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Real-Time Fluorescence Imaging of the DNA Damage Repair Response during Mitosis

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Running title: Imaging of DNA repair during mitosis

Key words: GFP, 53BP1, DNA damage, mitosis, time lapse imaging

Abstract

In this study, the response to DNA damage during mitosis was visualized using fluorescent protein-based real-time imaging of 53BP1 linked to GFP (GFP-53BP1) focus formation by the fusion protein in the MiaPaCa2 Tet-On Advanced cell line. To observe 53BP1 foci during mitosis, MiaPaCa2^{Tet-On} GFP-53BP1 cells were observed every 30 minutes with the FV1000 confocal laser microscope (Olympus Corp., Tokyo, Japan). Time lapse imaging demonstrated that $11.4 \pm 2.1\%$ of the mitotic MiaPaCa2^{Tet-On} GFP-53BP1 cells formed 53BP1 foci. Non-mitotic cells did not have an increase in GFP-53BP1 focus formation. Time lapse imaging showed that MiaPaCa2^{Tet-On} GFP-53BP1 cells with 53BP1 foci formation lead withdrawal of mitosis and induced apoptosis. These results indicate that DNA strand breaks occur during mitosis and can be repaired, at least to some extent. This is the first report of time-lapse imaging, which demonstrated increased expression of 53BP1 during mitosis and cell death after the increase of 53BP1. Time lapse imaging of 53BP1 labelled by GFP can be useful tool for the evaluation of response to DNA damages.

Introduction

Genome integrity is continuously challenged by DNA lesions. These lesions are induced by radiation and toxic environmental chemicals, and also occur as byproducts of normal cell metabolism or DNA replication [Jackson, 2009]. Genome integrity is preserved by DNA damage signaling and repair machineries, which prevent the adverse consequences of DNA lesions and prevent their transmission

to daughter cells [Hoeijmakers, 2001]. DNA double-strand breaks (DSB) are the most deleterious form of DNA damage because they do not leave an intact complementary strand to be used as a template for DNA repair. DSBs are generated in response to ionizing radiation (IR) or radiomimetic drugs by free radical attack of deoxyribose, and also arise in cells treated with topoisomerase II inhibitors that prevent religation of DNA strands broken by topoisomerase II activity. DSBs can also form upon replication of DNA molecules containing other DNA lesions, such as DNA SSBs [Jackson, 2009]. In the treatment of malignant tumors, DNA-targeting treatments, which include various anticancer drugs and radiation therapy, induce DSBs in DNA of cancer cells. DSB induces DNA damage response (DDR) in the injured cancer cells. Successful repair of the injured DNA enables the cancer cells to survive and to grow with resistance to the treatment. DDR comprises a network of pathways that coordinate cellular reactions to DNA damages. H2AX phosphorylation on Ser¹³⁹ (called γ -H2AX) has been shown to be an early step in the response of cells to DNA damage [Rogakou et al., 1998]. Phosphorylation of H2AX to γ -H2AX at DSBs promotes further chromatin modifications such as MRE11/RAD50/NBS1, MDC1, BRCA1, and p53 binding protein 1 (53BP1) [Bonner et al., 2008; van Attikum et al., 2009]. Therefore, the observation of γ -H2AX has become a common approach to detect the presence of DSBs. On the other hand, 53BP1, which was originally identified in a yeast two-hybrid screen looking for proteins that interact with the p53 tumor suppressor [Iwabuchi et al., 1994], has various key roles in DNA repair response and checkpoint control (Fig. 1). Like γ -H2AX, 53BP1 has been shown to relocalize into foci at DSBs site shortly after the damages and is an important sensor of DNA damage. Furthermore, 53BP1 is required for the phosphorylation of numerous ATM (ataxia-telangiectasia mutated) substrates during DSB response [Abraham RT, 2002]. Cells lacking 53BP1 are sensitive to DNA damaging agents and have defects in both S-phase and G2/M checkpoints [Fernandez-Capetillo, 2002].

Our laboratory pioneered in vitro and in vivo imaging with fluorescent proteins [Chishima et al., 1997; Yang et al., 2000; Hoffman, 2005]. Imaging with fluorescent proteins enables visualization of cellular and subcellular dynamics including cancer metastasis, cell cycle event, and apoptosis. Herein, by exploiting GFP fused to the chromatin-binding protein of 53BP1 as a live-cell imaging marker for DNA repair, 53BP1 foci were observed as an early marker of response to DNA damage during mitosis in MiaPaCa2 human pancreatic cancer cells. This study determined the fragility of the DNA during mitosis by imaging of 53BP1 foci formation as a marker of response to DNA damage.

