Received: 11 May 2020 | Revised: 19 August 2020 | Accepted: 23 August 2020

DOI: 10.1111/cas.14637

ORIGINAL ARTICLE

Cancer Science WILEY

Complex roles of the actin-binding protein Girdin/GIV in DNA damage-induced apoptosis of cancer cells

Chen Chen[1] | Atsushi Enomoto[1] | Liang Weng[2,3] | Tetsuro Taki[1] | Yukihiro Shiraki[1] | Shinji Mii[1] | Ryosuke Ichihara[1] | Mitsuro Kanda[4] | Masahiko Koike[4] | Yasuhiro Kodera[4] | Masahide Takahashi[1,5]

[1]Department of Pathology, Nagoya University Graduate School of Medicine, Nagoya, Japan

[2]Center for Molecular Medicine, Xiangya Hospital, Central South University, Changsha, China

[3]Key Laboratory of Molecular Radiation Oncology Hunan Province, Changsha, China

[4]Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya, Japan

[5]International Center for Cell and Gene Therapy, Fujita Health University, Toyoake, Japan

Correspondence

Atsushi Enomoto and Masahide Takahashi, Department of Pathology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan.
Emails: enomoto@iar.nagoya-u.ac.jp; mtakaha@med.nagoya-u.ac.jp

Funding information

Grant-in-Aid for Scientific Research (S), Grant/Award Number 26221304, and Grant-in-Aid for Scientific Research (B), Grant/Award Number 18H02638, Ministry of Education, Culture, Sports, Science and Technology of Japan; AMED-CREST (Japan Agency for Medical Research and Development, Core Research for Evolutional Science and Technology) Grant/Award Numbers 19gm0810007h0104 and 19gm1210008s0101; Project for Cancer Research and Therapeutic Evolution (P-CREATE) from AMED, Grant/Award Number 19cm0106332h0002.

Abstract

The actin-binding protein Girdin is a hub protein that interacts with multiple proteins to regulate motility and Akt and trimeric G protein signaling in cancer cells. Girdin expression correlates with poor outcomes in multiple human cancers. However, those findings are not universal, as they depend on study conditions. Those data suggest that multiple aspects of Girdin function and its role in tumor cell responses to anticancer therapeutics must be reconsidered. In the present study, we found that Girdin is involved in DNA damage-induced cancer cell apoptosis. An esophageal cancer cell line that exhibited high Girdin expression showed a marked sensitivity to UV-mediated DNA damage compared to a line with low Girdin expression. When transcriptional activation of endogenous Girdin was mediated by an engineered CRISPR/Cas9 activation system, sensitivity to DNA damage increased in both stationary and migrating HeLa cancer cells. High Girdin expression was associated with dysregulated cell cycle progression and prolonged G_1 and M phases. These features were accompanied by p53 activation, which conceivably increases cancer cell vulnerability to UV exposure. These data highlight the importance of understanding complex Girdin functions that influence cancer cell sensitivity to therapeutics.

KEYWORDS

apoptosis, cancer cell heterogeneity, cell cycle, cell migration, DNA damage, Girdin

Abbreviations: BTSC, brain tumor stem cell; CAF, cancer-associated fibroblast; Cdk5, Cyclin-dependent kinase 5; IF, immunofluorescent; IHC, immunohistochemistry; OE, overexpression; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; Rb, retinoblastoma; SAM, synergistic activation mediator; sgRNA, single guide RNA; TCGA, The Cancer Genome Atlas; UVC, ultraviolet C.

1 | INTRODUCTION

Cancer cells develop remarkable mechanisms to promote their uncontrolled proliferation, survival, and motility. These characteristics contribute to their invasion of neighboring tissues and metastasis to distant organs.[1] Treating cancers by various therapeutic techniques, such as radiation and chemotherapeutic reagents, is often unsatisfactory because the tumor cells gain therapeutic resistance, leading to subsequent recurrence.[1-4] The mechanisms of cancer therapeutic resistance have been the subject of intensive studies. Obviously, one of the most important causes of the therapeutic resistance is genetic and nongenetic heterogeneity and alterations of cancer cells, which becomes more prominent and elaborate by cancer cell evolution during the progression of the diseases and treatments.[5,6] The presence of cancer stem cells and dormant cancer cells is another cause of therapeutic resistance in multiple cancers.[7,8]

Girdin, which is also known as Gα-interacting vesicle-associated protein (GIV), was previously identified as an actin-binding protein and a substrate of Akt that is involved in the remodeling of the actin cytoskeleton.[9,10] It is essential for actin remodeling at the leading edge of migrating cancer cells.[9] Girdin is crucial for cell migration as well as cell polarization and membrane trafficking.[9,11,12] It also participates in Akt and heterotrimeric G protein signaling downstream of growth factors and cytokines through interactions with numerous proteins.[9,10,13] Girdin-deficient mice show a severe defect in collective migration of newborn neurons in postnatal and adult brains.[14] These findings gave rise to the idea that Girdin is a conserved regulator of collective behavior of cells across many cell types.[15] Indeed, we recently reported that Girdin plays an essential role in the collective invasion of human cancer cells.[16] Supporting this view, many studies have shown that high Girdin expression correlates with poor outcomes of patients with cancers of the breast, colon, and esophagus.[17-21]

