Original Article

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Redundant roles of extra-cellular signal-regulated kinase (ERK) 1 and 2 in the G1-S transition and etoposide-induced G2/M checkpoint in HCT116 cells

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SUMMARY The extracellular signal-regulated kinase (ERK) 1 and 2 intracellular signaling pathways play key roles in a variety of cellular processes, such as proliferation and differentiation. Dysregulation of ERK1/2 signaling has been implicated in many diseases, including cancer. Although ERK1/2 signaling pathways have been extensively studied, controversy remains as to whether ERK1 and ERK2 have specific or redundant functions. In this study, we examined the functional roles of ERK1 and ERK2 in cell proliferation and cell cycle progression using an auxin-inducible degron system combined with gene knockout technology. We found that ERK1/2 double depletion, but not ERK1 or ERK2 depletion, substantially inhibited the proliferation of HCT116 cells during G1-S transition. We further demonstrated that ERK1/2-double-depleted cells were much more tolerant to etoposide-induced G2/M arrest than ERK1 or ERK2 single-knockout cells. Together, these results strongly suggest the functional redundancy of ERK1 and ERK2 in both the G1-S transition under physiological conditions and the DNA damage-induced G2/M checkpoint. Our findings substantially advance understanding of the ERK1/2 pathways, which could have strong implications for future pharmacological developments.

Keywords Cell cycle, DNA damage, mitogen-activated protein kinase

1. Introduction

Proper response to extra- and intracellular stimuli is essential for the normal development and maintenance of homeostasis in living organisms, in which intracellular signaling pathways play important roles. The mitogen-activated protein kinase (MAPK) intracellular signaling cascade, consisting of MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAP3K), is highly conserved in eukaryotes, from yeast to mammals, and transduces signals *via* sequential phosphorylation (*1,2*). Extracellular signal-regulated kinase (ERK) 1 and 2 are the terminal components of the MAPK cascade. In mammals, ERK MAPK signaling regulates a variety of cellular processes, including proliferation and differentiation, and its dysregulation is implicated in many diseases, including cancer (*3-5*). Rat sarcoma virus (RAS), a small GTPase that activates ERK *via* rapidly accelerated fibrosarcoma (RAF) MAP3K and MAPK/ERK kinase (MEK) MAPKK, is one of the most frequently mutated oncogenes in human cancers (*3,6,7*).

Although ERK1 and ERK2 share over 80% amino acid sequence identity (*8*), the phenotypes of ERK1 and ERK2 knockout mice differ significantly. *Erk1* knockout mice are viable (*9*), whereas *Erk2* knockout mice are embryonic lethal (*10-12*). Further studies using a Cre-loxP system or short interfering RNA (siRNA) technology have shown different phenotypes of ERK1- and ERK2-deficient mice and cells (*13-16*), emphasizing that ERK1 and ERK2 possess specific functions. In contrast, recent studies have reported the functional redundancy of ERK1 and ERK2 (*17-19*). For example, Frémin *et al*. (*20*) showed that transgenic expression of ERK1 fully rescues mouse developmental defects associated with the loss of ERK2. However, whether ERK1 and ERK2 have specific or redundant functions remains controversial.

In this study, we investigated the functional roles

of ERK1 and ERK2 in cell proliferation and cell cycle progression using an auxin-inducible degron system combined with gene knockout technology. Our results suggest the functional redundancy of ERK1 and ERK2 in both the G1-S transition under physiological conditions and the DNA damage-induced G2/M checkpoint.

