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Lyn tyrosine kinase promotes silencing of ATM-dependent checkpoint signaling during recovery from DNA double-strand breaks

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ABSTRACT

DNA damage activates the DNA damage checkpoint and the DNA repair machinery. After initial activation of DNA damage responses, cells recover to their original states through completion of DNA repair and termination of checkpoint signaling. Currently, little is known about the process by which cells recover from the DNA damage checkpoint, a process called checkpoint recovery. Here, we show that Src family kinases promote inactivation of ataxia telangiectasia mutated (ATM)-dependent checkpoint signaling during recovery from DNA double-strand breaks. Inhibition of Src activity increased ATM-dependent phosphorylation of Chk2 and Kap1. Src inhibition increased ATM signaling both in G2 phase and during asynchronous growth. shRNA knockdown of Lyn increased ATM signaling. Src-dependent nuclear tyrosine phosphorylation suppressed ATM-mediated Kap1 phosphorylation. These results suggest that Src family kinases are involved in upstream signaling that leads to inactivation of the ATM-dependent DNA damage checkpoint.

1. Introduction

Genotoxic stress activates the DNA damage checkpoint and DNA repair machinery. After initial activation of DNA damage responses, damaged cells are eliminated by apoptosis, undergo permanent growth arrest (cellular senescence), or recover to their original states after completion of DNA repair and resumption of cell cycle progression. While the cell cycle is arrested by the checkpoint, checkpoint signaling is gradually silenced as DNA lesions are removed and checkpoint proteins become inactivated. Activation of the DNA damage checkpoint and DNA repair have been studied in depth, but at present little is known about the mechanism by which cells terminate checkpoint signaling and resume cell cycle progression, a process called checkpoint recovery. Currently, it is believed that removal of DNA lesions terminates activated checkpoint signaling. However, termination of checkpoint signaling also requires active involvement of the checkpoint recovery process, by which activated checkpoint proteins are dephosphorylated by phosphatases or degraded by ubiquitin-dependent proteasomal degradation [1,2].

DNA double strand breaks (DSBs) recruit and activate the checkpoint kinase ATM (ataxia telangiectasia mutated), and activated ATM phosphorylates multiple substrates. The C-terminal tail of the histone variant H2AX is predominantly phosphorylated by ATM. The resultant phosphorylated form of H2AX, γ-H2AX, is widely accepted as a marker of DSBs after ionizing radiation and exposure to radiomimetic agents. Chk2 is phosphorylated and activated by ATM, and activated Chk2 amplifies the DNA damage signal [3,4]. Kap1 (KRAB-associated protein 1) is another substrate of ATM [5,6].

During checkpoint recovery, Chk2 is regulated by protein phosphatases PP2A and Wip1/PPM1D [1,2], as well as by ubiquitin-dependent proteasomal degradation [7–9]. Kap1 is also regulated by protein phosphatase PP4 [10]. However, except for the results of previous studies describing reversal of phosphorylation and removal of activated checkpoint proteins, little is known about the upstream signaling that attenuates ATM signaling during checkpoint recovery.

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Src family kinases (SFKs) are the largest family of non-receptor tyrosine kinases [11]. SFKs are mainly located on the cytoplasmic side of the plasma membrane, but previous studies have shown that a small proportion of some SFKs is localized to the nucleus [12–16]. For example, the SFK Lyn is activated and translocated into the nucleus upon DNA damage induction [17,18]. During DNA damage responses, Lyn regulates apoptosis both negatively and positively [19–22]. These observations indicate that SFKs are involved in DNA damage responses; however, little is known about the involvement of the SFKs in the ATM-regulated checkpoint pathways.

Recently, we found that SFKs downregulate ATR-Chk1 signaling to promote recovery from the G2 DNA damage checkpoint [23]. We showed that SFK inhibition induces persistent activation of ATR-dependent checkpoint and prolonged cell cycle arrest in G2 phase. On the other hand, SFK inhibition decreases γ-H2AX signal, indicating that SFK inhibition suppresses generation of DSBs. These data raise a possibility that SFKs play a role in regulation of ATM signaling. We present here the results which show that SFKs promote inactivation of ATM-dependent checkpoint signaling during recovery from DSBs. In our experiments, SFK inhibition increased ATM-dependent phosphorylation of Chk2 and Kap1 after induction of DSBs. Furthermore, Src-dependent nuclear tyrosine phosphorylation suppressed Kap1 phosphorylation. These results suggest that SFKs are involved in the upstream signaling leading to inactivation of the ATM-dependent DNA damage checkpoint.