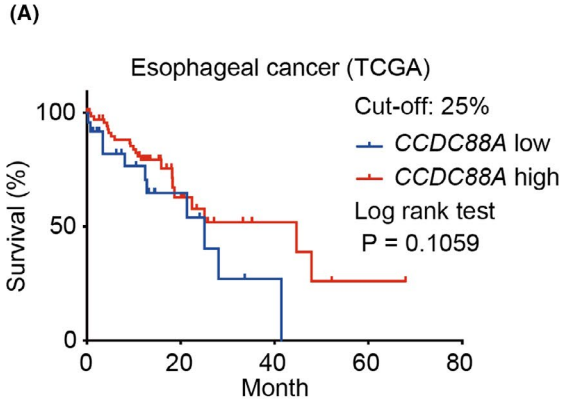
Other seminal studies have shown that Girdin functions as a hub protein that controls the migration-proliferation dichotomy ("go or grow" mechanism) in HeLa cancer cells.[22] Cyclin-dependent kinase 5-mediated phosphorylation of Girdin has a crucial role in promoting cell migration, whereas the nonphosphorylated form of Girdin promotes cell proliferation.[23] Given the general view that migratory cells are essentially not proliferative and are resistant to DNA damage and cytotoxic reagents,[24,25] the migration-proliferation dichotomy has a central role in tumor cells' resistance to anticancer therapeutics. Nonetheless, cell proliferation capacity is undoubtedly essential for cancer progression, rendering the role of high Girdin expression in cancer progression rather unclear.

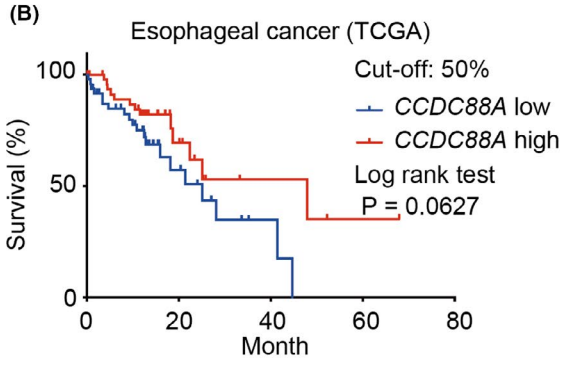
TABLE 1 Clinicopathological characteristics of esophageal cancer patients analyzed in the current study

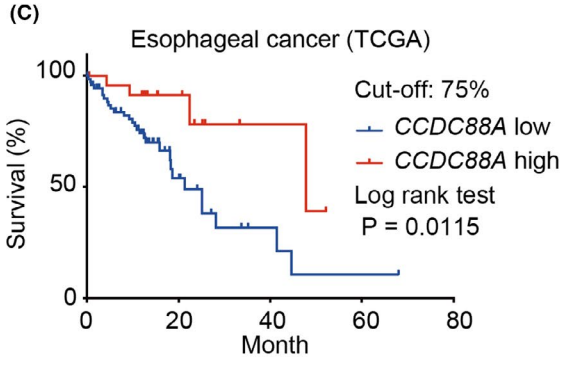
Characteristic	Total	Girdin-low (%)	Girdin-high (%)	*P* value (χ^2 test)
Number	29	11 (37.9)	18 (62.1)	
Age (y)				
<65	70	8 (27.6)	8 (27.6)	.1373
≥65	130	3 (10.3)	10 (34.5)	
Sex				
Male	24	8 (27.6)	16 (55.2)	.2636
Female	5	3 (10.3)	2 (6.9)	
Alcohol intake				
No	9	2 (6.9)	7 (24.1)	.2422
Yes	20	9 (31.0)	11 (37.9)	
Brinkman index				
<1000	24	9 (31.0)	15 (51.7)	.9165
≥1000	5	2 (6.9)	3 (10.3)	
Tumor location				
Cervix	1	1 (3.4)	0 (0.0)	.3145
Upper	7	3 (10.3)	4 (13.8)	
Middle	18	7 (24.1)	11 (37.9)	
Lower	3	0 (0.0)	3 (10.3)	
Histological grade				
Grade 1	8	2 (6.9)	6 (20.7)	.2284
Grade 2	17	6 (20.7)	11 (37.9)	
Grade 3	4	3 (10.3)	1 (3.4)	
Clinical stage				
I	2	1 (3.4)	1 (3.4)	.9849
II	3	1 (3.4)	2 (6.9)	
III	16	6 (20.7)	10 (34.5)	
IV	8	3 (10.3)	5 (17.2)	