2. Materials and Methods

2.1. Cell culture and reagents

HCT116 cells either expressing or not expressing *Oryza sativa* transport inhibitor response 1 (OsTIR1) (F74G) were cultured as previously described (*21,22*). In some experiments, HCT116 cells were synchronized at the G1/S boundary, as described by Boulay *et al.* (*23*), with minor modifications. Briefly, cells were seeded at 4×10^5 cells per 35-mm dish. The following day, the medium was replaced with fresh medium containing 2 mM thymidine and incubated for 16 h. The cells were then washed three times, and the medium was replaced with fresh thymidine-free medium. After 8 h, the medium was replaced with 2 mM thymidine for 16 h, washed thrice, and replaced with thymidine-free fresh medium. Propidium iodide and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Blasticidin S hydrochloride and etoposide were purchased from Wako (Osaka, Japan). The Click-iT 5-ethynyl-2′-deoxyuridine (EdU) kit (C10425), 5-phenyl-1H-indole-3-acetic acid (5-Ph-IAA), puromycin, and SCH772984 were obtained from Thermo Fisher Scientific (Waltham, MA, USA), BioAcademia (Oasaka, Japan), Nacalai Tesque (Kyoto, Japan), and Selleck (Houston, TX, USA), respectively.

2.2. Plasmids and viral vector preparation

The lentivirus expression plasmid for hemagglutinin (HA)-tagged protein, pCL20c-CMVΔ4-HA, has been previously described (*24*). The entire coding sequences of three ERK1 variants, variant 1 (accession number NM_002746), variant 2 (NM_001040056), and variant 3 (NM_001109891), and ERK2 (NM_ 002745) were inserted into pCL20c-CMVΔ4-HA to generate pCL20c-CMVΔ4-HA-ERK1_V1, pCL20c-CMVΔ4-HA-ERK1_V2, pCL20c-CMVΔ4-HA-ERK1_V3, and pCL20c-CMVΔ4-HA-ERK2, respectively. Lentiviral vectors were prepared as described previously (*25*).

The following single-guide RNA (sgRNA) target sequences were cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 (#42230; Addgene, Watertown, MA, USA): *ERK1*, 5′-GCGTAGCCACATACTCCGTC-3′ and *ERK2*, 5′-GCCTACAGACCAAATATCAA-3′. The resultant plasmids, pX330-ERK1-TG12 and pX330-ERK2-TG15, together with pUREF-EX, were used to knock out *ERK1* and *ERK2*, respectively. The pUREF-EX plasmid was constructed by introducing the simian virus 40 (SV40) promoter-driven puromycin resistance gene (*PuroR*) into the pEF-BOS-EX vector (*26*). The mini-auxin-inducible degron (mAID) tag (*27,28*) was knocked-in at the C-terminus of the endogenous *ERK2* gene through clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated homologous recombination with pX330-ERK2-T2 and donor DNA plasmids. The sgRNA target sequence in pX330-ERK2-T2 was 5′-CAAATTTAAGATCTGTATCC-3′. Donor plasmids were constructed using pBlueScript KS II as a backbone. The mAID tag sequence (derived from pMK381 (Addgene #140536)) and phosphoglycerate kinase (PGK)-blasticidin S deaminase (BSD) (or -PuroR) expression cassettes were flanked by 1.1- and 1.2-kb left and right homology arms, respectively. The left arm and mAID tag were connected with the following 30-bp linker by overlapping PCR: forward, 5′-GGCGCTGGTGCAGGCGCCGGATCCACTAGT-3′ and reverse, 5′-ACTAGTGGATCCGGCGCCTGCACCAGCGCC-3′. The PGK-*PuroR* sequence was derived from pMK381, and *PuroR* was replaced with *BSD* (derived from pMK347 (Addgene #121181)) to generate the PGK-*BSD* expression cassette.

2.3. Cell line generation

To generate *ERK1* and *ERK2* single-knockout cell lines, HCT116 cells were transiently co-transfected with pUREF-EX and pX330-ERK1-TG12 (for ERK1) or pX330-ERK2-TG15 (for ERK2). Twenty-four hours after transfection, cells were cultured with 3 μg/mL puromycin for another 24 h, washed with phosphate-buffered saline (PBS), and subsequently cultured in the presence of 1 μg/mL puromycin for 24 h and in the absence of puromycin for 48 h. The cells were then subjected to limiting-dilution cloning. Gene disruption in clones was confirmed by genomic DNA PCR using the primers listed in Table S1 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=130*) and by western blot analysis.

