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TECHNICAL NOTE

Derivation of a novel G2 reporter system

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Abstract Progression through G2 phase of the cell cycle is a technically difficult area of cell biology to study due to the lack of physical markers specific to this phase. The FUCCI system uses the biology of the cell cycle to drive fluorescence in select phases of the cell cycle. Similarly, a commercially available system has used a fluorescent analog of the Cyclin B1 protein to visualize cells from late S phase to the metaphase–anaphase transition. We have modified these systems to use the promoter and destruction box elements of Cyclin B1 to drive a cyan fluorescent protein. We demonstrate here that this is a useful tool for measuring the length of G2 phase without perturbing any aspect of cell cycle progression.

Keywords Cell cycle · G2 phase · Mitosis · DNA synthesis · Cyclin B1

Introduction

Cell cycle progression is tightly regulated by a series of phosphorylation events driven by the cyclin dependent kinases (CDKs), which require specific binding to their regulatory cyclin partners. Kinase-dependent control over the transition between G1 phase of the cell cycle into DNA synthesis (S phase) is heavily studied, however the events regulating the transition from G2 phase into mitosis (M phase) receive much less focus, especially in mammalian systems (Lim and Kaldis 2013). The lack of tools to precisely measure movement through G2 phase of the cell cycle is one technical hurdle to studying this aspect of cell cycle control.

Sakaue-Sawano et al. (2008) published the FUCCI reporter system for use in vitro and in vivo to measure the timing of G1 phase of the cell cycle and the relative timing in S/G2/M phase. This system relies on the cell cycle dependent oscillating activity of the E3 ligase complexes APC^{Cdh1} and SCF^{Skp2}. Fluorescent analogs of Cdt1 and Geminin are used to detect cells in either G1 phase or S/G2/M phase of the cell cycle, respectively. This system is not able to measure the timing of movement through G2 phase directly.

A commercially available the G2/M Cell Cycle Phase Marker developed by Amersham Biosciences [User manual # 25-8010 (50–53)] has come close to achieving this. This system expresses a large portion of the coding region of the G2/M cyclin, Cyclin B1, as well as the promoter from the Cyclin B1 gene (CCNB1). Cyclin B1 provides a valuable source for measuring the length of G2 phase of the cell cycle. Expression is first seen in late S-phase and the mRNA levels increase specifically during G2 phase (Pines and Hunter 1989; Piaggio et al. 1995). It is known that using −3,800 bp relative to the cap site of the Cyclin B1 promoter can confer G2-

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enhanced promoter activity (Cogswell et al. 1995). This system drives expression of the N-terminal domain of Cyclin B1 that includes a 9-amino-acid destruction box (D-box) motif (RTALGDIGN) that targets Cyclin B1 for ubiquitination and subsequent degradation (King et al. 1996a, b). This D-box sequence is recognized by the APC^{Cdt} ubiquitin system in metaphase and the protein is destroyed in anaphase of the cell cycle (Meyn et al. 2002; Passmore et al. 2003; Yamano et al. 2004). Hence, this is a valuable tool for measuring the length of G2 up to the metaphase-anaphase transition. However, the Cyclin B1 region used in this system also includes the cytoplasmic retention sequence (CRS) domain of the protein, a region known to interact with a number of intracellular proteins (Fidalgo da Silva et al. 2011, 2013; Hagting et al. 1998; Barnes et al. 2001). This poses potential problems for researchers studying or manipulating these specific proteins as the reporter may interact with their proteins of interest and may cause unexpected side effects.

In this work we develop an analogous system that uses the Cyclin B1 promoter to drive a cyan fluorescent protein (ECFP) fused to a small D-box domain to ensure degradation at the metaphase–anaphase transition. Using live cell imaging in conjunction with Hoechst staining we demonstrate that this system does not alter the timing of G2 phase of the cell cycle or relative cell numbers as assessed using a metabolic marker.