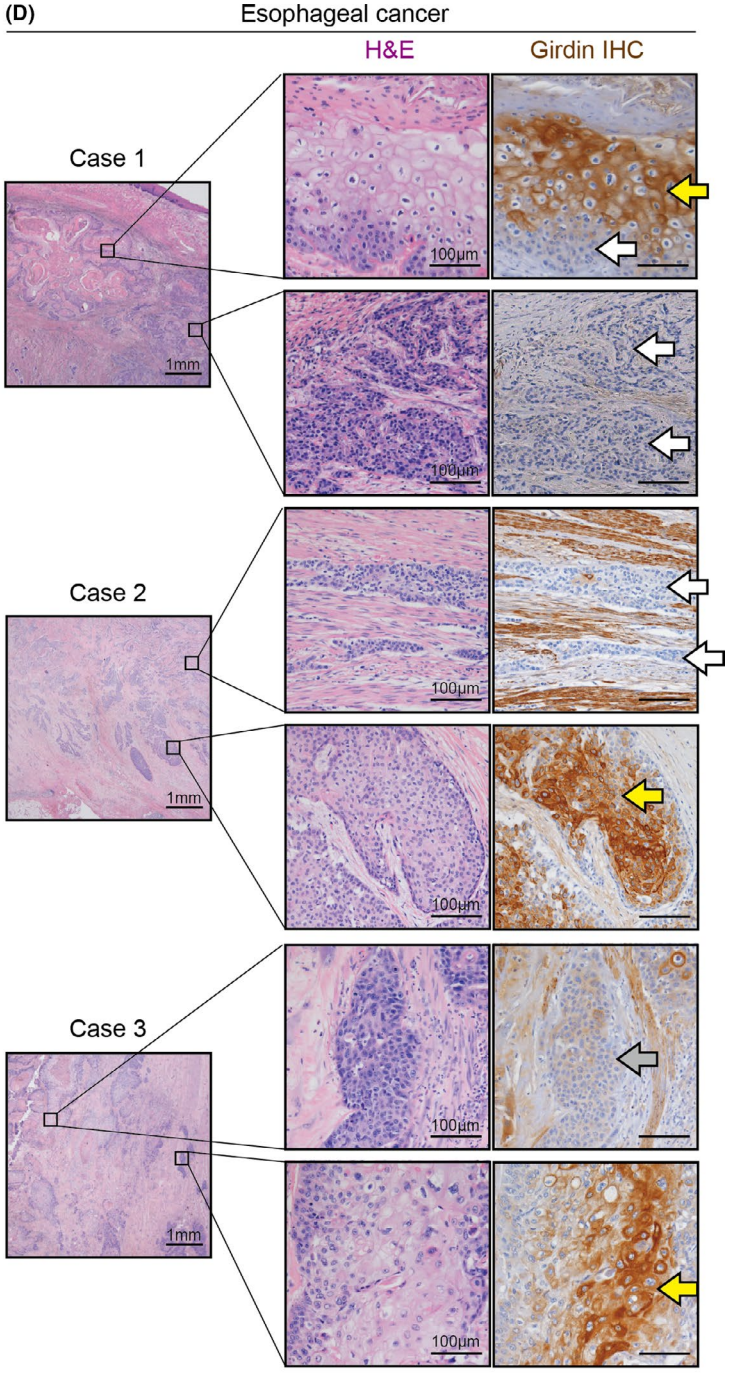
Another issue in evaluating the significance of Girdin expression in cancer progression is that its expression is not limited to tumor cells. That is, it is also found in endothelial cells and CAFs that constitute the tumor microenvironment, which confounds the interpretation of data obtained from mRNA extracted from whole tumors.[26,27] Indeed, in contrast to previous studies that showed a correlation of high Girdin expression in breast cancer cells to the poor outcome of the patients,[28] our previous study showed that Girdin activation in CAFs did not correlate with patient outcomes.[27] Interestingly, another study showed that

FIGURE 1 Prognostic value and intratumoral heterogeneity of Girdin expression in esophageal cancer. A-C, Comparison of the overall survival of Girdin-high and -low esophageal cancer samples available in The Cancer Genome Atlas (TCGA) database. Ninety-six cases were classified according to Girdin expression, and overall survival of the cases was plotted by Kaplan-Meier analysis. Cut-off values of 25%, 50%, and 75% were selected and used to classify tumors as Girdin-low or -high. D, Representative images of tissue sections of three independent cases with esophageal cancer stained by Girdin Ab. The presence of Girdin-negative cells (white arrows) and Girdin-positive cells (yellow arrows) indicates intratumoral heterogeneity of Girdin expression. IHC, immunohistochemistry. E, Comparison of the overall survival of Girdin-high and -low esophageal cancer cases, who were diagnosed in Nagoya University Hospital. Biopsy samples taken from 29 cases were classified according to Girdin expression following the scoring system shown in the left panel, and overall survival of cases was plotted by Kaplan-Meier analysis. HR, hazard ratio









(E)

Biopsy sample (Pre-radiation samples)

Intensity score (IS)	
Score 0: Negative	Girdin low
Score 1: Weakly positive	
Score 2: Positive	Girdin high
Score 3: Strongly positive	

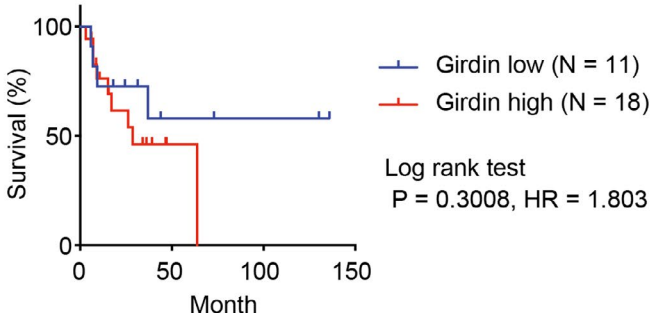


FIGURE 2 High Girdin expression is associated with increased vulnerability of cancer cells to ultraviolet C (UVC)-mediated DNA damage. A, KYSE140 and KYSE150 cell lines were exposed to UVC (100 J/m^2) and incubated for 3 h, followed by western blot analysis with the indicated Abs. B, C, Representative images of colonies of KYSE140 and KYSE150 cells formed 6 d after UVC irradiation are shown (B). Circles indicate locations of colonies. In (C), the surviving fractions, which were calculated by dividing the numbers of colonies by the numbers of seeded cells that were multiplied by plating efficiencies in three independent experiments, were evaluated and quantified. D, Schematic illustration of the generation of Girdin overexpression (OE) HeLa cells by the CRISPR/sgRNA-directed synergistic activation mediator system. TSS, transcription start site of the *CCDC88A* gene that encodes Girdin. E, Control (C) and Girdin OE HeLa cells were exposed to UVC irradiation (100 or 20 J/m^2) for the indicated periods, followed by western blot analysis. F, G, Control and Girdin OE HeLa cells were exposed to UVC irradiation for 7 d, followed by colony formation assay. The surviving fractions in three independent experiments were evaluated and quantified

