of these proteins in mitotic chromosomes, chromosome spreads can be prepared and analyzed by immnofluorescence. This protocol describes the preparation of metaphase chromosome spreads for immunofluorescence analysis. DT40 cells have 78 chromosomes, including 38 autosomal chromosome pairs and chromosomes Z and W, known as sex chromosomes.

Materials

5–10 × 10^6 knockout (Basic Protocol 1) and control DT40 cells in 10 ml of DT40 medium in 25-cm^2 tissue culture flasks
Nocodazole (e.g., Sigma, cat. no. M1404)
DT40 medium: Dulbecco's modified medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS; see *APPENDIX 2A*)/ 1% (v/v) chicken serum
0.56% hypotonic buffer (40 mM KCl/0.5 mM EDTA/20 mM HEPES, pH 7.5)
Phosphate-buffered saline (PBS; *APPENDIX 2A*)
Primary antibodies for proteins of interest (e.g., anti-CENP-T; generated in-house)
0.5% bovine serum albumin (BSA)/PBS (*APPENDIX 2A*)
Appropriate secondary dye–conjugated antibody (e.g., FITC- or Cy3-conjugated anti-rabbit IgG, Jackson Laboratory, cat. no. 111-097-003 or 111-167-003)
3% paraformaldehyde/250 mM HEPES (pH7.4)
0.2 μg/ml of 4,6-diamidino-2-phenylindole (DAPI)/Vectashield Antifade (Vector Laboratories)

15-ml polypropylene tubes (Falcon or Corning)
Glass microscope slides
Cytospin centrifuge (e.g., Cytospin 3, Shandon)
Coplin jars
Parafilm
Fluorescence light microscope (e.g., Olympus IX71) with CCD camera

Prepare cells

1. Treat 5×10^6 knockout and control DT40 cells with 500 ng/ml nocodazole (final concentration) for 2 hr at 5% CO_2, 38°C to enrich for mitotic cells.
2. Collect cells by centrifuging 5 min at 250 × *g*, room temperature.
3. Remove the supernatant, and resuspend the cell pellets in 300 µl DT40 medium.
4. Add 2 ml of 0.56% hypotonic buffer, and incubate 20 min at 37°.
5. Add 2 ml PBS, and shake gently.
6. Disperse 200 to 300 µl of the cell suspension onto a glass microscope slide using a Cytospin for 5 min at 250 × *g*, room temperature.
7. Insert the slide into a Coplin jar with 3% PFA/ 250 mM HEPES to fix the cells, and incubate 15 min at room temperature.

   This procedure should be performed in a Coplin jar, where appropriate.

8. Wash the slide briefly in 40 ml PBS, and incubate it 15 min at room temperature in 40 ml of 0.5% NP40/PBS.

Stain cells with primary antibody

9. Remove the slide from the Coplin jar, and add 100 µl primary antibody diluted 1:1,000 to1:10,000 in 0.5% BSA/PBS

   The optimal antibody dilution to use is determined by the final result, and can be adjusted in subsequent experiments. If background signals are high, you can use a high dilution.

10. Cover the antibody solution with Parafilm and incubate 1 hr at 37°C.
11. Wash the slide three times, 5 min each, in 40 ml of 0.5% BSA/PBS.

Stain cells with secondary antibody

12. Add to the slide 100 µl secondary antibody diluted 1:1,000 to 1:10,000 in 0.5% BSA/PBS.

    If background signals are high, you can use a high dilution.

13. Cover the antibody solution with Parafilm and incubate 45 min at 37°C.
14. Wash the slide three times, 5 min each, in 40 ml 0.5% BSA/PBS.
15. Carefully remove all the liquid from the slide using a soft tissue.

Counterstain cells and analyze

16. Add 30 µl DAPI/Vectashield Antifade, and cover with a cover slip.
17. Analyze the cells with a fluorescence microscope.