2. Material and methods

2.1. Plasmids, cell lines, and cell culture

Wild-type Lyn was tagged with FLAG-HA epitopes and a nuclear localization signal (NLS) at its N-terminus (NLS-Lyn) [16,23,24]. FH-NLS-Lyn retains the inhibitory tyrosine phosphorylation site at the C-terminal tail. shRNAs against SFKs were described previously [23]. Gene transfection was performed using acidified polyethylenimine [25,26]. HeLa S3 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% bovine serum.

2.2. Antibodies

The following antibodies were used: ATM-pS1981 (cl. 10H11. E12, Cell Signaling Technology, #4526), Chk2 (cl. DCS-273, Medical & Biological Laboratories, K0088-3), Chk2-pT68 (Cell Signaling Technology, #2661). Antibodies against cyclin A, Chk1, Chk1-pS345, Chk1-pS317, γ-H2AX, Hemagglutinin (HA), Hsc70, Kap1, Kap1-pS824, Lyn, and c-Yes were described previously [23,24].

2.3. Analysis of checkpoint proteins

Analysis of G2 checkpoint proteins was performed exactly as described previously [23]. For analysis of checkpoint proteins in asynchronously growing cells, cells were seeded and cultured for more than 24 h. Cells were then exposed to 200 nM Adriamycin (Sigma–Aldrich, D1515) for 1 h and allowed to recover. At the time of Adriamycin addition, 5 μM SU6656 (Sigma–Aldrich, S9692) or 40 μM PP2 (Sigma–Aldrich, P0042) were also added.

2.4. Flow cytometry

Flow-cytometric analyses were performed as described previously [23,27,28]. For analysis of Kap1-S824 phosphorylation, HeLa S3 cells were transfected 24 h after seeding. Eight hours after transfection, the cell-culture medium was replaced with fresh medium, and the cells were cultured for an additional 16 h. Cells were then exposed to 200 nM Adriamycin for 1 h, recovered for 4 h, and harvested. The cells were fixed with 1.5% paraformaldehyde, stained with the indicated antibodies, and analyzed on a BD FACSCanto II.

3. Results

3.1. SFK inhibition suppresses γ-H2AX but induces prolonged activation of ATM signaling

Recently, we showed that SFK inhibition decreases γ-H2AX signal after Adriamycin exposure [23]. Therefore, in this study, we examined the role of SFKs in ATM signaling. For this purpose, we used HeLa S3 cell line, because it can easily be adapted to cell synchronization method. We used Adriamycin since previous studies suggest the involvement of oncogenic tyrosine kinases in resistance against chemotherapy [29,30].

ATM phosphorylates Chk2 at Thr68 and Kap1 at Ser824, and phosphorylation of these sites can serve as an indicator of ATM activity *in vivo* [3–6]. Therefore, we examined ATM-mediated phosphorylation of Chk2 and Kap1 during recovery from the G2 DNA damage checkpoint. For this purpose, HeLa S3 cells were synchronized in early S phase by thymidine exposure, and then released from arrest. Six hours after release, cells entered late S and G2 phase and were exposed to Adriamycin, which resulted in immediate phosphorylation of Chk2 and Kap1 (Fig. 1). Accumulation of cyclin A and cyclin B indicates that the cells were in late S and G2 phase (Fig. 1) [23]. The reduction of Chk2 protein amount would be due to proteasomal degradation of Chk2 protein [7–9]. The phosphorylation of Kap1 was largely diminished 11 h after the end of Adriamycin exposure, but a significant phosphorylation

