

Fluorescence kinetics in HeLa cells after treatment with cell cycle arrest inducers visualized with Fucci (fluorescent ubiquitination-based cell cycle indicator)

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Abstract

Fucci (fluorescent ubiquitination-based cell cycle indicator) is able to visualize dynamics of cell cycle progression in live cells; G₁- and S-/G₂-/M-phase cells expressing Fucci emit red and green fluorescence, respectively. This system could be applied to cell kinetic analysis of tumour cells in the field of cancer therapy; however, it is still unclear how fluorescence kinetics change after various treatments, including exposure to anticancer agents. To explore this, we arrested live HeLa cells expressing the Fucci probes at various cell cycle stages and observed the fluorescence, in conjunction with flow cytometric analysis. X-irradiation, HU (hydroxyurea) and nocodazole arrest cells at G₂/M boundary, early S-phase and early M-phase, respectively. Although X-irradiation and HU treatment induced similar accumulation kinetics of green fluorescent cells, nocodazole treatment induced an abnormal red fluorescence at M phase, followed by accumulation of both red and green fluorescent cells with 4N DNA content. We conclude that certain agents that disrupt normal cell cycle regulation could cause unexpected fluorescence kinetics in the Fucci system.

Keywords: cell cycle; cell cycle arrest; fluorescent protein; nocodazole; ubiquitination-based cell cycle indicator

1. Introduction

Cdt1 is a DNA replication licensing factor expressed in G₁-phase; the expression of Cdt1 protein is strictly dependent on the cell cycle. The underlying regulatory mechanism is cell cycle-specific ubiquitination mediated by an E3 ligase, SCF^{Skp2}. The expression of a Cdt1 inhibitor, Geminin, is also regulated by cell cycle-specific ubiquitination, in this case, mediated by APC^{Cdh1}. Geminin expression is observed in S-/G₂-/M-phases; thus, protein levels of Cdt1 and Geminin oscillate inversely (Nishitani et al., 2000, 2004; Wei et al., 2004). Sakaue-Sawano et al. (2008) developed a Fucci (fluorescent ubiquitination-based cell cycle indicator) system, taking advantage of the aforementioned cell cycle-specific ubiquitination. The system employs red- and green-emitting fluorescent proteins [KO (Kusabira Orange) and AG (Azami Green)] fused to the E3 ligase substrates Cdt1 and Geminin, respectively; this arrangement enables expression of KO at G₁-phase and AG at S-/G₂-/M-phases. The Fucci system allows visualization of the expected behaviour in cultured cells *in vitro*, and developmental tissues and organs *in vivo*, under normal conditions (Sakaue-Sawano et al., 2008). Although this technology could be applied to cell kinetic analysis in tumour cells following treatment with antitumour agents, the correlations between fluorescence kinetics and actual cell cycle stages following agents have not yet been verified. Therefore, we examined the fluorescence kinetics in HeLa cells expressing the Fucci system, following treatment with agents that induce cell cycle arrest at different cell cycle stages. We demonstrate that one particular agent

deregulates the fluorescence kinetics and yields a visualization result that is inconsistent with the actual cell cycle stage.

2. Materials and methods

2.1. Cell line and culture conditions

HeLa cells expressing the Fucci probes were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan and maintained in DMEM (Sigma–Aldrich) supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ humidified atmosphere.

2.2. Treatments

Cells were seeded on to 60-mm dishes and subcultured for 24 h before treatments and were treated for the indicated times with 3 mM hydroxyurea (Sigma–Aldrich) or 50 ng/ml nocodazole (Wako). Cells were also irradiated using an HS-225 therapeutic X-ray machine (Shimadzu) (225 kVp, 15 mA, 1.0 mm Cu filtration) at a dose rate of 0.84 Gy/min.

2.3. Flow cytometric analysis

Two types of sample were subjected to flow cytometric analysis: non-fixed samples, for detecting fluorescence intensity of AG or

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Abbreviations: AG, Azami Green; Fucci, fluorescent ubiquitination-based cell cycle indicator; HU, hydroxyurea; KO, Kusabira Orange.