Materials and methods

Construction of the G2/M CFP reporter

The pcDNA3–CFP plasmid obtained from Addgene (Cambridge, MA, USA; # 13030) was used as a backbone vector for the construction of the pCCNB1–ECFP G2/M reporter vector. The ECFP protein in the pcDNA3–CFP is expressed under the CMV promoter. The human cytomegalovirus (CMV) promoter drives constitutive protein expression in cells (Qin et al. 2010). The CMV promoter was BglII–HindIII digested from the pcDNA3–CFP vector, and on its place a linker (BglII-GCACTTGCGGTGAGGCATAATTA-CAACT-HindIII) was used to close the resulting vector. The Cyclin B1 promoter was obtained from the pCycB(−3800)-CAT vector through HindIII–BamHI digestion (Cogswell et al. 1995). The 2.75 kb promoter fragment was ligated into the pcDNA3–CFP without the CMV promoter. The sequence 5′-ATGAG AACCGCCCTGGGCGACATCGGCAACC-3′ coding for the Cyclin B1 D-box sequence plus a 5′ ATG starting codon was inserted into the new vector, between the promoter and the ECFP cassette, through BamHI and XhoI site.

Cell culture and transfection

Human Embryonic Kidney (HEK293) cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 1 % penicillin–streptomycin (PS) (Sigma) and 10 % fetal bovine serum (FBS) (Hyclone). Cells were incubated at 37 °C in 5 % CO_2. HEK293 cells were transfected with pcDNA3–ECFP or pCCNB1–ECFP using linear polyethylenimines (PEI) (Polysciences Inc., Warrington, PA, USA) (Reed et al. 2006) and incubated at 37 °C 5 % CO_2 for 12–18 h after transfection.

Double thymidine block

Cells were arrested in S phase of the cell cycle with double thymidine block. HEK 293 cells were transfected and then incubated for ~24 h. Medium was removed and replaced with fresh DMEM 10 % FBS 1 % PS per well. Cells were incubated overnight in medium containing 2 mM thymidine (Sigma-Aldrich). The next day, the medium was removed and the plates were washed with PBS. Fresh medium was added to the cells to release then from the S-phase arrest. After 8 h the 2 mM overnight thymidine block was repeated and followed by the PBS wash and addition of fresh media to release the cells from S-phase arrest.

Tetrazolium assay (MTT)

HEK 293 cells, transfected as described above, were split onto a 96-well plate. 20 µl of MTT (Sigma-Aldrich) stock (5 mg/ml in PBS) was added to each well and incubated at 37 °C in 5 % CO_2 for 2 h. Then 100 µl of extraction buffer (20 % SDS in DMF/H_2O, 2.5 % of 80 % acetic acid, 2.5 % 1 M HCl, pH 4.7) was added to each well overnight and incubated at 37 °C in 5 % CO_2. The absorbance of each well was taken at 570 nm.

Flow cytometry analysis

HEK 293 cells were synchronized using double thymidine block, as described above and were carefully collected at indicated time-points. They were washed twice in PBS, and then either used immediately or fixed with 70 % ethanol for future analysis. Fresh or fixed cells were suspended in 1 ml of PBS and 50 µl of 500 µg/ml propidium iodide solution was added. A minimum of 300,000 cells were analyzed per treatment using a Beckman Coulter FC500 flowcytometer (Miami, FL, USA).

Immunofluorescence (IF) microscopy and quantification

HEK 293 cells were seeded onto 10 cm plates containing glass cover-slips. Cells were cultured and transfected as described above. 24h following transfection the cells were arrested using double thymidine block. Cells were released and the cover-slips were collected at determined time points. The cells on the cover-slips were fixed with 4 % paraformaldehyde (PFA) in phosphate-buffered saline for 2 h and permeabilized with 0.02 % Triton X-100 for 5 min. TO-PRO-3-iodide (Invitrogen, Carlsbad, CA, USA) was used as nuclear stain. Cell images were obtained using LAS AF6000 software with a LEICA DMI6000 fluorescent microscope (Wetzlar, Germany). The total number of cells and ECFP fluorescent cells were counted using Metamorph AF 1.4 software. The percent of cells expressing ECFP or phosphor-histone H3 (pH3) was calculated by dividing the number of cell expressing ECFP or pH3 by the total cell number, as indicated by TO-PRO-3 (red cells) staining, and multiplying by 100. A minimum of 10 fields of view for each slide were counted for each experiment and the mean ± SD were calculated over an average of 3 individual experiments.