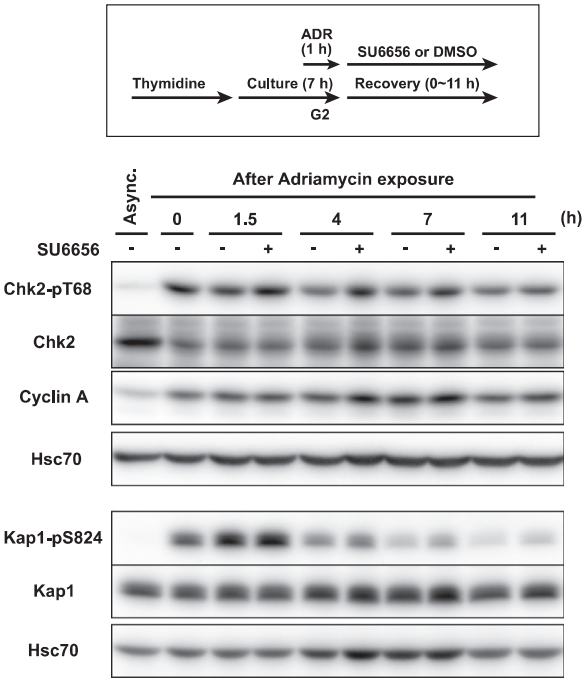

Fig. 1. SFK inhibition induces prolonged activation of ATM signaling after Adriamycin exposure in late S/G2 phase. HeLa S3 cells were synchronized in early S phase by exposure to 4 mM thymidine for 18 h, released from arrest, and cultured for 6 h to reach G2 phase. Next, cells were exposed to 110 nM Adriamycin for 1 h, allowed to recover for the indicated times, and harvested for Western blotting. At the end of Adriamycin exposure, 5 μM SU6656 was added. ADR, Adriamycin. Async., asynchronous cells.

of Chk2 was observed. The reason for this discrepancy is unclear, but the difference in the time course of the phosphorylation between Chk2 and Kap1 was also reported by others [31]. ATM autophosphorylation at Ser1981 is an indicative of activation of the kinase activity [32]. Eleven hours after the end of Adriamycin exposure, ATM-S1981 phosphorylation could still be observed, indicating that ATM was still active, but it was too weak to quantitate reliably (data not shown).

Since SFK inhibition induced a delay in checkpoint recovery, we focused on the time point of 11 h after the end of Adriamycin exposure. Treatment with SU6656, a specific SFK inhibitor [33] resulted in elevated phosphorylation of Chk2 and Kap1 over the time course (Figs. 1 and 2B, D and E), while SFK inhibition decreased γ-H2AX signal as we showed previously (Fig. 2A). The suppression of γ–H2AX signal during the time course was presented previously [23]. The addition of caffeine, a well-known inhibitor of ATM/ATR-dependent cell cycle checkpoints [34], abolished Chk2-T68 phosphorylation (Fig. 2B). The quantitative difference among Figs. 1 and 2B, D and E would be due to the difference in length of thymidine exposure and the timing of SU6656 addition. SU6656 promoted the accumulation of cyclin A (Fig. 1), suggesting that SFK inhibition delays the recovery from G2 checkpoint arrest as we showed previously [23]. Without Adriamycin exposure, SU6656 did not affect phosphorylation of Chk2 and Kap1 (Fig. 2C and F). ATM and ATR have similar substrate specificity, and ATM-independent Kap1 phosphorylation and DNA-PK-dependent Chk2 phosphorylation were reported [6,35]. To further clarify the involvement of ATM, we treated cells with a specific ATM inhibitor, KU-55933 [36]. This treatment abolished phosphorylation of Chk2-T68 and Kap1-S824 (Fig. 2D and E), indicating that phosphorylation of these sites are mediated by ATM as reported previously [3–5]. Although ATM activity is required for ATR activation by DSBs in late S and G2 phase [37], KU-55933 did not inhibit, but stimulated ATR-dependent Chk1 phosphorylation for unknown reason (Fig. 2G). These data indicate that SFK inhibition induces prolonged activation of ATM signaling during recovery from the ATM-dependent DNA damage checkpoint.