KO and, fixed samples, for DNA content analysis. After each treatment, collected culture medium and trypsinized cells were centrifuged together, and the pellets were washed in ice-cold PBS. For DNA content analysis, cells were fixed in ice-cold 70% ethanol in PBS for at least 30 min on ice. After fixation, cells were re-washed in ice-cold PBS and incubated in 0.5 µg/ml 7-AAD solution (BD Bioscience). Finally, both non-fixed and fixed single cell suspensions were strained through nylon mesh. Each sample was analysed with a FACScalibur flow cytometer (Becton Dickinson) using the FlowJo software (Tree Star).

2.4. Immunocytochemistry

Cells were cultured on Lab-Tek™ Chamber Slides (Nunc A/S) for 24 h before nocodazole treatments. After treatments, cells were washed in TBS-T (TBS containing Tween 20) and fixed with ice-cold methanol for 5 min. Cells were stained with a monoclonal anti- α -tubulin (dilution 1:500, Sigma–Aldrich) as the primary antibody for 1 h, followed by an Alexa Fluor 594 goat anti-mouse IgG (dilution 1:500, Molecular Probes) as the secondary antibody for 30 min. Finally, slides were washed in TBS-T and mounted with

ProLong Gold Antifade Reagent with DAPI (4',6-diamidino-2-phenylindole, Molecular Probes).

2.5. Fluorescence imaging

Images of fluorescent cells were taken using an Eclipse TE300 inverted microscope (Nikon).

3. Results and discussion

The structures of the Fucci probes are shown (Figure 1). In the red probe (Figure 1A, left), amino acids 30–120 include the Cy motif of Cdt1, which is a binding site of E3 ligase, SCF^{Skp2} (Nishitani et al., 2006); this domain is fused with mKO2 (monomeric KO 2) (Karasawa et al., 2004). The region does not contain the binding site (QXRVTDF motif) of DDB1-Cul4 E3 ligase. In the green probe (Figure 1A, right), amino acids 1–110 include the Destruction box of Geminin, which is a binding site of the E3 ligase APC^{cdh1}; this domain is fused with mAG (monomeric Azami Green) (Karasawa et al., 2003). HeLa cells expressing both fusion proteins were used