Girdin is also expressed by BTSCs derived from human glioblastomas and is involved in the maintenance of BTSC stemness.[29] Girdin interacts with the 4F2 heavy chain, a subunit of multiple amino acid transporters, to negatively regulate amino acid signaling involving mTORC1, further showing the complexity of Girdin function.[30] Consistent with these findings, a recent study showed that mTORC1 signaling is suppressed in BTSCs rather than activated as nontumor stem cells in human gliomas,[31] implying that Girdin might contribute to the resistance of BTSCs to existing therapeutics by suppressing their metabolism.

In the present study, we examined the effect of Girdin expression on cancer cells' sensitivity to cytotoxic therapeutics. We selected UVC-induced DNA damage as a model of radiation therapy. We first found that high Girdin expression was associated with an increased sensitivity of cancer cells to UVC-mediated DNA damage. Interestingly, migratory cells, which are known to show significant resistance to DNA damage-induced apoptosis, became prone to that by Girdin overexpression. This result suggested that high Girdin expression counteracts or eliminates the DNA damage-protective effect of Girdin-mediated cell migration. Finally, we attempted to address the mechanism of this observation by identifying a novel role of Girdin in cell cycle regulation. These data suggested the presence of complex positive and negative roles of Girdin in cancer progression that depended on the cancer type and therapeutic context. These finding should be considered in the development of therapeutics that target pathways involving Girdin.

2 | MATERIALS AND METHODS

2.1 | Human tissue samples

Biopsy and surgically resected esophageal tissue samples from 28 esophageal squamous cell carcinoma patients, who had provided informed consent, were obtained at Nagoya University Hospital from 2006 to 2017 (Table 1). This study was carried out in accordance with the Helsinki Declaration for Human Research and approved by the Ethics Committee of Nagoya University Graduate School of Medicine (approval no. 2017-0127).

2.2 | Antibodies and reagents

The following Abs were used in this study: anti-Girdin (R&D Systems), anti-Girdin (IBL), anti-Girdin phospho S1647 (ECM Biosciences), anti-histone H3 (1B1B2) (Cell Signaling Technology), anti-histone H3 phospho S10 (Abcam), anti-histone H3 phospho S28 (Abcam), anti-cleaved PARP1 (Abcam), anti-cleaved PARP1 (Cell Signaling Technology), anti-Rb phospho Ser795 (New England BioLabs), anti-Rb (4H1) (Cell Signaling Technology), anti-p53 (Cell Signaling Technology), anti-p53 phospho S15 (Cell Signaling Technology), anti-p53 phospho S46 (Cell Signaling Technology), anti-Mad2 (C-10) (Santa Cruz Biotechnology), anti-α-tubulin (Sigma-Aldrich), anti-γ-tubulin (Sigma-Aldrich), Alexa Fluor 488 goat anti-mouse IgG (Thermo Fisher Scientific), Alexa Fluor 488 goat anti-rabbit IgG (Thermo Fisher Scientific), rabbit anti-sheep IgG (H + L), Human SP ads-HRP (Southern Biotech), and rabbit anti-rat IgG H&L (HRP) (Abcam) Abs.