Materials and Methods

Cell cultures and constructs

GFP fused to the human 53BP1 IRIF binding domain was cloned into the pLVX-Tight-Puro

lentiviral vector (Clontech), transduced into the MiaPaCa2 Tet-On Advanced cell line (Clontech), and cultured in high-glucose DMEM (Invitrogen) with 10% Tet system-approved fetal bovine serum (Clontech) [Efimova, 2010]. MiaPaCa2 Tet-On Advanced is certified by Clontech as derived from MiaPaCa2 (American Type Culture Collection) by viral transduction and was used without further authentication. After induction for 48 h with 1 µg/mL doxycycline (Sigma), GFP-positive cells were sorted to establish a stable MiaPaCa2^{Tet-On} GFP-53BP1 cell line.

Mice

Transgenic nude C57/B6-RFP mice (RFP nude mice) were used in this study [Yang, 2009]. Mice were bred and maintained in a barrier facility under HEPA filtration at AntiCancer, Inc. (San Diego, CA, USA). Mice were fed with an autoclaved laboratory rodent diet. All animal studies were conducted in accordance with the principles and procedures outlined in the National Research Council's Guide for the Care and Use of Laboratory Animals under PHS Assurance Number A3873-01.

Fluorescent imaging of 53BP1 foci formation during mitosis

MiaPaCa2^{Tet-On} GFP-53BP1 cells were seeded in 35 mm dishes and treated with 1 µg/mL doxycycline for 48 h. To observe the cellular response to DNA damage by 53BP1, MiaPaCa2^{Tet-On} GFP-53BP1 cells were observed every 30 min by the FluoView FV1000 confocal laser microscope (Olympus Corp., Tokyo, Japan). High-resolution images were captured directly on a personal computer (Fujitsu Siemens Computers, Munich, Germany). Images were analyzed with the use of Cell® software (Olympus Biosystems).

Evaluation of 53BP1 foci formation in MiaPaCa2^{Tet-On} GFP-53BP1 cells.

To evaluate the 53BP1 foci formations, MiaPaCa2^{Tet-On} GFP-53BP1 cells were divided into foci-positive cells and foci-negative cells according to number of the foci [Miwa, 2013, Uehara, 2014]. The cells which showed 5 or more foci were considered as positive cells. The experimental data are expressed as the mean ± SE.

Fluorescent imaging of 53BP1 foci formation in vivo

RFP nude mice were anesthetized with a ketamine mixture (10 µl ketamine HCl, 7.6 µl xylazine, 2.4 µl acepromazine maleate, and 10 µl H₂O). An arc-shaped incision was made in the abdominal skin, and the subcutaneous connective tissue was separated to free the skin flap without injuring the epigastric cranialis artery and vein. MiaPaCa2^{Tet-On} GFP-53BP1 cells (1×10^6 cells in 5 µl Matrigel) were implanted in skin flap of RFP nude mouse [Yamauchi, 2006]. Three days after the implantation, 2 mg/mL doxycycline was added to the drinking water for 48 h. Then, 53BP1 foci formations were

in the tumor were observed by FV1000.

Results

Distribution of 53BP1 expression in MiaPaCa2^{Tet-On} GFP-53BP1 cells.

To observe response to DNA damage in cancer cells, MiaPaCa2^{Tet-On} GFP-53BP1 cells were treated with 1 $\mu\text{g/mL}$ doxycycline for 48 h (Fig. 2). Then, 53BP1 foci were observed with FV1000. Only a part of the cells showed strong expression of 53BP1 (Fig. 3). Fluorescent imaging of MiaPaCa2^{Tet-On} GFP-53BP1 cells revealed that $2.8 \pm 0.8\%$ of the cells were foci positive, and $81.5 \pm 11.3\%$ of the foci positive cells showed mitotic change, including symmetric nuclei and two nuclei in each cells. Overexpression of 53BP1 in symmetric nuclear cells and binuclear cells suggested that response to DNA damage frequently occurs during mitosis.

Time-lapse imaging of 53BP1 expression during mitosis in MiaPaCa2^{Tet-On} GFP-53BP1 cells.

To visualize time course of the responses to DNA damage in cancer cells, MiaPaCa2^{Tet-On} GFP-53BP1 cells were treated with 1 $\mu\text{g/mL}$ doxycycline for 48 h. Then, 53BP1 foci were observed every 30 min with FV1000 for 24 h. Time-lapse single cell imaging of MiaPaCa2^{Tet-On} GFP-53BP1 cells demonstrated that 53BP1 foci were increased in mitotic phase (Fig. 4, Video 1), and the increased foci formation lasted after the mitosis. The increased expression of 53BP1 during mitosis indicates that DNA repair is needed during replication of DNA. Furthermore, time-lapse imaging revealed cell death after the increased 53BP1 foci during mitosis. During the time-lapse imaging, mitotic changes were observed 1.1 ± 0.1 times in each cell, and apoptotic changes were observed in $7.3 \pm 1.9\%$ of the cells during the 24 h. During the time-lapse imaging, foci formations of GFP-53BP1 were observed in $11.4 \pm 1.2\%$ of the mitotic cells during the 24 h.