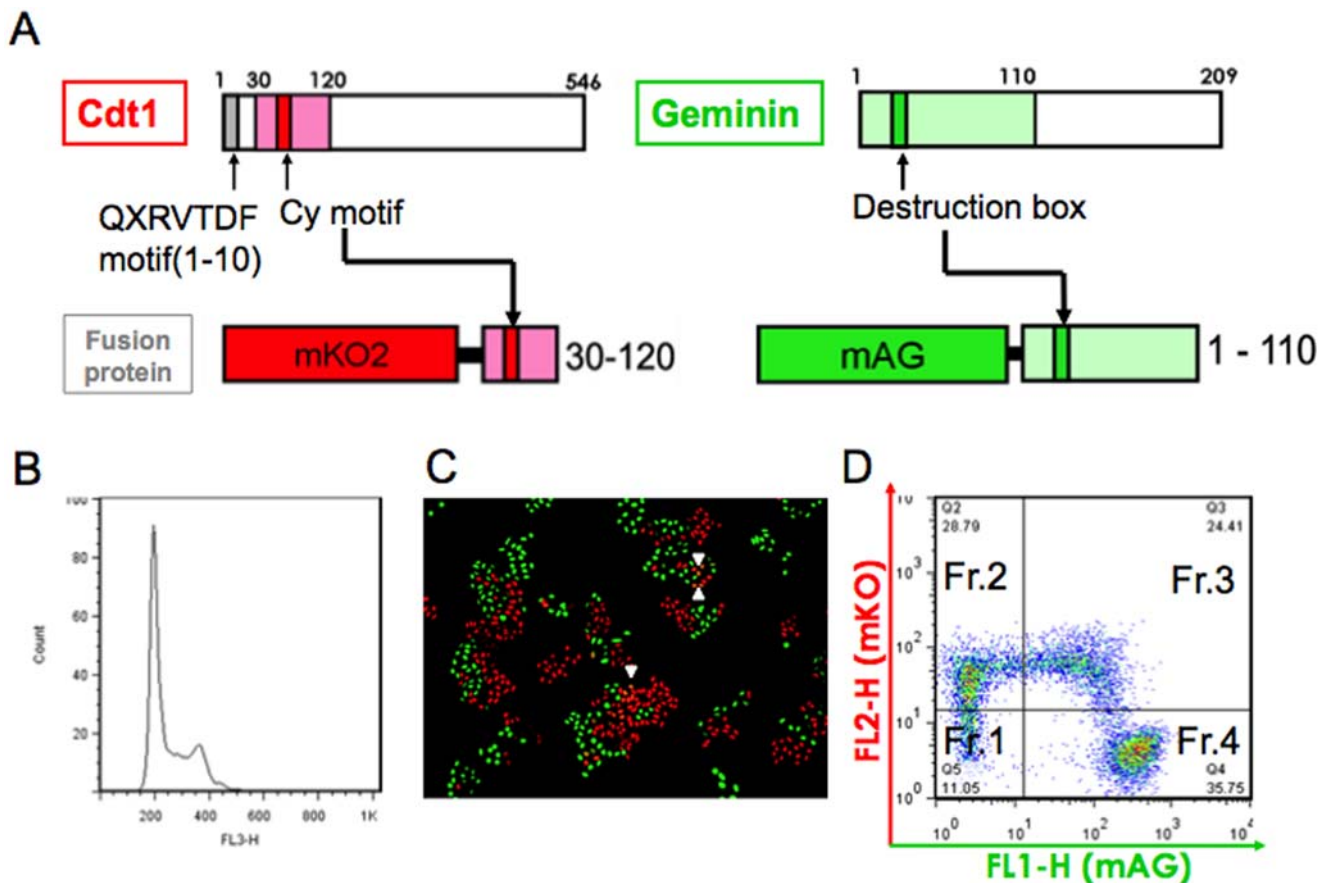


Figure 1 Structures and expression of Fucci probes expressed in HeLa cells

(A) Structures of fusion proteins in the Cdt1 and Geminin probes. (B) Flow cytometric analysis of DNA contents using 7-AAD in exponentially growing HeLa cells expressing the Fucci probes. (C) A typical example of fluorescence microscopic image in exponentially growing HeLa cells expressing the Fucci probes. Red, Cdt1 probe; green, geminin probe; arrowheads, representative cells expressing both probes. (D) 2D flow cytometric analysis of Cdt1 and Geminin probes. Fr., Fraction.