Statistical analysis

Data are presented as means ± the standard deviation. The statistical significance was determined by using Student *t* test (unpaired) through the http://www.physics.csbsju.edu/stats/t-test.html. *p* values ≤0.05 were considered significant and *p* values >0.05 were not considered significant.

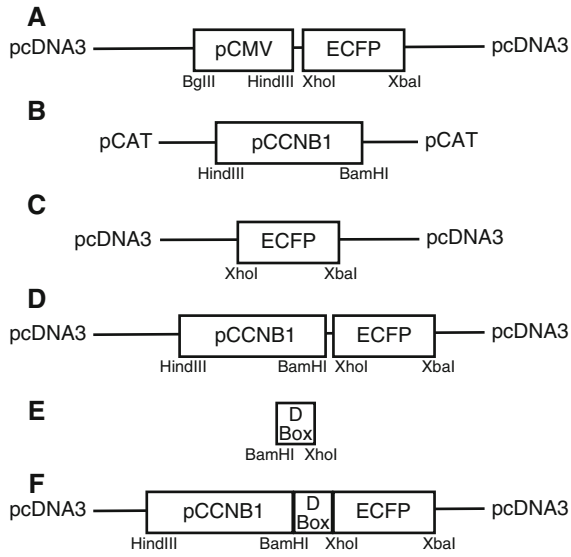

Fig. 1 Construction of pCCNB1–ECFP vector. **a** The backbone vector pcDNA3–CFP, **b** pCycB(−3800)-CAT contains the CCNB1 promoter. **c** The pCMV promoter was digested with BgIII–HindIII from pcDNA3–CFP, and a BgIII–HindIII linker was inserted to close the vector. **d** The pCCNB1 was digested using HindIII–BamHI from the pCycB(−800)-CAT vector and ligated into the vector shown in **c**. **e** The *D-box* was ligated by BamH1–XhoI into the vector depicted in **d**. **f** pCCNB1–ECFP vector—ECFP sensor is expressed under the control of the Cyclin B1 (CCNB1) promoter, the destruction of this reporter is controlled by the destruction box (*D-box*)

Results and discussion

We have developed a new cell cycle fluorescent reporter to specifically measure the timing of cell movement through G2 phase of the cell cycle. This reporter can be used in state-steady experiments or during live-cell imaging. To create the new G2/M reporter, the CMV promoter cassette from pcDNA3–CFP was substituted by the Cyclin B1 promoter obtained from the pCycB (−3800)-CAT vector constructed by Dr. Cogswell (Cogswell et al. 1995). Cogswell et al. (1995) have shown that the Cyclin B1 promoter extending to -3800 bp, relative to the cap site, can confer G2-enhanced promoter activity. Furthermore, they have demonstrated that the upstream stimulatory factor (USF)-binding site and its cognate transcription factor(s) are critical for expression of Cyclin B1. This −3,800 bp promoter was inserted upstream of the ECFP open reading frame, between the BamHI and HindIII restriction sites. The fragment corresponding to the Cyclin B1 D-box and the start codon (ATGAGAACCGCCCTGGGCGACATC