Refusion of 53BP1 expressing nuclei during mitosis.

Time-lapse imaging of 53BP1 foci formation using MiaPaCa2^{Tet-On} GFP-53BP1 cells demonstrated division of 53BP1 foci-positive nucleus, reunion of the divided nuclei, and induction of apoptosis (Fig. 5, Video 2). This phenomenon indicates that response to DNA damage is activated during mitosis, and that fatal DNA damage leads withdrawal of mitosis and apoptosis. It is considered that DNA damage signaling from unrepairable lesions can activate apoptosis or can trigger cell cycle withdrawal to prevent spread of aberrant DNA [Giunta S, 2011]. Our time-lapse imaging of 53BP1 visualized the phenomenon of withdrawal of mitosis and induction of apoptosis by fatal DNA damage.

Fluorescent imaging of 53BP1 foci formation in vivo

To observe the DNA damage response in vivo, MiaPaCa2^{Tet-On} GFP-53BP1 cells (1×10^6 cells in 5

µl Matrigel) were implanted in skin flap of RFP nude mouse [Yamauchi, 2006]. Five days after the implantation, the skin flap was opened and 53BP1 foci formations were observed by FV1000. Fluorescent imaging demonstrated that a large number of 53BP1 foci were formed in the tumor (Fig. 6). This result indicates that the DNA damage and repair are constantly repeated during the growth of tumor.

Discussion

Aberrant or damaged DNA commonly occurred by free radical, ultraviolet light, and replication of DNA. To prevent the spread of the abnormal DNA, the abnormal site of DNA is detected and repaired. DNA repair is tightly coordinated with cell cycle progression through the activation of orchestrated signaling pathways that are often termed DNA damage checkpoints [Harrison, 2006]. In response to unrepaired DNA damage, these pathways delay or stop the cell cycle at critical stages before or during DNA replication (G1/S and intra-S checkpoints) and cell division (G2/M checkpoint), thereby preventing duplication and segregation of damaged DNA. The rapid ubiquitination of chromatin surrounding DNA double-stranded breaks (DSB) recruit many DNA damage response (DDR) proteins. 53BP1, one of the pivotal DDR proteins, is an important sensor of DNA damage, and forms foci at DSB sites shortly after the damage of DNA. It is known that 53BP1 functions downstream of γ H2AX-dependent hierarchy of proteins that collectively establish IRIF at DSB sites. This hierarchy includes the Mre11/Rad50/NBS1 (MRN) complex, ATM, MDC1, RNF8, RNF168 and HERC2 [Lavin et al., 2008; Stewart et al., 2009; Doil et al., 2009; Bekker-Jensen et al., 2010]. In this study, 53BP1, one of the most important DDR proteins was visualized and observed by time-lapse imaging system. During single cell time-lapse imaging, increased 53BP1 foci were observed just before mitosis, and the 53BP1 foci lasted until next mitosis. Our time-lapse imaging of 53BP1 foci enabled the observation of fate, and is useful tool for the evaluation of response to DNA damages. To elucidate mechanisms of regulation of early response to DNA damages, single cell time-lapse imaging of DDR proteins using fluorescent proteins can greatly contribute to the investigation.

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Figure legend

Figure 1. Effects of 53BP1 on maintenance of genomic stability.

53BP1 bound to damaged chromatin carries out several functions. Upon DNA double strand breaks (DSBs), 53BP1 rapidly redistributes from a diffuse nuclear localization to discrete foci that co-localize with phosphorylated histone H2AX and other repair proteins including BRCA1 [Schultz, 2000; Ward, 2003]. 53BP1 function is also required for class-switch recombination and has been implicated in non-homologous end-joining (NHEJ) [Ward, 2004; Xie, 2007]. Overexpression of dominant-negative 53BP1 construct suppressed NHEJ and increased frequency of HR-mediated repair, suggesting 53BP1 is involved in regulating the choice between NHEJ and HR-mediated repair of DNA DSBs [Xie, 2007].

Figure 2. MiaPaCa2^{Tet-On} GFP-53BP1 cells.

- a. Diagram of 53BP1 IRIF binding domain reporter which includes a glycine-arginine rich (RG) motif, tandem Tudor (T) domains, a nuclear localization sequence (NLS), and two BRCT domains. The GFP-IBD construct incorporates a tetracycline-responsive element (TRE), green fluorescent protein (GFP) and the 53BP1 RG motif, tandem Tudor domains, and NLS.
- b. 53BP1 foci positive and negative cells. The cells were divided into foci-positive and foci-negative cells according to the number of foci. The cells which had 5 or more foci were considered as foci positive cells [Miwa, 2013].

Figure 3. Distribution of 53BP1 foci in MiaPaCa2^{Tet-On} GFP-53BP1 cells.