ERK1 knockout clone cells were co-transfected with pX330-ERK2-T2 and two donor DNA plasmids containing *PuroR* and *BSD* expression cassettes, as described above. The medium was changed 24 h after transfection, and the cells were cultured for another 24 h. The transfected cells were incubated with 0.5 μg/mL puromycin and 10 μg/mL blasticidin S hydrochloride for 10 days, and then, single-cell colonies were picked and cultured for further analyses. mAID tagging at the C-terminus of the endogenous *ERK2* gene was confirmed by genomic DNA PCR using the primers listed in Table S1 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=130*) and by western blot analysis. This established cell line was named ERK1-KO/ERK2-mAID.

2.4. Western blot analysis and immunocytochemistry

Total cell lysates were prepared and analyzed by western blotting as previously described (*25*) using anti-actin (1:5,000; A6050; Sigma-Aldrich), anti-ERK1/2 (1:2,000; #4695; Cell Signaling Technology, Danvers, MA, USA), and anti-HA (1:2,000; 11867423001, Sigma-Aldrich) primary antibodies. Immunocytochemistry was carried out as described previously (*25*) using anti-HA (1:100; 11867423001, Sigma-Aldrich) primary antibody and Alexa Fluor 568-conjugated goat anti-mouse IgG (1:500; Thermo Fisher Scientific) secondary antibody. Fluorescent images were captured using an IX71 inverted microscope (Olympus, Tokyo, Japan) attached to a DP50 digital CCD camera (Olympus).

2.5. Cell proliferation and cell cycle analysis

For the cell proliferation assay, cells were seeded at 2 × 10^4 cells/well in a 24-well plate, grown for 1 d, and cultured in the presence or absence of SCH772984 or 5-Ph-IAA for the indicated times and concentrations, as described in the legends of the corresponding figures. The cells were fixed with 4% paraformaldehyde in PBS for 20 min and stained with DAPI. Five images were randomly captured for each well using a Keyence BZ-X800 fluorescence microscope (Keyence, Osaka, Japan), and the cell numbers were counted using ImageJ software.

The phase distribution of the cell cycle was analyzed by flow cytometry using propidium iodide, with or without EdU. Wild-type, *ERK1* single knockout, and *ERK2* single knockout clones of HCT116, as well as parental HCT116 cells, were seeded at 4 × 10^5 cells per 35-mm dish and grown for 1 d. The cells were labeled with 10 μM EdU for 1 h, trypsinized, washed once with PBS containing EDTA (0.5 mM), and fixed with 70% cold ethanol for 20 min. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 20 min, treated with Click-iT EdU Reaction Cocktail (prepared according to the manufacturer's instructions) for 20 min, washed thrice with PBS, stained with 10 μg/mL propidium iodide, and analyzed by flow cytometry using a BD FACSLyric Flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). HCT116 ERK1-KO/ERK2-mAID clones were seeded at 2 × 10^5 cells per 35-mm dish and grown for 1 d. Cells were then treated with or without 5 μM 5-Ph-IAA for 48 h, labeled with 10 μM EdU for 1 h, and subjected to flow cytometric analysis, as described above. For cell cycle analysis using propidium iodide, but not EdU, cells were treated with or without 20 μM etoposide for 1 h and subjected to flow cytometry analysis as described above, except that cells were treated with 5 μg/mL RNase A after the ethanol-fixed cells were washed with PBS.

2.6. Statistical analysis

Statistical significance was determined using two-tailed unpaired Student's *t*-test. Statistical significance was set at a p value of < 0.05.

3. Results

3.1. Effect of ERK1 and ERK2 inhibition on cell proliferation

First, we examined the effects of ERK inhibition on cell proliferation. The near-diploid cell line HCT116 was cultured with six different concentrations (0-256 nM) of SCH772984, an ERK1/2 inhibitor, for 3 d. As shown in Figure 1A, cell numbers decreased in a dose-dependent manner. Of the different concentrations, we chose 64 nM for the kinetic analysis, because an appropriate inhibitory effect was observed at this concentration. We treated cells with 64 nM SCH772984 and monitored cell proliferation by counting cells every day for 3 d. Compared with vehicle-treated cells, SCH772984-treated cells showed lower numbers of cells at all examined time points (Figure