    A typical image for centromere staining on mitotic chromosomes with an anti-CENP-T antibody is shown in Figure 8.7.6. DT40 cells have many mini chromosomes, which are difficult to detect by DAPI staining. All of the red dots shown in Figure 8.7.6 are kinetochore signals, not background.

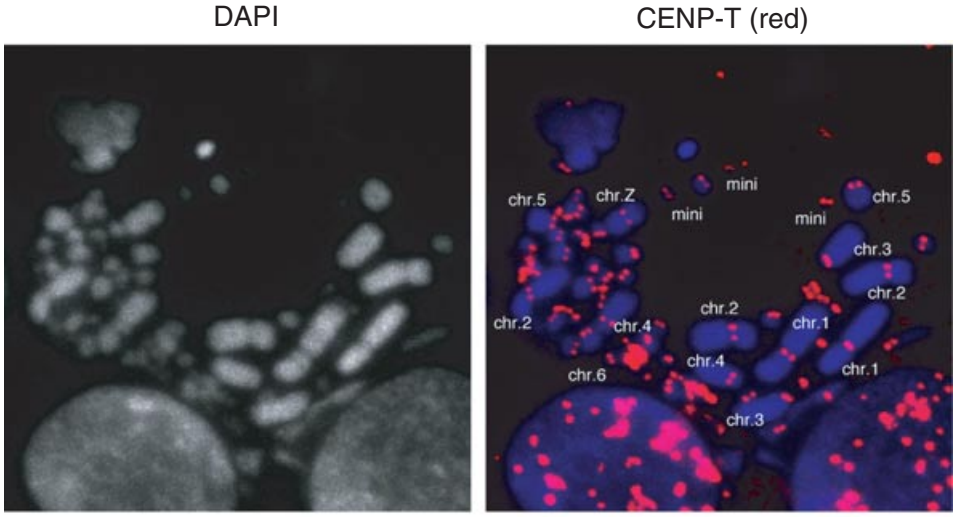

Figure 8.7.6 Localization of CENP-T on mitotic chromosomes CENT-P signals generated by FITC-conjugated secondary antibodies are shown in red. Chromosomes are visualized by counterstaining with DAPI (blue). The scale bars correspond to 10 μm. For the color version of this figure go to *http://www.currentprotocols.com*.

IMMUNO-ELECTRON MICROSCOPY (EM) ANALYSIS IN DT40 CELLS

BASIC PROTOCOL 5

The size of the kinetochore is very small, and ultrastructural analysis with electron microscopy (EM) is a powerful means of understanding the phenotype generated in DT40 cells knocked out for kinetochore proteins (Hori et al., 2008, Amano et al., 2009). As in the case of immunofluorescence analysis, it is very important to examine localization of kinetochore proteins in cells where a kinetochore protein has been disrupted because the ultrastructure of the kinetochore may be changed. This protocol describes a standard method for immuno-EM analysis of kinetochore protein; it is applicable to other proteins, as well.

Materials

5–10 × 10^6 knockout (Basic Protocol 1) and control DT40 cells in 10 ml of DT40 medium in 25-cm^2 tissue culture flasks
Phosphate-buffered saline (PBS, *APPENDIX 2A*)
3% (w/v) paraformaldehyde (PFA)/250 mM HEPES, pH7.4
0.5%-NP-40 (Sigma)/PBS
Antibodies to proteins of interest (e.g., Santa Cruz, Abcam, or MBL)
0.5% (v/v) bovine serum albumin (BSA)/PBS
Appropriate secondary nanogold-conjugated antibody (e.g., Nanogold conjugated anti-rabbit IgG, Nanoprobes)
2% (v/v) glutaraldehyde (e.g., Sigma cat. no. G7776)/100 mM sodium cacodylate buffer, pH7.3 (e.g., TAAB Laboratories)
7% (w/v) sucrose (e.g., Wako)/100 mM sodium cacodylate buffer, pH7.3
Silver enhancer kit (e.g., HQ silver, Nanoprobes)
0.5% (w/v) osmium tetroxide (OsO_4; e.g., PGM Chemical)
Uranyl acetate (UA, e.g., Mallinckrodt)
20%, 30%, 40%, 60%, 80%, 95%, and 100% ethanol
50% (v/v) ethanol/propylene oxide (e.g., Wako)
100% propylene oxide