MiaPaCa2^{Tet-On} GFP-53BP1 cells were seeded in 35 mm dish and treated with 1 µg/mL doxycycline for 48 h. Then, 53BP1-foci formations were observed by FV1000. Small part of the cells showed positive foci formation of 53BP1. Large part of the foci-positive cells showed two nuclei or symmetrical form of foci-positive nuclei.

Figure 4. Time-lapse imaging of 53BP1 during mitosis in MiaPaCa2^{Tet-On} GFP-53BP1.

MiaPaCa2^{Tet-On} GFP-53BP1 cells were seeded in 35 mm dish and treated with 1 µg/mL doxycycline for 48 h. Then, 53BP1-foci formations were observed every 30 min for 24 h by FV1000 (Supplementary movie 1). Time-lapse imaging demonstrated that only a small part of the cells formed 53BP1 foci during mitosis (arrows), and the foci formation continued after the mitosis, and that one of the foci positive cell induced apoptosis (solid arrow). Formations of 53BP1 foci were increased in mitotic phase, and the increased foci formation lasted after the mitosis.

Figure 5. Time-lapse imaging of withdrawal of mitosis and induction of apoptosis in MiaPaCa2^{Tet-On} GFP-53BP1 cells with increased expression of 53BP1.

MiaPaCa2^{Tet-On} GFP-53BP1 cells were seeded in 35 mm dish and treated with 1 µg/mL doxycycline for 48 h. Then, 53BP1-foci formations were observed every 30 min for 24 h by FV1000 (Supplementary movie 2). Time-lapse imaging demonstrated that 53BP1 foci-positive cell (arrow) divided the nucleus (7–8 h), but the divided nuclei fused again (9 h), and the cell induced apoptosis (15 h). This time-lapse imaging showed that fatal DSBs occurring during mitosis lead cell cycle arrest and withdrawal of the mitosis.

Figure 6. Fluorescent imaging of 53BP1 foci formation in vivo.

RFP nude mice were anesthetized with a ketamine mixture (10 µl ketamine HCl, 7.6 µl xylazine, 2.4 µl acepromazine maleate, and 10 µl H₂O). An arc-shaped incision was made in the abdominal skin,

and the subcutaneous connective tissue was separated to free the skin flap without injuring the epigastric cranialis artery and vein. MiaPaCa2^{Tet-On} GFP-53BP1 cells (1×10^6 cells in 5 μ l Matrigel) were implanted in skin flap of RFP nude mouse [[Yamauchi, 2006](#)]. Three days after the implantation, 2 mg/mL doxycycline was added to the drinking water for 48 h. Then, 53BP1 foci formations were in the tumor were observed by FV1000.