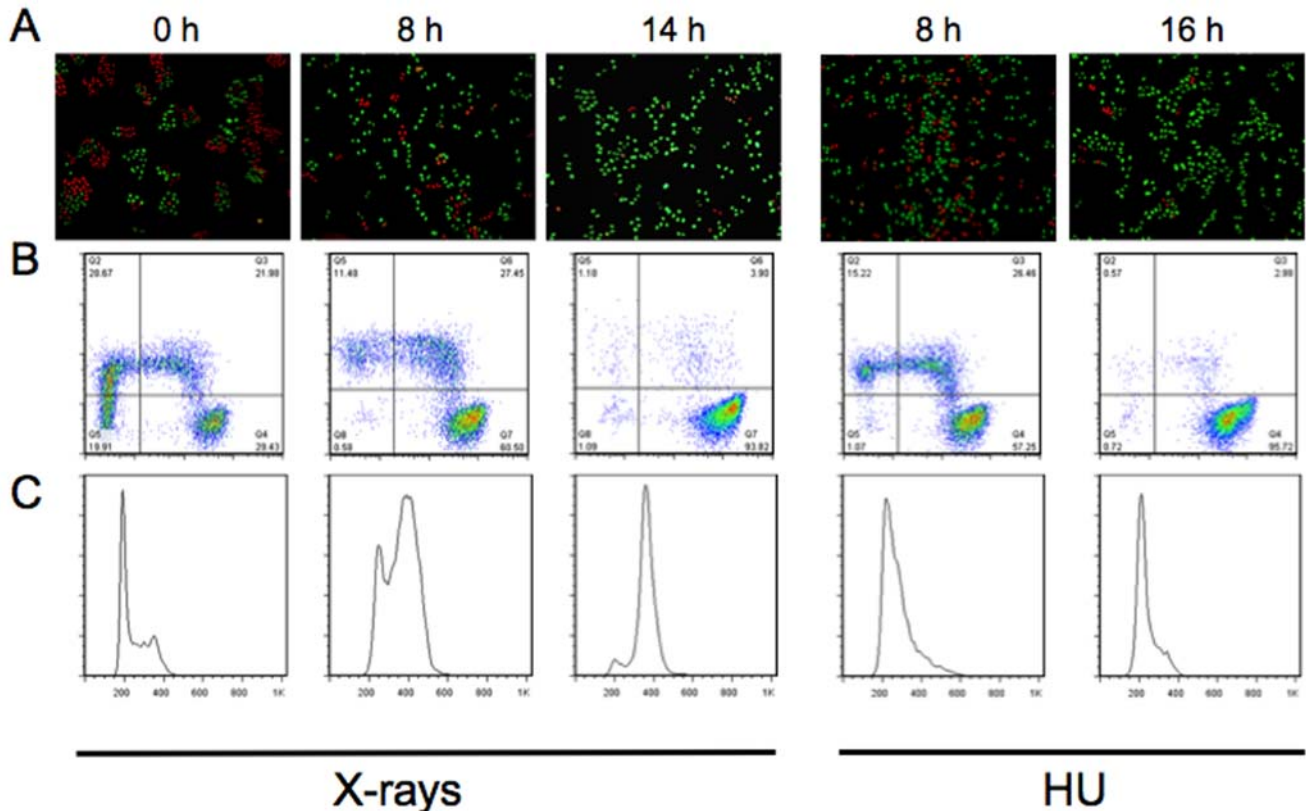


Figure 2 Fluorescence and cell cycle kinetics in HeLa cells observed using the Fucci system following X-irradiation or HU treatment (A) Typical examples of fluorescence microscopic images in cells following X-irradiation or HU treatment. Fluorescence images were taken at the indicated times after 10 Gy X-irradiation or 3 mM HU treatment. (B) 2D flow cytometric analysis of both probes following X-irradiation or HU treatment. (C) Flow cytometric DNA content analysis following X-irradiation or HU treatment.

in this study. In exponentially growing cells expressing the Fucci probes, fluorescence microscopic observation revealed clear distinct red, green and a small fraction of double fluorescent cells (Figure 1C), confirming the previous report (Sakaue-Sawano et al., 2008). Double fluorescent cells expressing both fluorescent proteins are considered to be at the G_1 -/S-phase. Depending on the difference in intensity between red and green fluorescence, double fluorescent cells look like different colours in merged fluorescent images (Figure 1C). Cells expressing neither protein are considered to be at early G_1 -phase. Cells treated with 7-AAD after fixation in 70% ethanol were also analysed by FCM (flow cytometry). 2D (Two-dimensional) histogram (green and red fluorescence intensity) exhibiting an inverse U-shaped pattern and a cell cycle histogram of DNA content stained with 7-AAD were obtained (Figure 1B), which is typical for exponentially growing cells. Fractions 1–4 represent early G_1 -, G_1 -, G_1 -/S- and S-/G₂-/M-phases, respectively, in the 2D histogram (Figure 1D). Fractions 1 and 3 are clearly depicted in this histogram.

After X-irradiation, cells showed gradual clockwise cell cycle progression and accumulation into Fraction 4 in the 2D histogram (Figures 2A, 2B). Disappearance of the Cdt1 probe correlated with cell cycle progression. This is inconsistent with the finding reported by Higa et al. (2003) that X-irradiation rapidly degrades Cdt1 as determined by Western blotting. This phenomenon is

mediated by DDB1-Cul4 E3 ligase via binding to the QXRVTDF motif, which is present in the first 10 amino acids of Cdt1 (Nishitani et al., 2006). Because the Cdt1 probe contains only the 30–120 amino acid region excluding the QXRVTDF motif as described above, the red fluorescence is unlikely to quickly disappear, even after X-irradiation. DNA content analysis using 7-AAD revealed that cells accumulated in G₂-/M-phase, consistent with previous reports describing G₂ arrest in HeLa cells after irradiation (Bernhard et al., 1994). Very similar fluorescence kinetics was obtained after HU treatment, except that the accumulation occurred in early S-phase (Figure 2C) (Nishitani et al., 2001). Up to 20 h after X-irradiation, release from the G₂ arrest was observed, and redistribution to the control state was detected by 24 h after irradiation (data not shown). When cells treated with 3 mM HU for 24 h were incubated in culture medium without HU after extensive washing, cells showed a similar time course of redistribution (data not shown). Again, cells exhibited clockwise cell cycle progression in both cases. These results suggest that the fluorescence kinetics in the Fucci system seem to reflect the cell cycle distribution, even after cell cycle arrests at G₂-/M- or early S-phase or DNA damage by X-irradiation or HU treatment. At least up to 24 h after the treatments, untreated control cells did not show a significant change of the cell cycle distribution (data not shown).