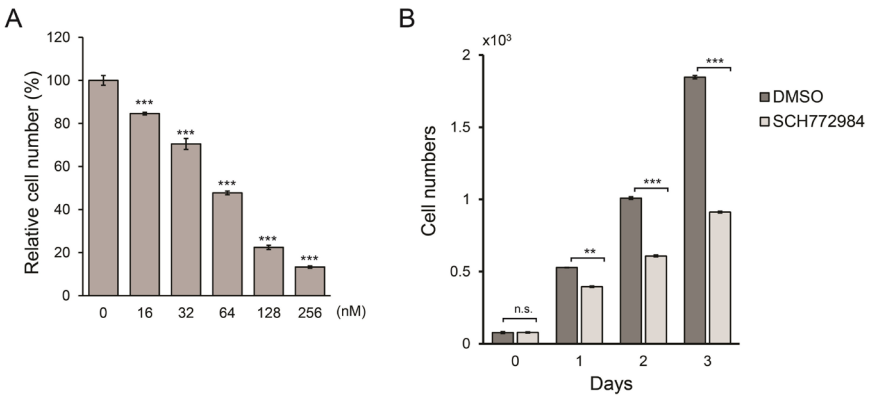

Figure 1. Inhibition of ERK1 and ERK2 prevented cell proliferation. (**A**) HCT116 cells were plated and grown for 1 d and treated with the ERK1/2 inhibitor SCH772984 (dissolved in dimethyl sulfoxide (DMSO)) at the indicated concentrations for 3 d. Cell numbers were normalized to those of control vehicle-treated (0 nM) cells. (**B**) HCT116 cells were plated and grown for 1 d (Day 0) and then cultured in the presence or absence of SCH772984 (64 nM) for the indicated days. Average cell numbers per captured image are shown. Quantitative data are expressed as mean ± S.E.M of three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not significant.

1B). These results suggest that ERK1 and/or ERK2 play a role in the proliferation of HCT116 cells.

3.2. Overlapping functions of ERK1 and ERK2 in cell proliferation

We examined whether ERK1 and ERK2 had redundant functions in cell proliferation. To this end, we first generated *ERK1* or *ERK2* single-knockout HCT116 cells (Figure 2A) and analyzed their proliferation. The results showed that depletion of either ERK1 or ERK2 had little effect on cell proliferation (Figure 2B), suggesting a functional redundancy of ERK1 and ERK2. To address this issue, we employed a conditional depletion system. The mAID tag was inserted at the C-terminus of endogenous *ERK2* in *ERK1* knockout HCT116 cells expressing OsTIR1(F74G). After screening with two different antibiotics (puromycin and blasticidin), we identified biallelic knock-in cell line that stably expressed the mAID-tagged ERK2 (ERK2-mAID) protein. The resulting cell line was named HCT116 ERK1-KO/ERK2-mAID. As shown in Figure 2C, the electrophoretic mobility of ERK2-mAID proteins was notably delayed, and no endogenous ERK2 proteins were detected, indicating mAID tag knock-in at both alleles. The expression levels of ERK2-mAID proteins were lower than those of endogenous ERK2 proteins, for unknown reasons. However, there were no defects in the proliferation of the HCT116 ERK1-KO/ERK2-mAID cell lines (Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=130*). Therefore, these cell lines were used for further analysis. Western blot analysis showed that the ERK2-mAID protein disappeared rapidly upon 5-Ph-IAA treatment (Figure 2D). Notably, the inhibition of proliferation was induced by depleting ERK2-mAID protein in the HCT116 ERK1-KO/ERK2-mAID cell lines (Figure 2E). Taken together, these results strongly suggest that ERK1 and ERK2 play a role in the functional redundancy of cell proliferation.