Figure 1

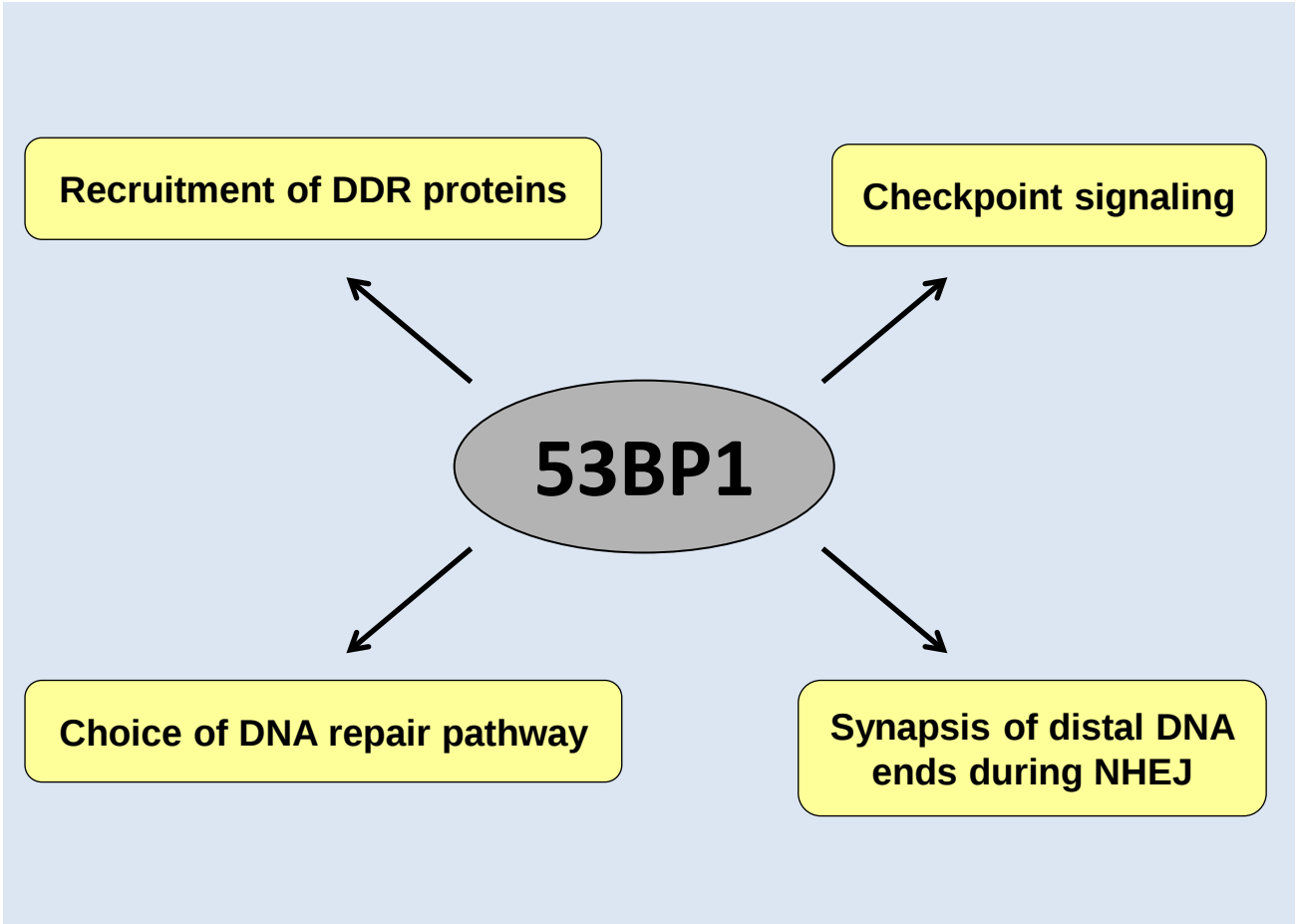
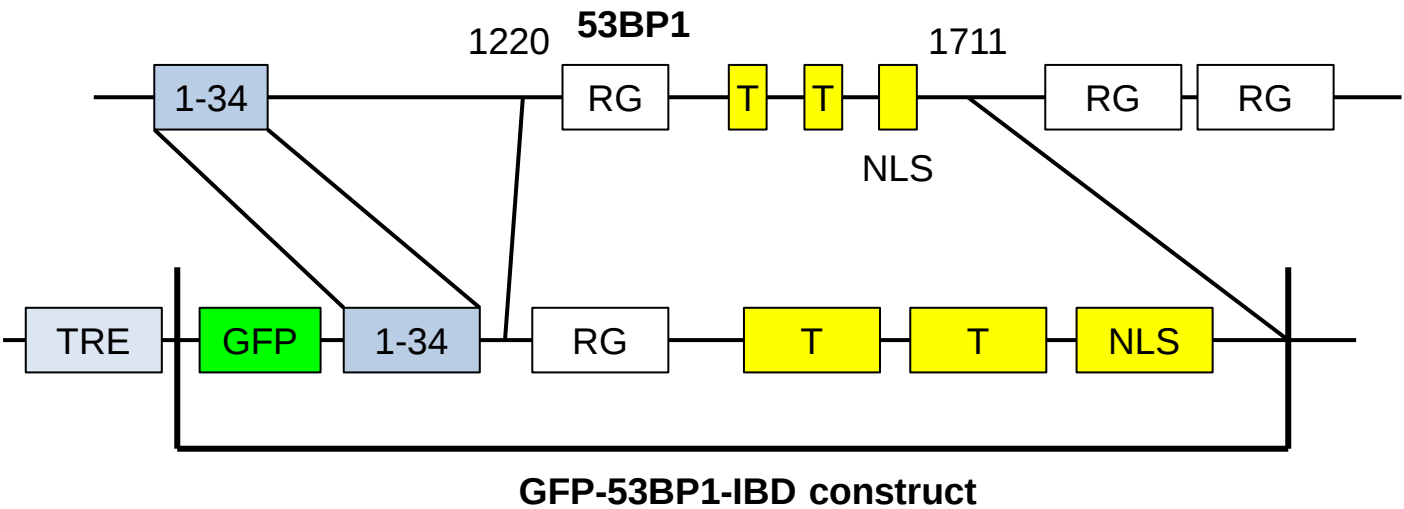