50% (v/v) epoxy resin (EPON; e.g., EPON 812, MNA, DDSA, DMP-30; TAAB Laboratories)/propylene oxide
100% epoxy resin

Glass microscope slides
Parafilm
Cytospin centrifuge (e.g., Cytospin 3, Shandon)
Coplin jars
Ultramicrotome (e.g., EM UC6+FC6, Leica)
Grid with formvar membrane (e.g., Veco)
Electron microscope (e.g., JEM1010, JEOL)

Prepare cells

1. Centrifuge ~5 × 10^6 DT40 knockout cells and control DT40 cells 5 min at 250 × *g*, room temperature.
2. Remove the supernatant, and resuspend the cells in 10 ml PBS. Centrifuge 5 min at 250 × *g*, room temperature.
3. Remove the supernatant, and resuspend the cells in 300 µl PBS. Adjust the cell concentration to 1–10 × 10^5/ml.
4. Disperse 300 µl cell suspension onto a glass microscope slide using a Cytospin device by centrifuging 5 min at 250 × *g*, room temperature.
5. Insert the slide with the cells into a Coplin jar, and incubate 15 min at room temperature with 40 ml 3% PFA in 250 mM HEPES to fix the cells.
6. Wash the slide briefly in PBS and incubate it 15 min at room temperature in 40 ml of 0.5% NP40/PBS.

Stain with primary antibody

7. Remove the slide from the Coplin jar, and add 100 µl of a primary antibody at an appropriate dilution.
8. Cover the antibody solution with Parafilm and incubate 1 hr at 37°C.
9. Wash the slide three times, 5 min each, in 40 ml of 0.5% BSA/PBS.

Stain with secondary antibody

10. Add to the slide 100 µl of secondary nanogold-conjugated antibody diluted in 0.5% BSA/PBS to an appropriate dilution.
11. Cover the antibody solution with Parafilm, and incubate 1 hr at 37°C.
12. Wash the slide three times, 5 min each, in 40 ml 0.5% BSA/PBS
13. Insert the slide in 3% PFA/250 mM HEPES, and incubate 10 min at room temperature.
14. Insert the slide in 2.5% glutaraldehyde/100 mM sodium cacodylate buffer to fix cells, and incubate for at least 1 hr at room temperature.
15. Wash the slide five times, 5 min each, in 7% sucrose/100 mM sodium cacodylate buffer, pH 7.3.

Prepare cells for EM

16. Silver-stain samples using a silver enhancer kit, according to the manufacturer's directions.
17. Incubate the slides in 0.5% OsO_4 for 30 min on ice.

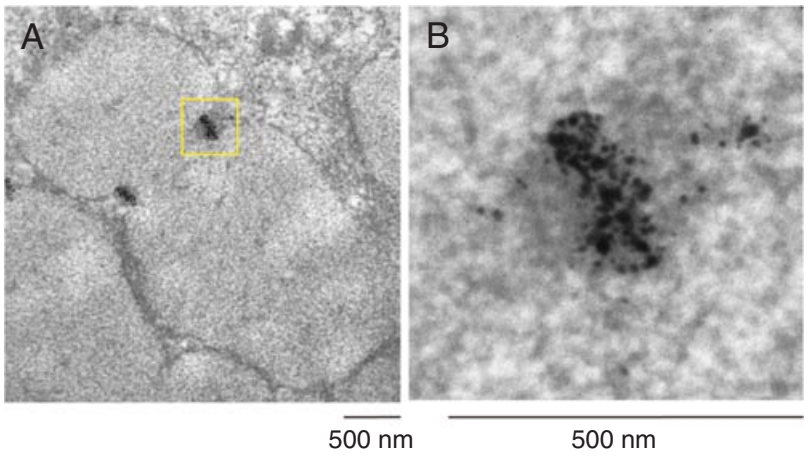

Figure 8.7.7 Immuno-electron microscopy (EM) image of mitotic chromosomes stained with anti-CENP-E antibodies. (**A**) The dark signals are the result of gold labeling. CENP-E localizes to the kinetochore plate (**B**) Magnified image of the region in the yellow box in panel A. The scale bars correspond.