3.3. Functional evaluation of ERK1 variants in cell proliferation

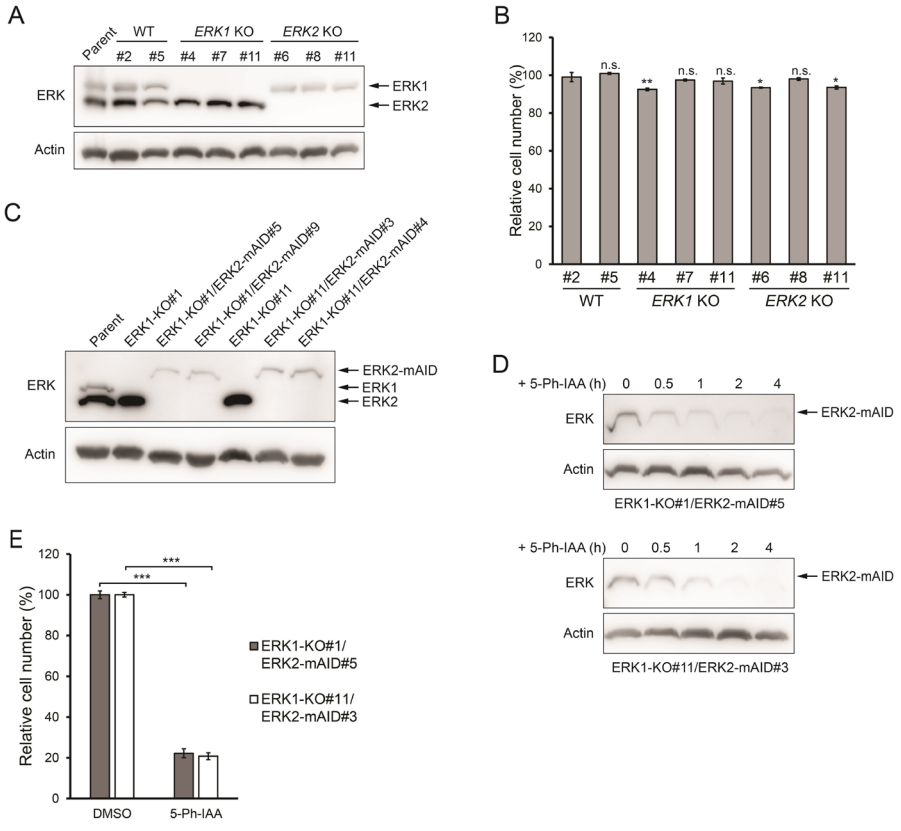

Figure 2. ERK1 and ERK2 played a redundant role in cell proliferation. (**A**) Parental HCT116 cells (Parent), two wild-type (WT) clones (#2 and #5), three *ERK1* knockout (KO) clones (#4, #7 and #11), and three *ERK2* KO clones (#6, #8, #11) were analyzed using western blotting with an anti-ERK1/2 antibody. (**B**) The WT and KO clones, as well as the parental cells, were cultured for 3 d, and cell proliferation was examined, as shown in Figure 1B. (**C**) ERK1-KO/ERK2-mAID cell lines were generated using ERK1-KO#1 and ERK1-KO#11. Obtained clones, ERK1-KO#1/ERK2-mAID#5, ERK1-KO#1/ERK2-mAID#9, ERK1-KO#11/ERK2-mAID#3, and ERK1-KO#11/ERK2-mAID#4, were analyzed using western blotting, as shown in (A). (**D**) ERK1-KO#1/ERK2-mAID#5 and ERK1-KO#11/ERK2-mAID#3 clones were treated with 5 μM 5-phenyl-1H-indole-3-acetic acid (5-Ph-IAA). Cell lysates were collected at the indicated time points and subjected to western blotting, as shown in (A). (**E**) Cell proliferation was analyzed in ERK1-KO#1/ERK2-mAID#5 and ERK1-KO#11/ERK2-mAID#3 clones treated with vehicle (DMSO) or 5 μM 5-Ph-IAA as shown in (B). Actin was utilized as the loading control. Quantitative data are expressed as mean ± S.E.M of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not significant.