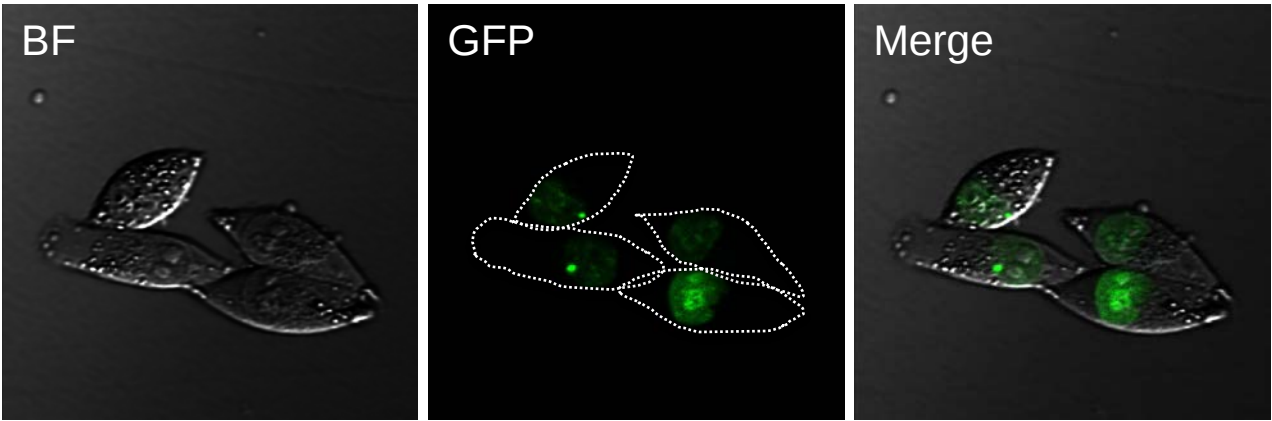
Figure 2

a.



b. 53BP1 foci positive and negative cells.