Since cell cycle synchronization by thymidine exposure induces γ-H2AX signal [23,38], we also examined the role of SFKs in ATM regulation in asynchronously growing cells. Adriamycin exposure induced ATM-mediated phosphorylation of Chk2 and Kap1 in asynchronously growing HeLa S3 cells (Fig. 3A, data not shown). Six hours after the end of Adriamycin exposure, SU6656 treatment increased Kap1-S824 phosphorylation (Fig. 3B), and the phosphorylation was inhibited by KU-55933 (Fig. 3C). By contrast, treatment with NU7441, a specific DNA-PK inhibitor, did not inhibit, but rather stimulated Kap1-S824 phosphorylation (data not shown). Another SFK inhibitor, PP2, also increased Kap1-S824 phosphorylation (Fig. 3D). In HeLa S3 cells, at least three family members, Lyn, c-Yes, and Fyn are expressed [39]. To identify the critical SFK that is involved in the suppression of ATM signaling, each SFK member is inhibited by shRNA-mediated gene knockdown. HeLa S3 cells depleted of Lyn showed an increase in Kap1 phosphorylation (Fig. 3E). By contrast, there is no effect of c-Yes knockdown on KAP1 phosphorylation. Because the level of Chk2 protein varied

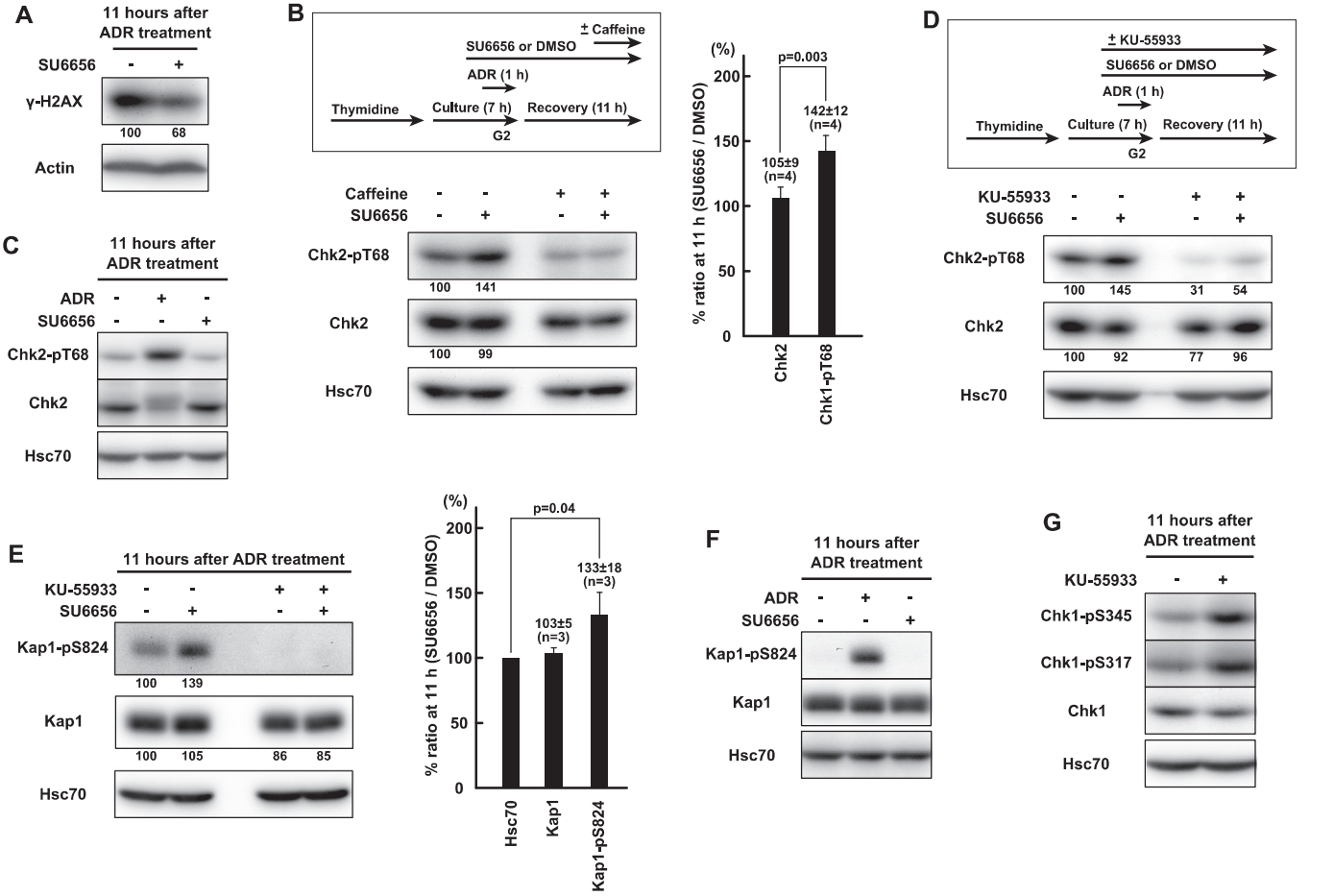

Fig. 2. SFK inhibition upregulates checkpoint signaling from ATM to Chk2 and Kap1 in G2 checkpoint recovery. (A–G) The same experiment as in Fig. 1, except that cells were arrested by thymidine for 24 h, and that 5 μM SU6656 was added 1 h before Adriamycin exposure. Cells were harvested 11 h after the end of Adriamycin exposure. One hour before harvest, 5 mM caffeine was added (B). One hour before Adriamycin exposure, 10 μM KU-55933 was added (D, E and G). The graphs represent results from at least three independent experiments. *P*-values were calculated using *t*-test.