Of three transcript variants of ERK1, variants 1-3, variant 1 (NM_002746) is predominantly expressed in HCT116 cells (Figure S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=130*). Transcripts 1-3 encode proteins of 379 amino acid residues (aa) (ERK1_V1), 357 aa (ERK1_V2), and 335 aa (ERK1_V3), respectively. In addition, although ERK2 has two transcript variants (NM_ 002745 and NM_138957), they encode proteins with the same aa. We lentivirally expressed HA-tagged ERK1_V1 (HA-ERK1_V1), ERK1_V2 (HA-ERK1_V2), or ERK1_V3 (HA-ERK1_V3) in HCT116 ERK1-KO/ERK2-mAID cells and analyzed the cell proliferation. The levels of exogenously expressed HA-ERK1s were similar to those of endogenous ERK1 (Figure 3A), where the percentage of HA-positive cells in cells infected with lentiviruses was nearly 100% (Figure S3, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=130*). As shown in Figure 3B, HA-ERK1_V1, but not HA-ERK1_V2 or HA-ERK_V3, almost completely rescued the impaired proliferation of ERK1/2-depleted cells, indicating that the predominantly expressed ERK1_V1 was functional in cell proliferation.

3.4. Role of ERK1 and ERK2 in cell cycle progression

To assess the effects of ERK inhibition on cell cycle progression, we cultured HCT116 cells in the presence or absence of SCH772984 and analyzed them using flow cytometry. The proportion of cells in the G1 phase was significantly increased, and the number of these cells was significantly reduced in the S and G2/M phases after treatment with SCH772984 (Figure 4A and Figure S4A, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=130*). Similar results were obtained when we examined HCT116 ERK1-KO/ERK2-mAID cells with or without 5-Ph-IAA treatment (Figure 4B and S4B, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=130*). In contrast, the cell cycle distribution of *ERK1* or *ERK2* single-knockout cells was similar to that of control SCH772984-untreated cells (Figure S5, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=130*). These results suggest that ERK1 and ERK2 were functionally redundant in regulating G1/S transition.

ERK1 and ERK2 play a role in the G2/M checkpoint in response to DNA damage (*29*). We examined the effect of etoposide, a DNA-damaging agent, on cell cycle progression. As expected, G2/M arrest was observed in HCT116 cells (Figure 5A). The proportion of G2/M phase cells was moderately but significantly decreased in *ERK1* and *ERK2* single knockout cells than in parental HCT116 cells (Figure 5A). We investigated HCT116 ERK1-KO/ERK2-mAID cells with or without 5-Ph-IAA treatment, focusing on the G2/M phase. To avoid inhibition of cell proliferation by ERK1/2 depletion, we synchronized cells at the G1/S phase using a double thymidine block (Figure S6, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=130*), treated them with or without etoposide, and performed flow cytometric analysis. G2/M accumulation induced by etoposide was largely attenuated in ERK1/2-double-depleted cells (Figure 5B). Collectively, these results suggest that ERK1 and ERK2 were functionally redundant at the etoposide-induced G2/M checkpoint.