Foci negative cells



Foci positive cell

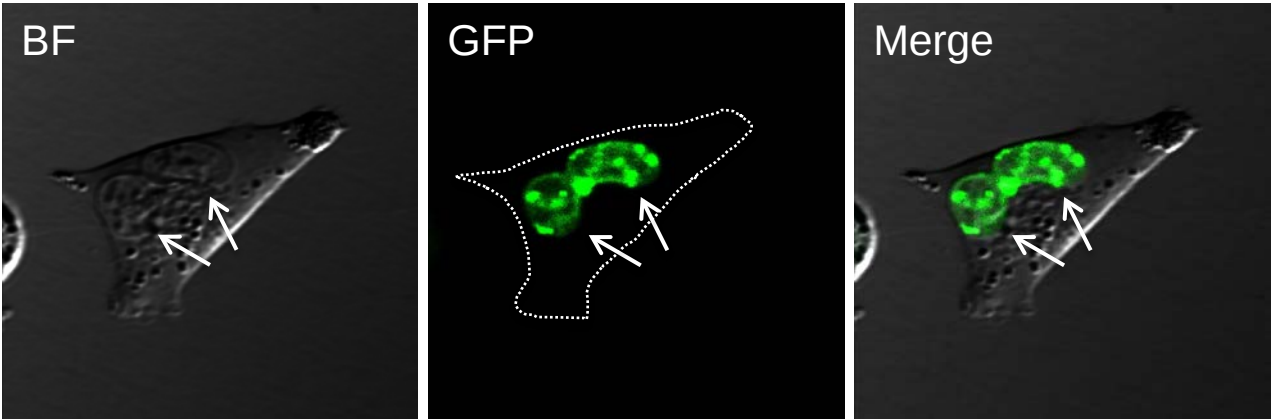


Figure 3

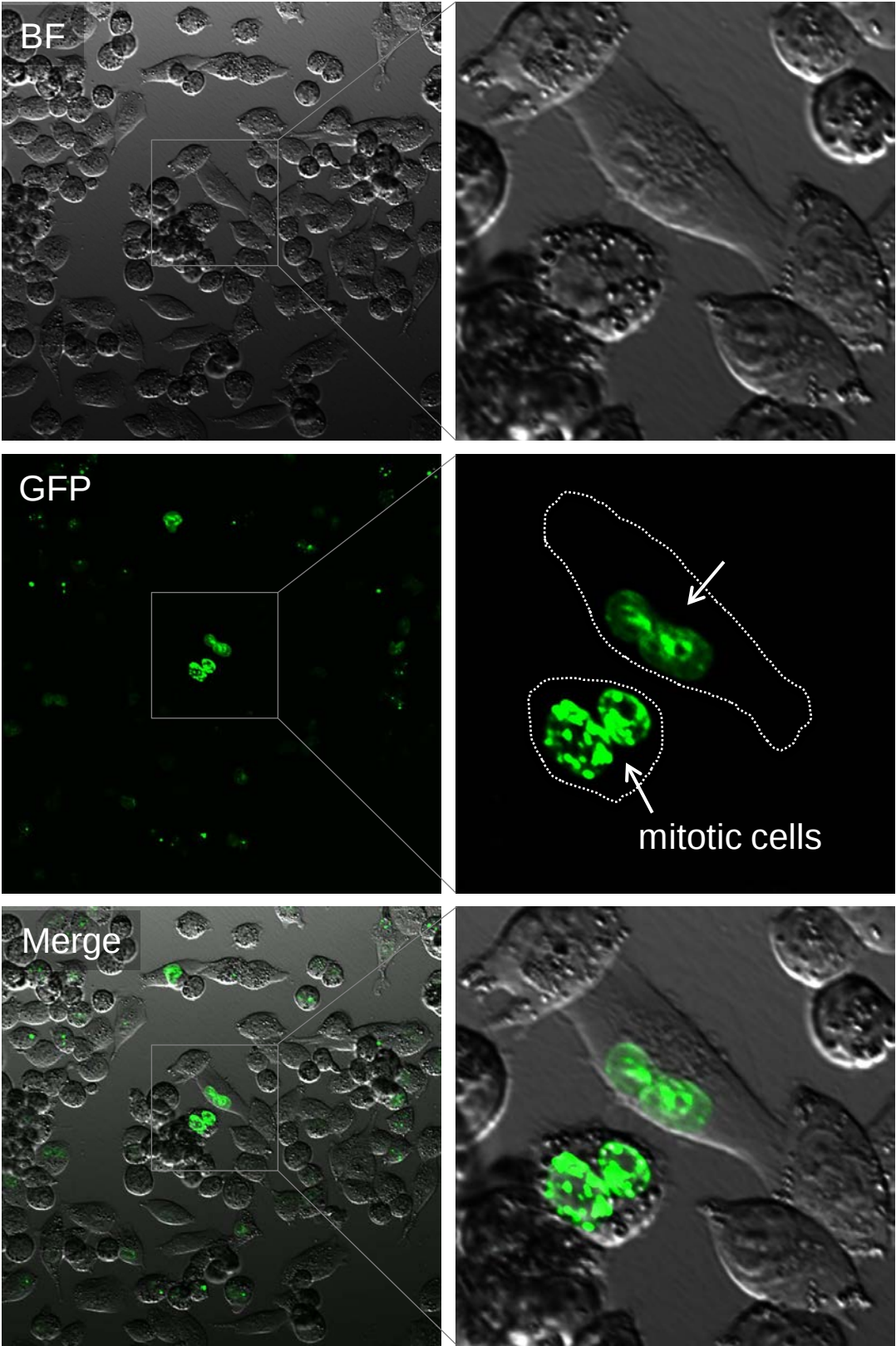