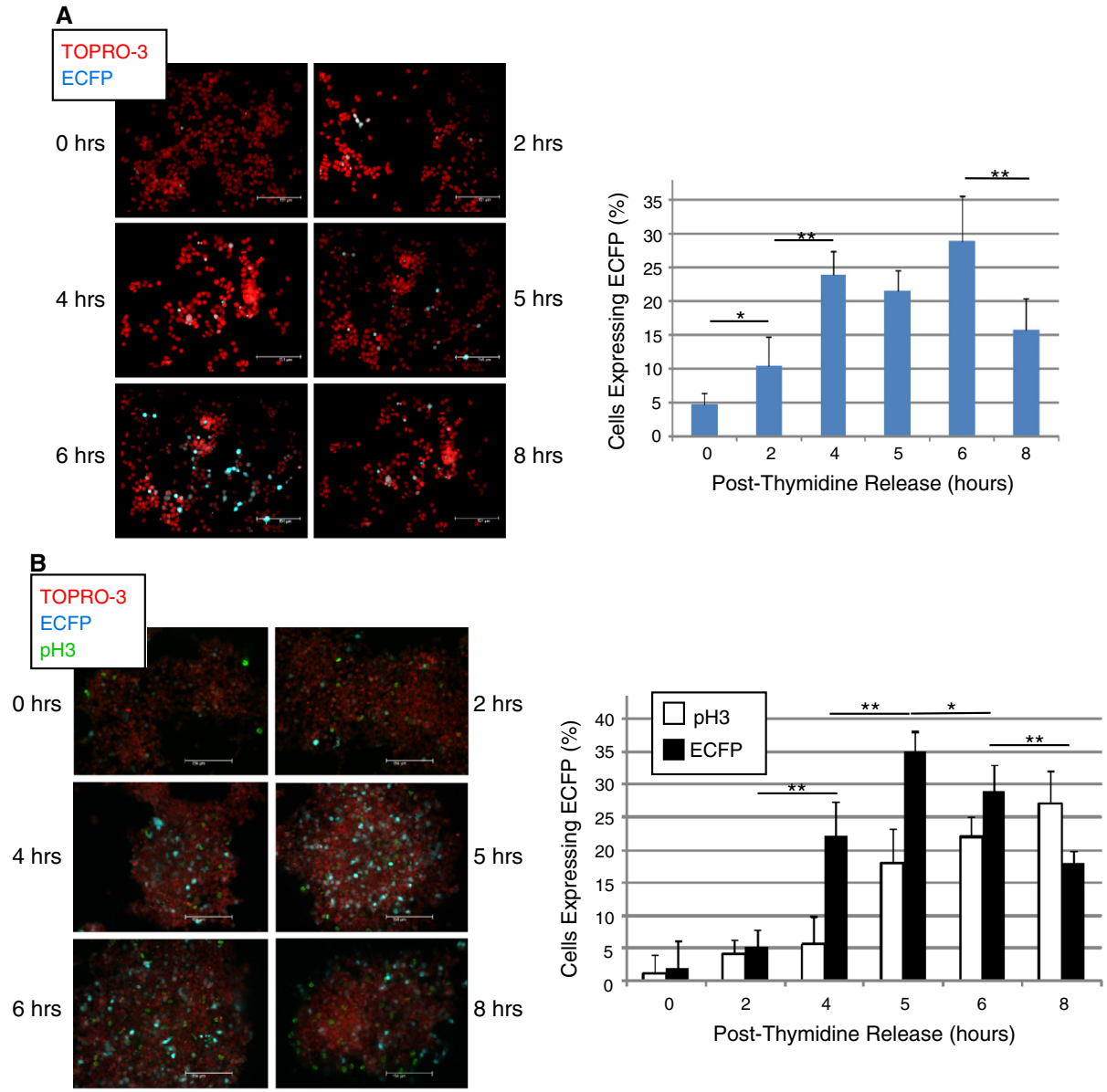

Fig. 2 pCCNB1–ECFP is expressed in a cell cycle dependent manner. HEK293 cells were transfected with pCCNB1–ECFP vector. 24 h after transfection the cells were arrested in S phase with double thymidine block. Cells were released from the block and collected at the indicated time points. TOPRO-3 stain (*Red*) and ECFP (*Blue*) are indicated as a merge. *Error bars* represent SD over 3 separate transfections. Unpaired t-test, $*p > 0.05$ and $**p < 0.05$. **a** *left* The panels are presented at × 10 magnification. This is one representative experiment of more than 3 repeats. **a** *right* Graph represents the percentage of cells staining positive for ECFP divided for the total of cells staining positive for TOPRO-3 scored for each sample (Y-axis). **b**: *left* pH3 (*green*) is added to the merge. The panels are presented at × 10 magnification. This is one representative experiment of more than 3 repeats. **b** *right* Graph represents the percentage of cells staining positive for ECFP (*solid bars*) or pH3 (*hollow bars*) divided for the total of cells staining positive for TOPRO-3 scored for each sample (Y-axis). Scored over 1,000 cells/transfection. (Color figure online)

GGCAAC) were inserted between the Cyclin B1 promoter and the ECFP through a BamHI–XhoI ligation, creating the new reporter vector pCCNB1–ECFP (Fig. 1).