2.3 | Cell lines and cell culture

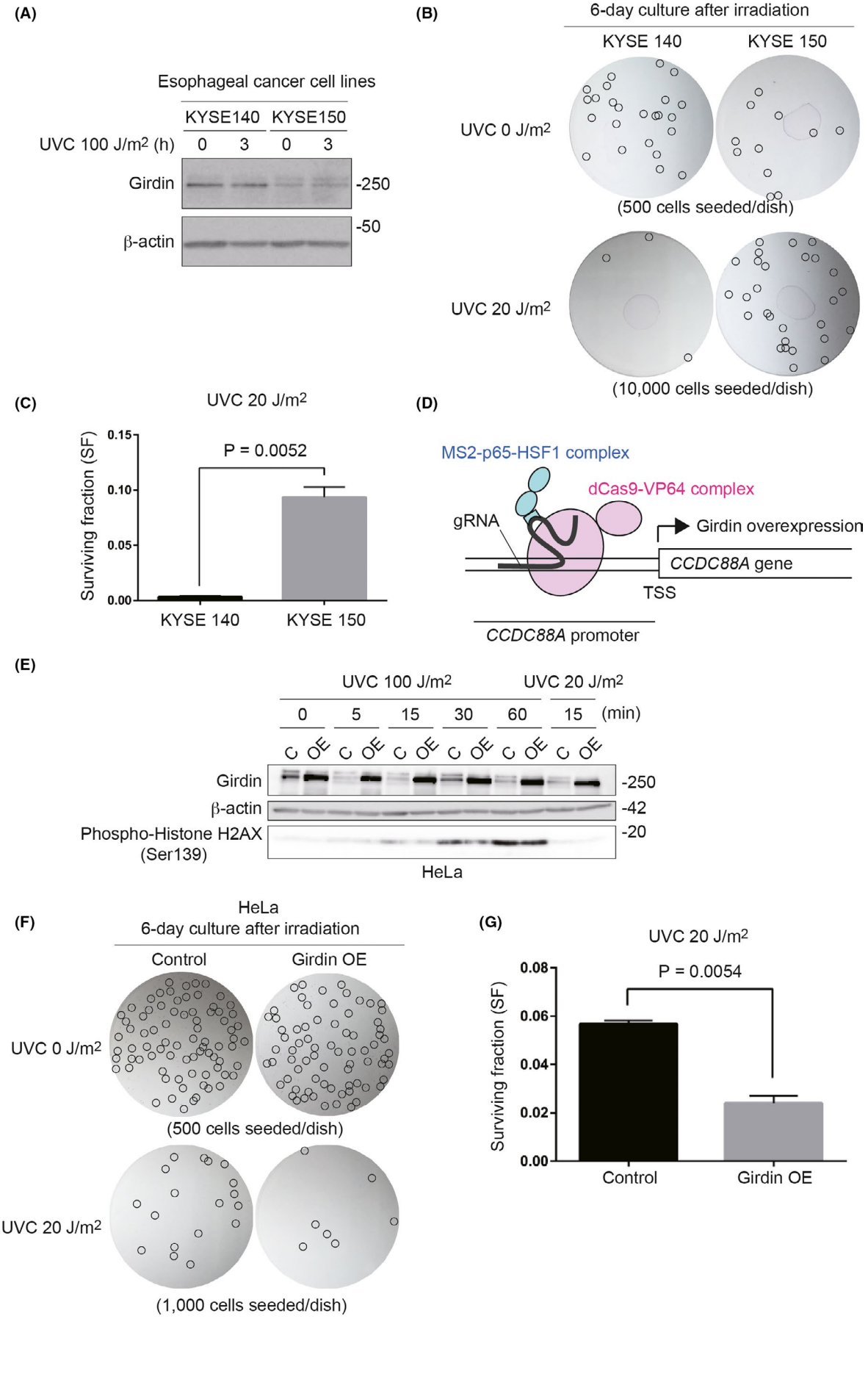
KYSE140 and KYSE150 cell lines[32] were purchased from the JCRB Cell Bank and cultured in Ham's F-12 Nutrient Mix, GlutaMAX medium (Gibco 31765035; Thermo Fisher Scientific) supplemented with 5% FBS (Gibco 10270-106). The HeLa cell line was purchased from ATCC. HEK293T cells were purchased from Invitrogen. HeLa and HEK293T cells were cultured in DMEM (#08458-16; Nacalai Tesque) supplemented with 10% FBS. Cells were cultured at 37°C in 5% CO_2 humidified air. The authenticity of HeLa cells (STR profile analysis) was verified by BEX Co., Ltd. Cell lines were routinely tested for mycoplasma contamination by staining with DAPI every 3 months.

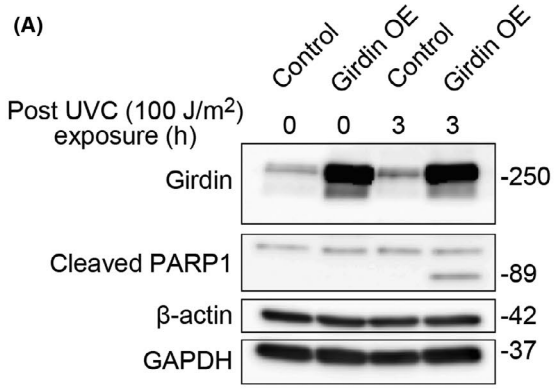
2.4 | Induction of DNA damage by UVC radiation

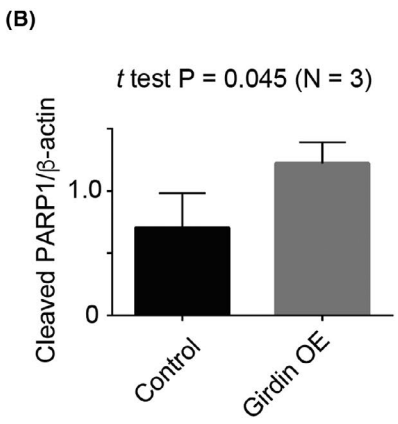
The culture medium was aspirated, and the cells were exposed at a dose of 20 or 100 J/m^2 of UVC radiation with Microprocessor-Controlled UV Crosslinkers (Spectroline) or were mock treated. Following the exposure, fresh medium was added, and the cells were incubated at 37°C with 5% CO_2 for indicated periods of time.

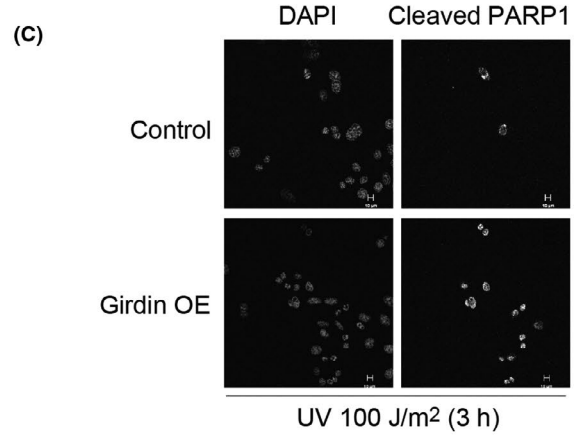
2.5 | Establishment of a cell line that stably overexpresses Girdin