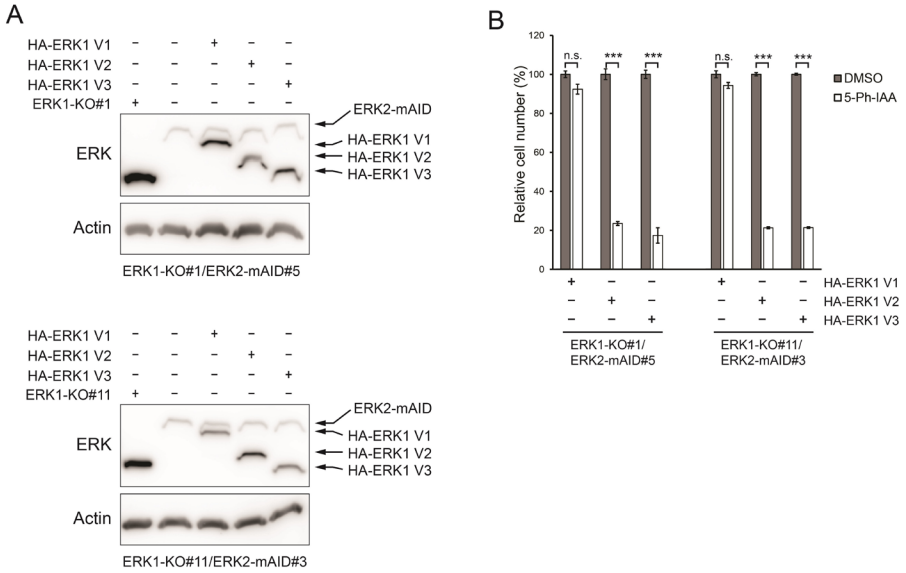

Figure 3. ERK1 variant 1, but not variants 2 and 3, rescued impaired cell proliferation of ERK1/2-depleted cells. ERK1-KO#1/ERK2-mAID#5 and ERK1-KO#11/ERK2-mAID#3 clones lentivirally expressing HA-ERK1 variants 1 (V1), 2 (V2), or 3 (V3), as well as ERK1-KO#1 and ERK1-KO#11 clones, were analyzed using western blotting, as in Figure 2A (A), and were subjected to a cell proliferation assay, as in Figure 1B (B). Actin was utilized as the loading control. Quantitative data are expressed as mean ± S.E.M of three independent experiments. $^{***}p < 0.001$; n.s., not significant.

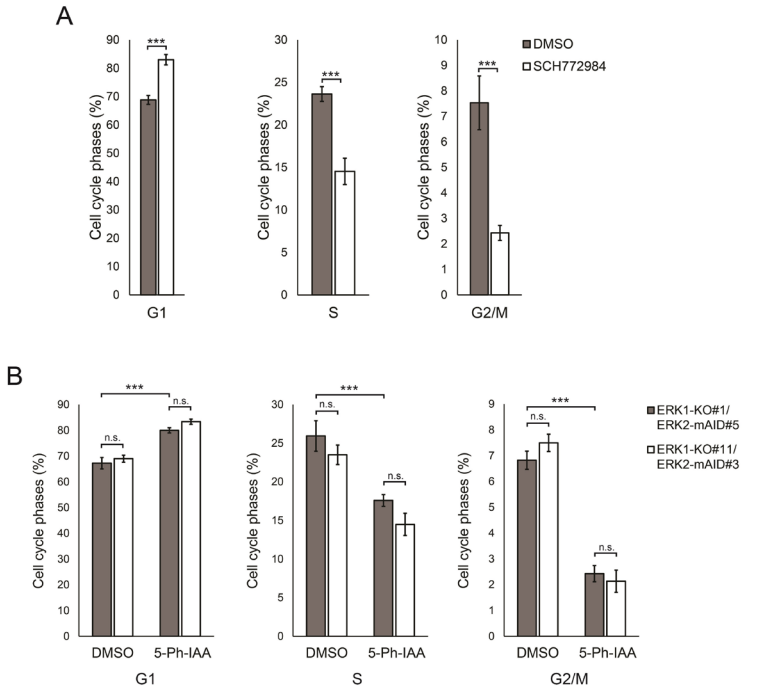

Figure 4. ERK1 and ERK2 had functional role in cell cycle progression. (**A**) HCT116 cells were cultured in the presence of vehicle (DMSO) or 100 nM SCH772984, and cell cycle distribution was analyzed by flow cytometry using EdU. (**B**) ERK1-KO#1/ERK2-mAID#5 and ERK1-KO#11/ERK2-mAID#3 clones treated with vehicle (DMSO) or 5 μM 5-Ph-IAA were analyzed as shown in (A). Quantitative data are expressed as mean ± S.E.M of three independent experiments. $^{***}p < 0.001$; n.s., not significant.