It was expected that the reporter would express ECFP fluorescent protein at the end of the S phase, fluorescence would peak during the G2/M transition and the signal would be degraded at the anaphase

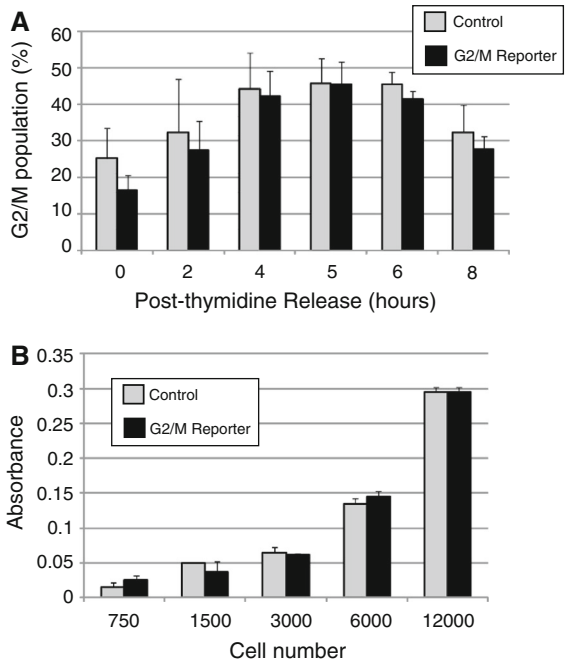

Fig. 3 pCCNB1–ECFP does not interfere with G2/M cell cycle transition. **a** Flow cytometry analysis was conducted using ~300,000 transfected HEK293 cells per data point. Percentage of cells in G2/M phase was determined by CPX analysis. *Gray bars* represent cells transfected with control (pcDNA3–CFP vector) and *black bars* with the pCCNB1–ECFP G2/M reporter vector. *Error bars* reflect SD of triplicate transfections, $p > 0.05$ between control and G_2M reporter vectors in all time points. **b** MTT analysis of HEK 293 cells expressing control pcDNA3–CFP (*gray bars*) or pCCNB1–ECFP G_2M reporter vector (*black bars*). Serial dilutions of transfected 293 cells were submitted to the Tetrazolium assay and absorbance taken at 570 nm to determine cell proliferation. *Error bars* reflect SD of triplicate transfections, $p > 0.05$ between control and G_2M reporter in all cell concentrations point

transition. To test this we transfected HEK 293 cells with pCCNB1–ECFP vector (G2/M reporter), arrested these cells in S-phase using double thymidine block (Bostock et al. 1971). Cells were released from the block by removing the medium containing thymidine and adding fresh medium. The cells were fixed for IF at different time-points (0–8 h), and the ECFP fluorescence (Exc- 434 nm, EM- 477 nm) was detected using fluorescent microscopy. We observed that at 0 h approximately 5 % of fluorescence was detected and the cells were arrested in S phase. Fluorescence increases with time, reaching a peak at 4–6 h, and decreasing at 8 h after the thymidine block release (Fig. 2a). We found that ECFP expression came on just prior to the phosphorylation of histone H3 (pH3; Fig. 2b indicated in green). Hence the G2/M reporter comes on during G2 phase as one would expect and is degraded toward the end of mitosis.

Flow Cytometry and MTT analysis were used to ensure that the region of the Cyclin B1 promoter (2.7 Kb) being used does not alter the G2/M cell cycle phase transition and cell proliferation. We arrested HEK293 cells using the double thymidine block, as above, and performed flow-cytometry analysis. As a control we used the pcDNA3–ECFP vector that contains the CMV promoter and constitutively drives ECFP in all phases of the cell cycle. According to our analysis approximately 50 % of the cells were in G2/M transition at the time of the fluorescence peak (4–6 h), and no significant difference was observed when cells were transfected with the CMV or our G2/M reporter (Fig. 3a).

We also used the tetrazolium assay (MTT) (Mosmann 1983; Berridge et al. 2005) to measure the proliferation of HEK 293 cells transfected with the vectors above. We observed that there is no significant difference in the metabolic status of the cells transfected with the CMV promoter or the G2/M reporter construct (Fig. 3b). We conclude that the pCCNB1–ECFP reporter is indeed expressing the ECFP protein under the control of the Cyclin B1 promoter and that the ECFP protein is being degraded after the G2/M transition. We see no adverse effects on the timing of M onset or on cell cycle phase or metabolic activity of the cells.

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Conflict of interest The authors have no conflicts to declare.

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