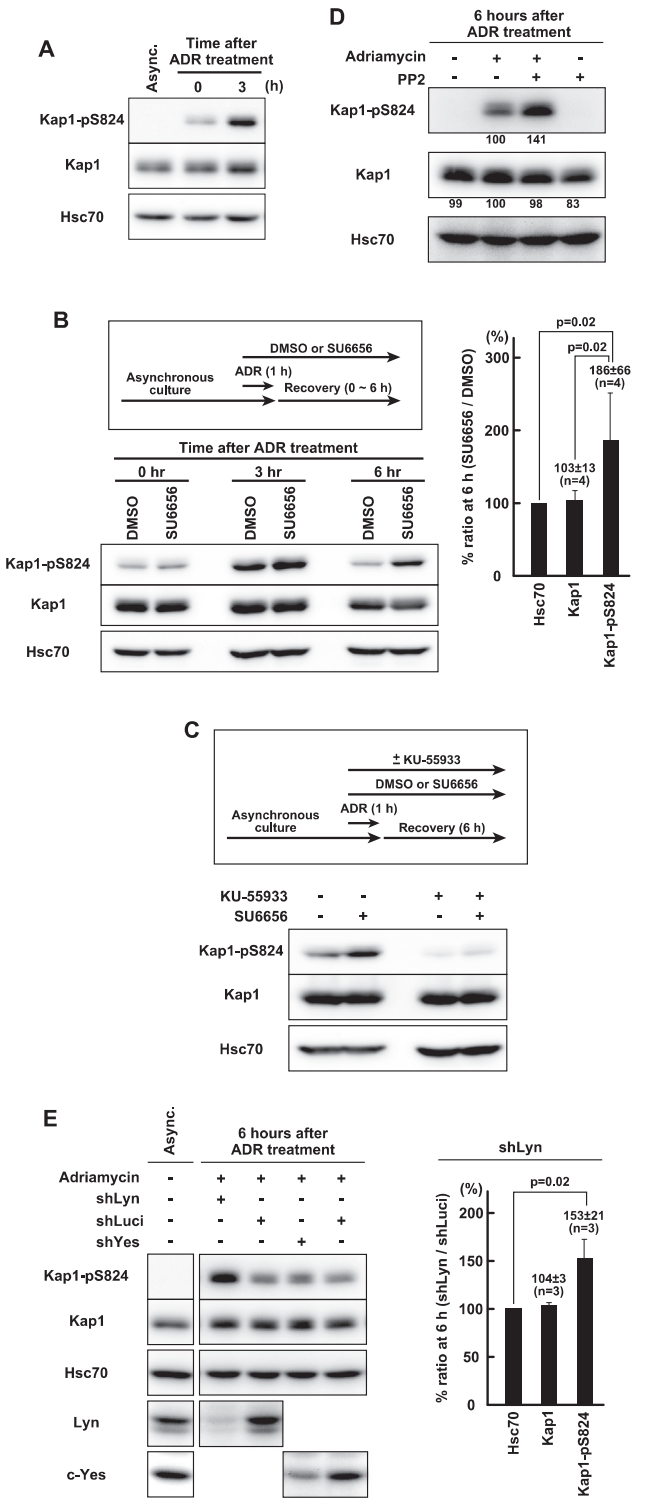

Fig. 3. SFK inhibition induces prolonged activation of checkpoint signaling from ATM in asynchronously growing cells. (A–C) Asynchronously growing HeLa S3 cells were exposed to 200 nM Adriamycin for 1 h, recovered for the indicated times, and harvested. SDS lysates were prepared and probed with the indicated antibodies. At the time of addition of Adriamycin, 5 μM SU6656 and/or 10 μM KU-55933 were added. The graph represents results from at least three independent experiments. *P*-values were calculated using *t*-test. (D) The same experiment as in A except that 40 μM PP2 was used instead of SU6656. Cells were harvested at 6 h after the end of Adriamycin exposure. (E) HeLa S3 cells were transfected with shRNA targeting Lyn, c-Yes, or Luciferase (Luci.). After 48–64 h after transfection, cells were exposed to 200 nM Adriamycin for 1 h, recovered for 6 h, and harvested for Western blotting. All lanes were rearranged from the same picture to match panel E. Async., asynchronous cells. The graph represents results from two independent experiments. *P*-values were calculated using *t*-test.

during checkpoint recovery, it was difficult to obtain reproducible results in Chk2-T68 phosphorylation (data not shown). These data support the conclusion described above, i.e., that SFKs promote recovery from the ATM-dependent DNA damage checkpoint.

3.2. Enforced expression of SFKs suppresses ATM signaling

To further exclude the possibility that the SFK inhibitors may affect ATM signaling nonspecifically, the effect of ectopic expression of Lyn on Kap1 phosphorylation was examined. We tagged the SFK Lyn with a nuclear localization signal (NLS-Lyn) and transiently expressed this protein in HeLa S3 cells. Nuclear