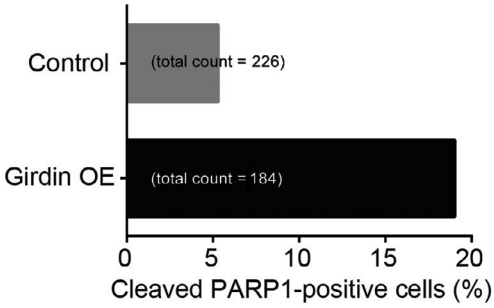
A HeLa cell line that stably overexpresses Girdin through the endogenous *CCDC88A* promoter was established by the

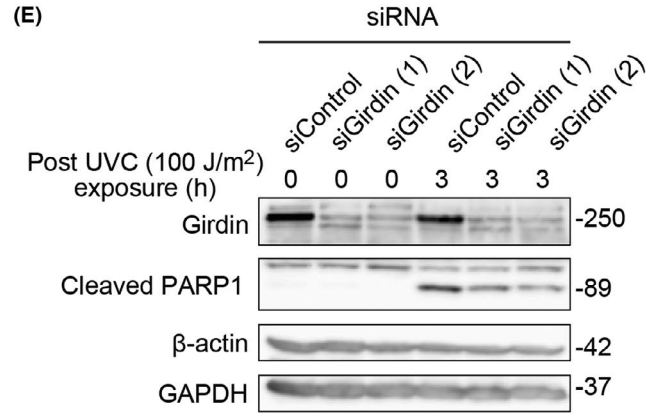

(A)
Esophageal cancer cell lines
KYSE140 KYSE150
UVC 100 J/m2 (h) 0 3 0 3
Girdin
-250
β-actin
-50
(B)
6-day culture after irradiation
KYSE 140 KYSE 150
UVC 0 J/m2
(500 cells seeded/dish)
UVC 20 J/m2
(10,000 cells seeded/dish)
(C)
UVC 20 J/m2
Surviving fraction (SF)
0.15
0.10
0.05
0.00
P = 0.0052
KYSE 140 KYSE 150
(D)
MS2-p65-HSF1 complex
dCas9-VP64 complex
gRNA
Girdin overexpression
CCDC88A gene
TSS
CCDC88A promoter
(E)
UVC 100 J/m2
UVC 20 J/m2
0 5 15 30 60 15 (min)
C OE C OE C OE C OE C OE C OE
Girdin
-250
β-actin
-42
Phospho-Histone H2AX (Ser139)
-20
HeLa
(F)
HeLa
6-day culture after irradiation
Control Girdin OE
UVC 0 J/m2
(500 cells seeded/dish)
UVC 20 J/m2
(1,000 cells seeded/dish)
(G)
UVC 20 J/m2
Surviving fraction (SF)
0.08
0.06
0.04
0.02
0.00
P = 0.0054
Control Girdin OE

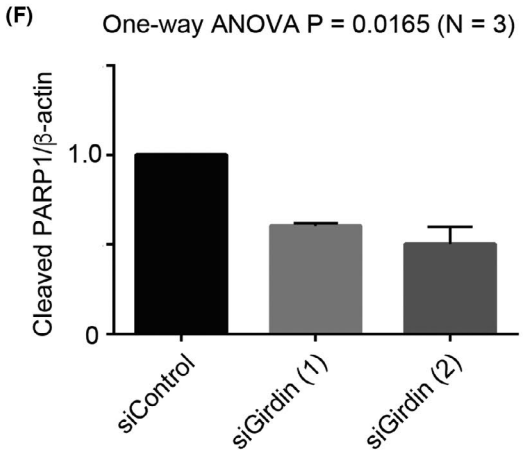

(A)
Control
Girdin OE
Control
Girdin OE
Post UVC (100 J/m2) exposure (h)
0 0 3 3
Girdin
-250
Cleaved PARP1
-89
β-actin
-42
GAPDH
-37

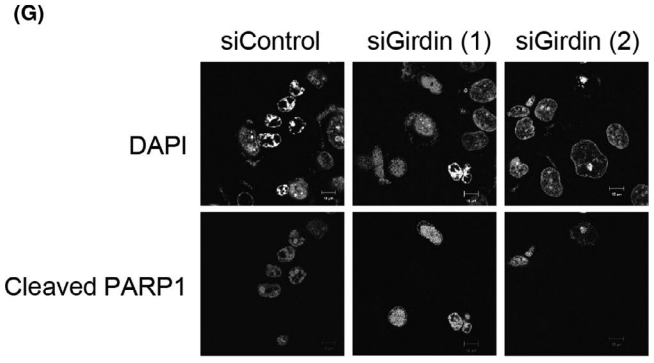

(B)
t test P = 0.045 (N = 3)
Cleaved PARP1/β-actin
1.0
0
Control
Girdin OE


(C)
DAPI
Cleaved PARP1
Control
Girdin OE
UV 100 J/m2 (3 h)


(D)
Control
(total count = 226)
Girdin OE
(total count = 184)
0 5 10 15 20
Cleaved PARP1-positive cells (%)


(E)
siRNA
siControl
siGirdin (1)
siGirdin (2)
siControl
siGirdin (1)
siGirdin (2)
Post UVC (100 J/m2) exposure (h)
0 0 0 3 3 3
Girdin
-250
Cleaved PARP1
-89
β-actin
-42
GAPDH
-37