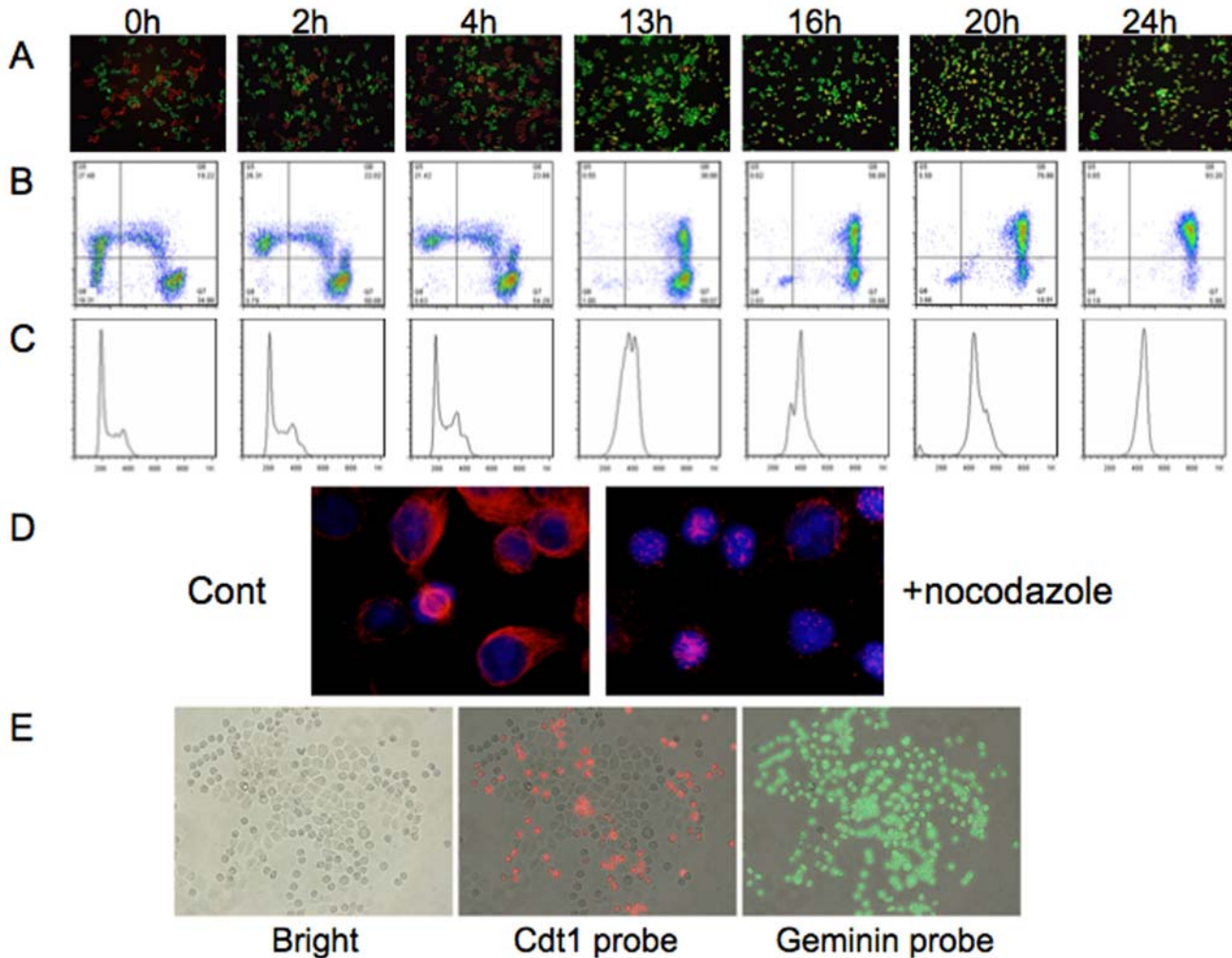


Figure 3 Fluorescence and cell cycle kinetics in HeLa cells with the Fucci system following nocodazole treatment

(A) Typical examples of fluorescence images in cells following nocodazole treatment. Fluorescence images were taken at the indicated times after 50 ng/ml nocodazole treatment. (B) 2D flow cytometric analysis of both probes following nocodazole treatment. (C) Flow cytometric DNA content analysis following nocodazole treatment. (D) Typical examples of immunofluorescence images for α -tubulin. Cont, non-treated cells; +nocodazole, cells 24 h after 50 ng/ml nocodazole treatment. (E) Typical examples of fluorescence images for both probes in cells after nocodazole treatment. Fluorescence was visualized 14 h after 50 ng/ml nocodazole treatment. Green fluorescence was observed in most cells, but red fluorescence was only observed in the round type of cells.