localization of NLS-Lyn protein and nuclear tyrosine phosphorylation by NLS-Lyn were described previously [16,23,24]. The transfected cells were exposed to Adriamycin during asynchronous growth, and the effect of SFK activity was examined by flow-cytometric analyses. Although low-level expression of NLS-Lyn did not affect Kap1-S824 phosphorylation (Fig. 4, [R1]), high-level expression of NLS-Lyn suppressed it (Fig. 4 [R3]). The kinase-inactive mutant NLS-Lyn-K275R/Y508F did not affect Kap1-S824 phosphorylation as much as wild-type NLS-Lyn. These results further confirm the conclusion that SFKs suppress checkpoint signaling from ATM to its substrates.

4. Discussion

The results of this study reveal that SFKs promote inactivation of checkpoint signaling from ATM to its substrates during checkpoint recovery. SFK inhibition by two different chemical inhibitors and shRNA increased ATM-dependent phosphorylation of Chk2 and Kap1 (Figs. 1, 2B, D, E and 3B–E), whereas expression of NLS-Lyn suppressed ATM-dependent Kap1 phosphorylation (Fig. 4). The increased ATM signaling is not due to a delay in DSB repair since SFK inhibition decreased γ-H2AX signal (Fig. 2A), as we showed previously [23]. Although the precise underlying molecular mechanisms are unclear, our data demonstrate that SFKs, especially Lyn, are negative regulators of ATM signaling. Because ATM is a master regulator of the DNA damage checkpoint, our findings shed light on the upstream signaling that leads to inactivation of the ATM-dependent DNA damage checkpoint. Although completion of DNA repair is necessary in order to bring about the end of checkpoint signaling, our data suggest that another layer of regulation is involved in the inactivation of ATM-dependent checkpoint.