(F)
One-way ANOVA P = 0.0165 (N = 3)
Cleaved PARP1/β-actin
1.0
0
siControl
siGirdin (1)
siGirdin (2)


(G)
siControl
siGirdin (1)
siGirdin (2)
DAPI
Cleaved PARP1

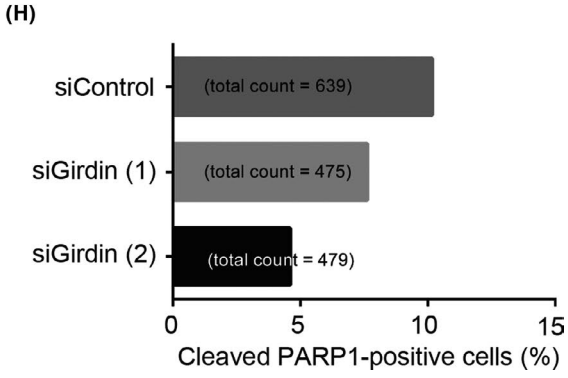

(H)
siControl
(total count = 639)
siGirdin (1)
(total count = 475)
siGirdin (2)
(total count = 479)
0 5 10 15
Cleaved PARP1-positive cells (%)

FIGURE 3 High Girdin expression is associated with increased apoptosis of HeLa cells after ultraviolet C (UVC) irradiation. A, B, Control and Girdin overexpression (OE) HeLa cells were exposed to UVC irradiation (100 J/m^2) and incubated for 3 h, followed by western blot analysis (A). The intensity of cleaved poly(ADP-ribose) polymerase 1 (PARP1) signals was normalized against β-actin, and the data from three independent experiments is shown (B). C, D, Control and Girdin OE HeLa cells were exposed to UVC irradiation (100 J/m^2) and incubated for 3 h, followed by immunohistochemistry (IHC) for cleaved PARP1 (C). The percentage of cleaved PARP1-positive cells was determined, and the data from three independent experiments is shown (D). E-H, HeLa cells transfected with either control or Girdin siRNA were exposed to UVC irradiation and incubated for 3 h, followed by western blot analysis (E, F) and IHC for cleaved PARP1 (G, H)

CRISPR/sgRNA-directed SAM system.[33] The sgRNA was designed using the CRISPR design website (http://sam.genome-engineering.org/database_request/) and the guide sequence (5′-TTTCTTCTCCCACAATCCAG-3′) was selected and cloned into the lenti-sgRNA (MS2)-pure vector (#7379;, Addgene) using the Golden-Gate sgRNA cloning protocol described on http://sam.genome-engineering.org/protocols/. Sequencing for the constructed plasmid was done before use. Lentiviruses expressing dCas9-VP64 and MS2-P65-HSF1 were generated by transfection of the packaging plasmids psPAX2 (#12260; Addgene), pMD2.G (#12259; Addgene), and lenti dCAS-VP64_Blast (#61425; Addgene) or lenti MS2-P65-HSF1_Hygro (#61426; Addgene) into HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific). HeLa cells were infected with the viruses, followed by selection in the presence of blasticidin (Wako) and hygromycin (Invitrogen). Afterwards, the cells expressing the SAM components were transduced with lentiviruses expressing the *CCDC88A* sgRNA. After 48 hours of infection, the cells were selected with puromycin (Sigma-Aldrich) for 14 days, replacing the puromycin every 3 days. All of the experiments using lentivirus vectors were undertaken in a BSL2 environment approved by Nagoya University.

2.6 | Cell synchronization

Cells were treated with 60 ng/mL nocodazole (Sigma-Aldrich) for 16 hours. The mitotic cells were collected by mechanical shake-off,[34] washed with PBS, and seeded on plates. The cells were harvested at different time points for cell cycle analysis. For cell synchronization at the G_1/S boundary, cells were treated with 2 mmol/L thymidine for 15 hours, washed with PBS, grown for 10 hours in a regular medium, and then treated again with 2 mmol/L thymidine for 15 hours, followed by washing with PBS. This marks time 0, after which the cells were collected at the indicated times for analysis.

2.7 | Flow cytometric analysis

For the quantitation of mitotic cells, cells were probed with anti-histone H3 (phospho S10) Ab (Abcam). Cells were collected and incubated with anti-histone H3 (phospho S10) Ab for 1 hour at room temperature in the dark. Cells were fixed with 4% paraformaldehyde for 15 minutes, followed by resuspension in solution with Alexa Fluor 488-conjugated rabbit anti-mouse IgG (Thermo Fisher Scientific) for 30 minutes at room temperature in the dark. Data acquisition was carried out using FACS Canto2 (BD Biosciences) and results were analyzed with FlowJo software (BD Biosciences).

To quantitate the DNA content by flow cytometry, the PI flow cytometry kit (Abcam) was used according to the manufacturer's instructions. Cells were collected and fixed by the addition of 66% ethanol at 4°C. On the following day, cells were treated with PI and RNase at 37°C for 30 minutes. The modeling of DNA content histograms was done by using ModFitLT software (Verity Software House).

2.8 | Statistical analysis

Significant differences were determined by two-tailed *t* tests for comparison of the means between two sets of data, or one-way ANOVA for comparison of the means among three or more sets of data using GraphPad Prism (GraphPad Software). All graphs represent mean ± SD. $P < .05$ was regarded as significant. For the analysis of overall survival of the patients, data were plotted using Kaplan-Meier analysis in GraphPad Prism and the significant differences were evaluated with a log rank test.