Figure 4

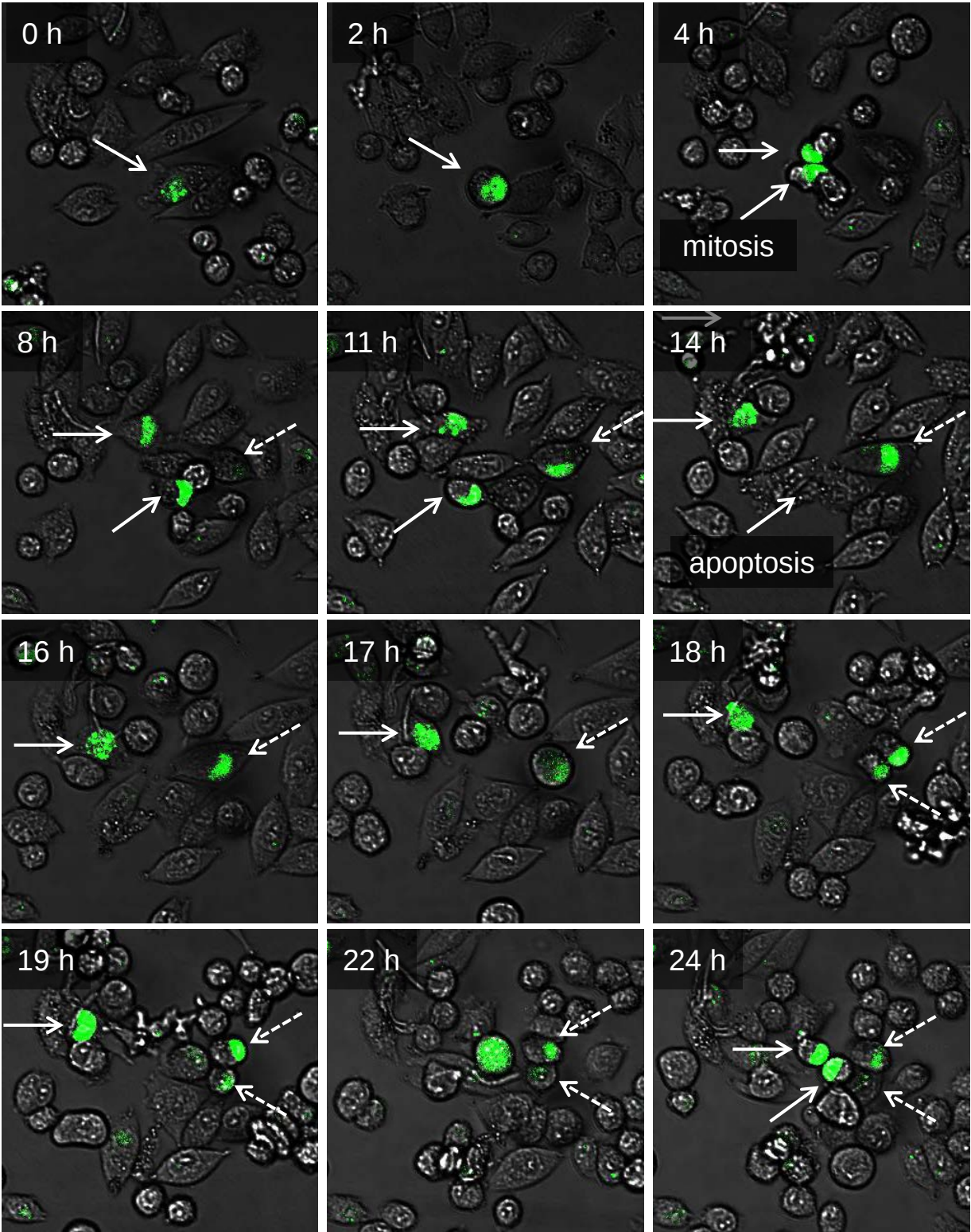


Figure 5

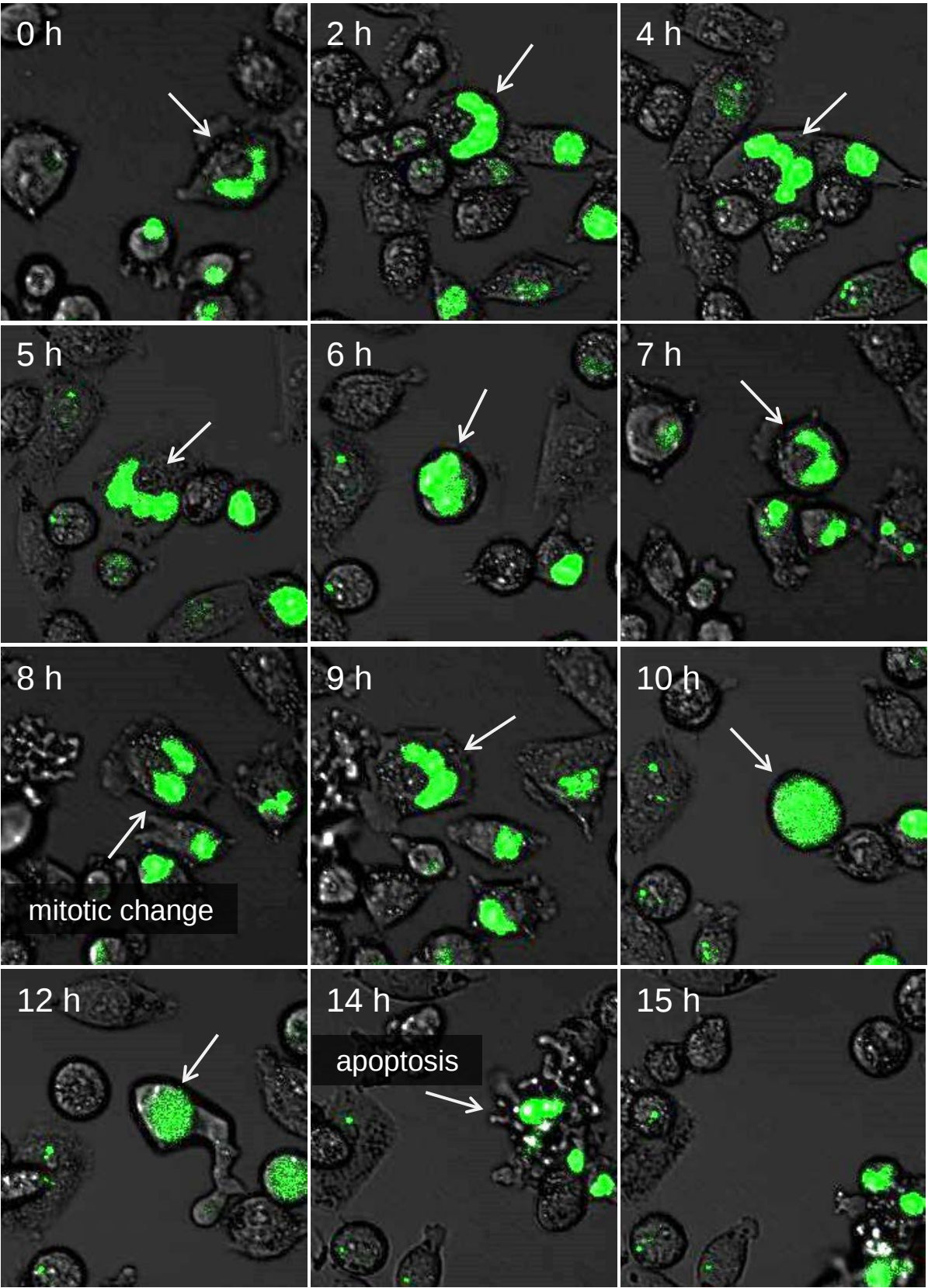


Figure 6