It is unclear whether SFKs regulate ATM signaling directly or indirectly. Recently, we showed that Kap1 is regulated by tyrosine phosphorylation that promotes ATM-dependent Kap1-S824 phosphorylation [24]. By contrast, the results of this study indicate that SFKs suppress the ATM-dependent Kap1-S824 phosphorylation (Figs. 1, 2E, 3B–E and 4). These data indicate that SFKs regulate the ATM-dependent Kap1 phosphorylation via a mechanism that does not involve tyrosine phosphorylation of Kap1. SFK inhibition delays recovery from G2 checkpoint arrest [23]. However, the effect of SFKs in regulating ATM signaling would not be due to alteration of cell cycle distribution, since SFK inhibition increased ATM signaling over the time course (Fig. 1). By the way, we showed that the regulation of interaction between Rad17 and Rad9 is a key event in the regulation of ATR-dependent checkpoint by SFKs [23,40]. Since ATM-dependent checkpoint should be largely independent of the interaction between Rad9 and Rad17 [3,4], ATM signaling is regulated by SFKs through a mechanism different from that of ATR signaling.

Previous studies have demonstrated that maintenance of G2 checkpoint arrest is largely dependent on ATR-Chk1 signaling, whereas ATM activity is mostly dispensable once DSB ends are resected and ATR is activated [1,2,37,41,42]. However, the contribution of ATM-Chk2 signaling in the G2 checkpoint maintenance,

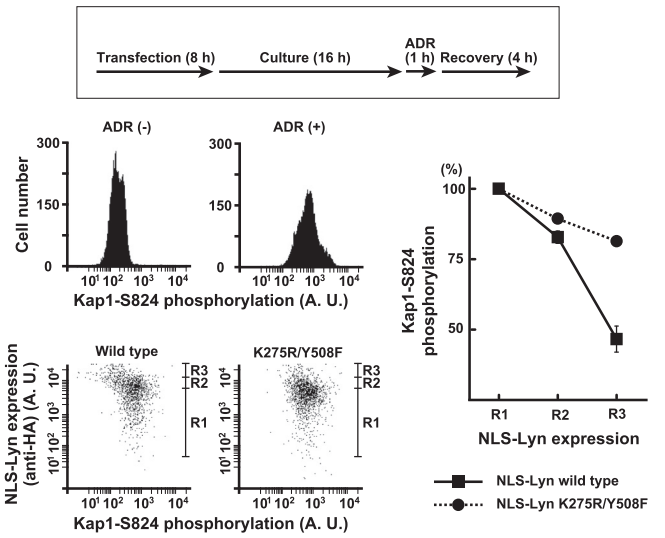

Fig. 4. Enforced expression of SFKs suppresses Kap1 phosphorylation. HeLa S3 cells were transfected with Lyn tagged with a nuclear localization signal (NLS-Lyn). Twenty-four hours after transfection, cells were exposed to 200 nM Adriamycin for 1 h. Four hours after the end of Adriamycin exposure, cell were fixed for flow-cytometric analyses. The intensity of the Kap1-pS824 signal was quantitated in each region (Rn), and is expressed relative to the intensity in cells expressing NLS-Lyn at a low level (R1). The graph represents the average of data from two independent experiments. A. U., arbitrary unit.

especially in the absence of functional ATR-Chk1 pathway, was reported [41,42]. In our experiments, after Adriamycin exposure, cells were arrested at G2 phase in an ATR- and Chk1-dependent manner, and inhibition of ATR or Chk1 inactivates the G2 checkpoint arrest and induces forced mitotic entry [23] as reported previously [1,2,34,37,42,43]. However, SFK inhibition induced a slight but significant delay in the mitotic entry induced by a specific Chk1 inhibitor Gö6976 [44] [Fig. 2E and F in Ref. [23]], indicating that the effect of SFK inhibition is also dependent on additional mechanisms other than ATR-Chk1 signaling. Since caffeine inhibits both ATM and ATR signaling, and SFK inhibition did not affect the caffeine-induced mitotic entry at all in the same condition [Fig. 2A and B in Ref. [23]], the SFK inhibition-induced delay in Gö6976-induced mitotic entry would be dependent on the SFK inhibition-induced Chk2 activation. These data raise the possibility that SFK inhibition affects checkpoint recovery through ATM-Chk2 signaling as well as ATR-Chk1 signaling, indicating that SFKs regulate checkpoint recovery through at least two different molecular mechanisms.

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