Nocodazole is a microtubule-depolymerizing agent and induces accumulation in early M-phase (Rieder and Maiato, 2004). Indeed, after this treatment, depolymerized α -tubulin was detected by immunofluorescence staining in cells showing chromosome condensation (Figure 3D). We therefore expected fluorescence kinetics similar to those resulting from the two agents described above. Indeed, cells first accumulated in Fraction 4 in a clockwise manner; however, simultaneously, cells began to progress from Fraction 4 to Fraction 3 (Figures 3A, 3B, 3C). From the morphological observation, M-phase cells were likely to express red fluorescence (Figure 3E), suggesting that cells entering M-phase began to express the red fluorescence and shifted to Fraction 3 by maintaining green fluorescence. This fraction gradually increased; eventually, most cells accumulated at this fraction 24 h after treatment. Although Fraction 3 would normally represent the G_1/S transition phase, DNA content analysis clearly demonstrated that the cells possess 4N DNA

content. When cells are released from nocodazole treatment, cells started to divide with the abnormal red fluorescence and seemed to proceed to normal G_1 -phase with red fluorescence; thereafter, normal fluorescence kinetics was observed (data not shown).

The Fucci system is an innovative technology that allows visualization of the spatiotemporal dynamics of cell cycle progression in multiple live cells (Sakaue-Sawano et al., 2008). This system could be applied to the cancer biology field, helping us to analyse cell kinetics following anticancer agents, assuming that the fluorescence kinetics faithfully reflects the cell cycle distribution even after drug treatments. We previously reported that this was the case for the Geminin probe, following X-irradiation using a tongue cancer cell line (SAS) transfected only by a plasmid encoding the Geminin probe (Ishikawa et al., 2009). In this study, we showed that the two probes could function normally following X-irradiation or HU treatment. However, we could not rule out the possibility that the Cdt1 probe is abnormally

degraded at the stage arrested following HU treatment because some early S-phase cells seem to express both Cdt1 and Geminin probes (Sakaue-Sawano et al., 2008). The precise definition of the early S-phase is not determined; therefore, this problem should be further studied to elucidate the exact mechanism. On the other hand, nocodazole treatment unequivocally resulted in abnormal fluorescence kinetics.

Cdt1 probe lacks the QXRVTDF motif present in the 1–10 amino acid region because stable transformants expressing a Cdt1 probe that included this region could not be obtained (Sakaue-Sawano et al., 2008). The DNA damage responsive element mediated by DDB1-Cul4 is contained in this region (Nishitani et al., 2006); therefore, the lack of amino acids 1–10 might incidentally render the probe stable even after DNA damage like X-irradiation or HU treatment. The Cy motif is a binding site for Cdk/cyclin, and subsequent phosphorylation causes SCF^{Skp2}-mediated ubiquitination from the onset of S-phase to the end of M-phase (Nishitani et al., 2004). Therefore, it is possible that nocodazole somehow inhibits SCF^{Skp2}-mediated ubiquitination of the Cdt1 probe during M-phase, independent of normal cell cycle regulation. Further study is definitely required for elucidation of its molecular mechanism.

In this study, we demonstrated for the first time that the Geminin probe is likely to reflect the actual cell cycle stages of cells exposed to at least the three agents used here; however, Cdt1 probe can give misleading results following treatment with certain type of agents. Therefore, when cells are treated with particular agents such as nocodazole, care should be taken when using the Fucci system for cell cycle analysis.

Author contribution

Atsushi Kaida carried out most of the study and participated in its design. Naoki Sawai carried out the experiments regarding HU treatment. Kengo Sakaguchi participated in the study design and data discussion. Masahiko Miura jointly conceived of the study and participated in its design and drafted the manuscript.

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