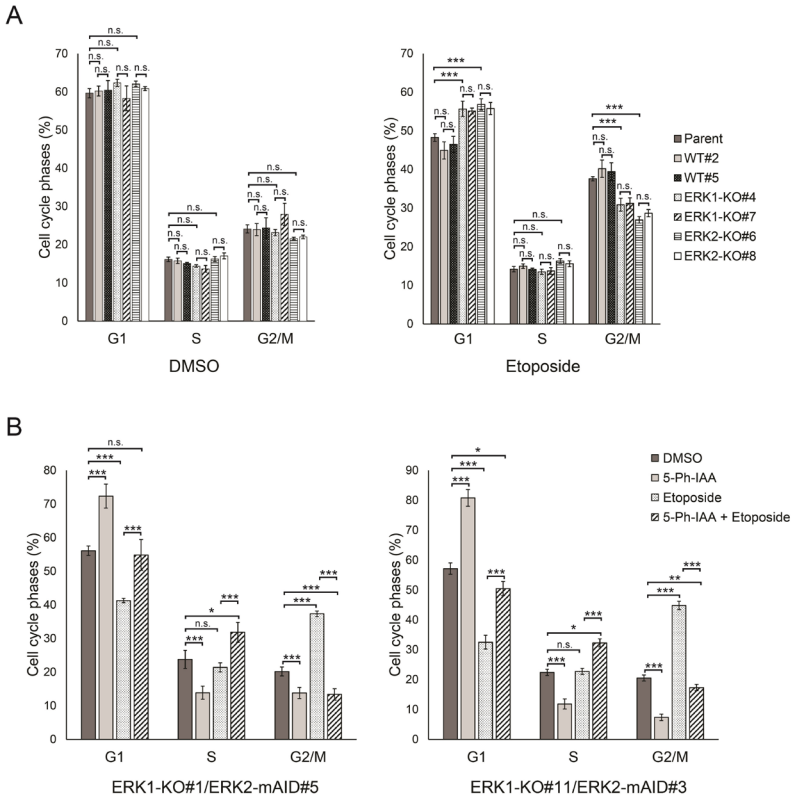

Figure 5. ERK1 and ERK2 had redundant roles in etoposide-induced G2/M accumulation. (**A**) The parental HCT116 cells (Parent), wild-type (WT) (#2 and #5), *ERK1* knockout (KO) (#4 and #7), and *ERK2* KO (#6 and #8) clones described in Figure 2A were treated with vehicle (DMSO) or 20 μM etoposide for 1 h, and cell cycle distribution was analyzed using flow cytometry. (**B**) The ERK1-KO#1/ERK2-mAID#5 and ERK1-KO#11/ERK2-mAID#3 clones described in Figure 2C were synchronized at the G1/S boundary, cultured in the presence or absence of 20 μM etoposide for 1 h with or without 5 μM 5-Ph-IAA for 4 h, as indicated, and subjected to flow cytometry analysis. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not significant.

4. Discussion

In this study, we explored the role of ERK1 and ERK2 in cell proliferation and cell cycle progression using the near-diploid cell line HCT116. At present, selective pharmacological inhibitors to distinguish between ERK1 and ERK2 are not available, and inhibition of ERK1/2 has been reported to prevent cell proliferation (*3,30*). Here, we employed an auxin-inducible degron system, called AID2 (*22*), in combination with gene knockout technology and provided evidence that ERK1 and ERK2 play redundant roles in cell proliferation (Figure 2) and cell cycle progression during the G1-S transition (Figure 4). To date, several *in vivo* and *in vitro* studies have reported that ERK1 and ERK2 have distinct functions (*13-16*). However, recent studies have suggested

redundant functions for ERK1 and ERK2 (*17-20*). Our present study using the AID2 system supports the latter hypothesis, that is, the interchangeable functions of ERK1 and ERK2. To the best of our knowledge, this is the first study to examine the functional redundancy of ERK1/2 in a rapid protein depletion system.

Moreover, we examined the functional roles of ERK1 and ERK2 in DNA damage response using the AID2 system (Figure 5). Wei *et al*. (*29*) reported that ERK1 and ERK2 are involved in etoposide-induced G2/M arrest and suggested the possibility of redundant roles of ERK1/2 in the G2/M checkpoint; however, this possibility was not addressed, most likely due to technical limitations, in which the authors employed an siRNA-mediated knockdown approach. Here, we generated a conditional depletion system of ERK2 in an ERK1 knockout background and showed that ERK1/2-double-depleted cells were much more tolerant to etoposide-induced G2/M arrest than ERK1 or ERK2 single knockout cells (Figure 5A and B). These findings strongly suggest the functional redundancy of ERK1 and ERK2 in G2/M checkpoint activation in response to DNA damage. Our findings substantially advance understanding of the ERK1/2 pathways, which could have strong implications for future pharmacological developments.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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