18. Wash the slides in water, and incubate in 0.5% UA overnight at 4°C.
19. Dehydrate the sample by incubating sequentially for 5 min each at room temperature in 20%, 30%, 40%, 60%, 80%, and 95% ethanol, and finally three times, 10 min each, in 100% ethanol.
20. Incubate the slide 10 min in 50% ethanol in propylene oxide.
21. Incubate the slide three times, 10 min each, in propylene oxide.
22. Incubate the slide overnight in 50% EPON/propylene oxide.
23. Embed the samples in EPON, and incubate for 2 nights at 65°C.
24. Separate the samples from the slide, cut them into 170-nm serial sections with a microtome, and place the sections on a grid with a formvar membrane to examine by EM.

    If you insert samples into liquid nitrogen a few seconds, you can easily separate the EPON with the cells from the slide.

    Typical EM images for centromere protein are shown in Figure 8.7.7.

COMMENTARY

Background Information

Buerstedde and Takeda (1991) originally found that DT40 cells show a high ratio of targeted to random integration upon transfection of a plasmid with a sequence from the chicken genome. After this discovery, DT40 became widely used in the field of cell biology. Although RNAi technology is popular means of suppressing gene expression in vertebrate cells, genetic disruption of a desired gene using DT40 or mouse ES cells is a useful and reliable way to characterize phenotype. The ratio of homologous recombination is higher in DT40 cells than in mouse ES cells (Buerstedde and Takeda, 1991). In addition, the growth rate of DT40 cells is fast, and cells are easily available for cell biological and biochemical studies (Okada et al., 2006; Fukagawa, 2008; Hori et al., 2008; Amano et al., 2009). For analysis of the phenotype at the level of the whole organism, DT40 is not appropriate system; however, for analysis of the function of genes required for cell cycle regulation, DT40 cells provide a very powerful system.

It is most important to understand the phenotype of knockout cells using cell biological methods. In this unit we introduced useful protocols for cell biological analysis in

DT40 cells. As we are mainly studying the function of the centromere and kinetochore (Fukagawa, 2008), the protocols were described for analysis of cells mutant for kinetochore proteins, including FACS, immunofluorescence, and immuno-EM. However, these techniques are certainly applicable to analysis of mutations in other genes required for cell cycle progression, e.g., those related to DNA replication, recombination, and DNA-repair.

Critical Parameters and Troubleshooting

The two main types of failures that may occur when using the protocols described in this unit are noted below, along with suggestions for preventing them.

Failure to isolate knockout cell lines

Possible solutions: (1) Check the knockout construct to make sure it contains, the drug resistance marker gene. (2) Check the concentration of selection drugs. (3) Check the quality of DNA. (4) Check the electroporation conditions. (5) Check the growth condition of the DT40 cells. Overgrowth of cells often reduces efficiency of transfection.

If all of the above criteria are correct and approximately 100 clones are not obtained, homologous recombination may not have occurred. In that case, the genomic region used to make the target construct is not appropriate, and should be changed. In the Basic Protocol 1 introduction paragraph about creation of the knockout vector, we mentioned that a 3 to 4 kb region, which corresponds to several exons, should be replaced with an appropriate drug resistance gene. If colonies resulting from homologous recombination are not obtained, we would change the region completely (at least 5 kb away from the previous region). There are regions in which homologous recombination does not occur, although the reason is unclear. Another possibility is that the homologous region within the knockout vector is too short. We recommend that a total region of >10 kb be used.

Absence of signals after immunofluorescence analysis

Possible solutions: (1) Check the dilution of antibodies. (2) Change the fixative from paraformaldehyde to methanol. Paraformaldehyde solution is used to fix cells in the protocol described above. For some antibodies it may not be appropriate to use paraformaldehyde. (3) Try a longer incubation with the antibodies (e.g., overnight). (4) Use a CCD camera with higher sensitivity.

Anticipated Results

If you screen 100 clones, you should get more than 30 positive colonies. In a 2nd round of knockout, efficiency will be reduced from 30% to $>10\%$. Typical results for FACS, immunofluorescence analysis, and immuno-EM are shown in Figures 8.7.4 to 8.7.7.

Time Considerations

After introduction of a knockout construct into DT40 cells, it takes 7 to 10 days to obtain colonies. It takes an additional 10 days to isolate DNA and perform a Southern hybridization analysis to identify the knockout allele. As DT40 cells have a diploid genome, requiring a 2nd round of transfection, it takes $\sim$2 months to generate a knockout cell line. If a gene of interest is essential for cell growth, it is necessary to introduce a cDNA expression construct under control of tetracycline repressive promoter (Fig. 8.7.3). In this case, it takes an additional month to generate a conditional knockout cell line. In total, it takes 2 (for a nonessential gene) or 3 (for an essential gene) months to generate a DT40 knockout cell line.

Once a knockout cell line is established, it takes 1 day to perform each standard immunofluorescence or FACS analysis. Iimmuno-electron microscopy experiments take 3 to 5 days to carry out.

Literature Cited

Amano, M., Suzuki, A., Hori, T., Backer, C., Okawa, K., Cheeseman, I.M., and Fukagawa, T. 2009. The CENP-S complex is essential for the stable assembly of outer kinetochore structure. *J. Cell Biol.* 186:173-182.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds.) 2011. Current Protocols in Molecular Biology. John Wiley & Sons, Hoboken, N.J.

Brown, T. 2004. Analysis of DNA sequences by blotting and hybridization. *Curr. Protoc. Mol. Biol*. 68:2.9.1-2.9.20.

Buerstedde, J.M. and Takeda, S. 1991. Increased ratio of targeted to random integration after transfection of chicken B cell lines. *Cell* 67:179-188.

Fukagawa, T., Mikami, Y., Nishihashi, A., Regnier, V., Haraguchi, T., Hiraoka, Y., Sugata, N., Todokoro, K., Brown, W., and Ikemura, T. 2001. CENP-H, a constitutive centromere component, is required for centromere targeting of CENP-C in vertebrate cells. *EMBO J.* 20:4603-4617.

Fukagawa, T. 2008. The kinetochore and spindle checkpoint in vertebrate cells. *Front. Biosci.* 13:2705-2713.

Hori, T., Amano, M., Suzuki, A., Backer, C.B., Welburn, J.P., Dong, Y., McEwen, B.F., Shang, W.H., Suzuki, E., Okawa, K., Cheeseman, I.M.,

and Fukagawa, T. 2008. CCAN makes multiple contacts with centromeric DNA to provide distinct pathways to the outer kinetochore. *Cell* 135:1039-1052.

Nishihashi, A., Haraguchi, T., Hiraoka, Y., Ikemura, T., Regnier, V., Dodson, H., Earnshaw, W.C., and Fukagawa, T. 2002. CENP-I is essential for centromere function in vertebrate cells. *Dev. Cell* 2:463-476.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P.S. 2003. Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnol.* 21:635-637.

Okada, M., Cheeseman, I.M., Hori, T., Okawa, K., McLeod, I.X., Yates, J.R. III, Desai, A., and Fukagawa, T. 2006. The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres. *Nature Cell Biol.* 8:446-457.

Voytas, D. 2000. Resolution and recovery of large DNA fragments. *Curr. Protoc. Mol. Biol.* 51: 2.5A.1-2.5A.9.