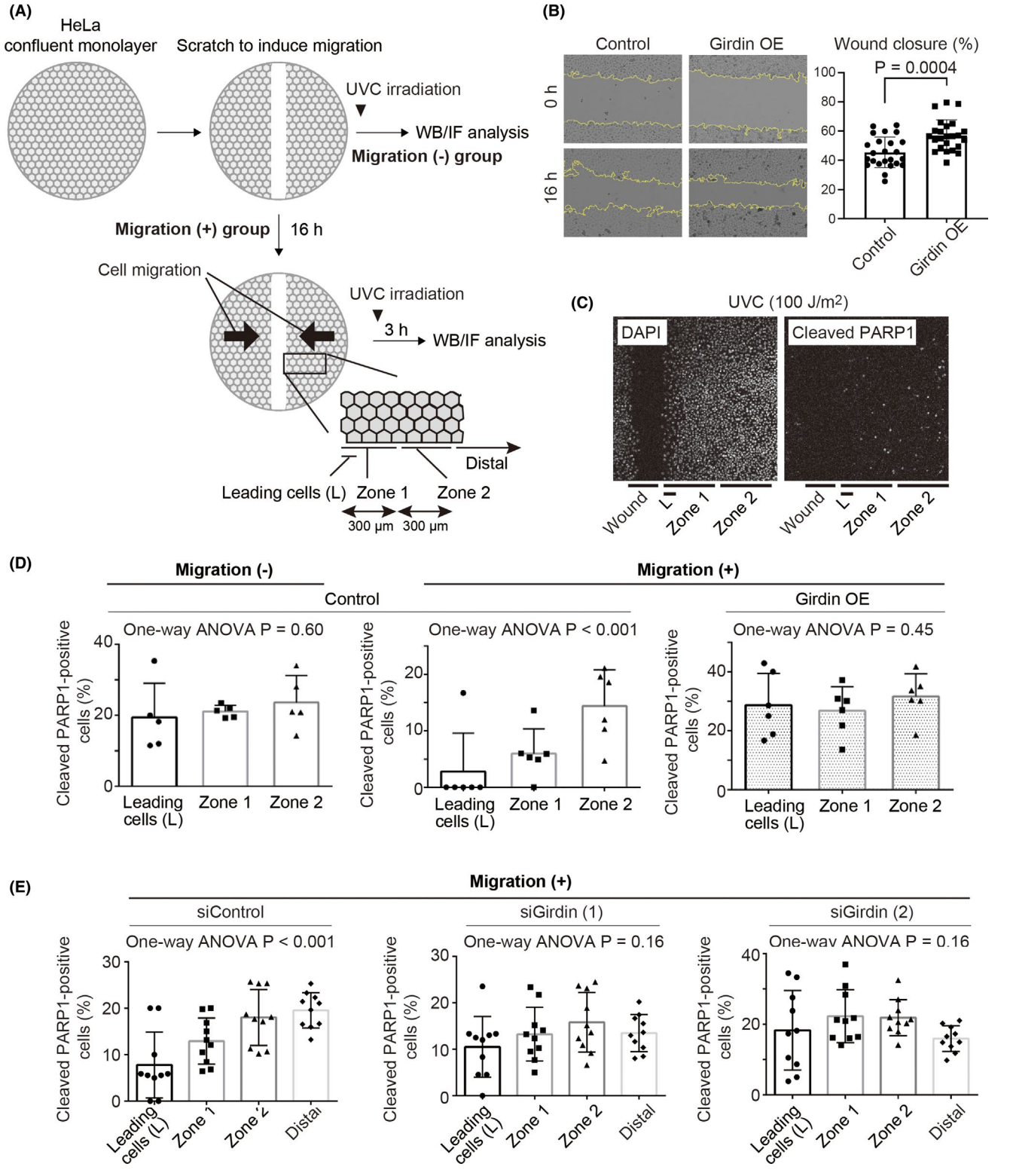
2.9 | Data analysis, histology, cell biology, biochemistry, and real-time quantitative PCR

Detailed protocols for TCGA database analysis, immunohistochemistry, cell biological and biochemical experiments, and real-time quantitative PCR are described in Document S1.

3 | RESULTS

3.1 | Prognostic values of Girdin expression levels in esophageal cancer: variations and inconsistencies

Previous studies showed a significant correlation between Girdin (*CCDC88A*) mRNA expression levels and poor clinical outcomes in esophageal cancer.[17] Those data were consistent with other studies showing correlations of Girdin expression with poor outcomes of many types of cancer.[18-21,28] Our analysis of esophageal cancer cases in the TCGA dataset, however, resulted in variable Kaplan-Meier survival curves and log rank *P* values that fluctuated depending on cut-off values (Figure 1A-C). Girdin gene expression



levels correlated with favorable prognosis of the patients with an empirically determined cut-off value (75%). However, with other values (25% and 50%), they showed no correlation with the prognosis.

We used IHC to examine Girdin protein expression in tissue sections of surgically resected tumors obtained from esophageal cancer patients in our institution (Table 1). The results showed varying degrees of Girdin expression between cancer cells (Figure 1D). We also examined Girdin expression in biopsy samples taken from the patients before treatment and stratified their outcome by a scoring system based on Girdin expression levels determined by IHC, but found no correlation between the patients' survival and Girdin expression (Figure 1E). Interestingly, even in the same tumor, Girdin expression was different between different tumor lesions,

FIGURE 4 Girdin-mediated sensitization of HeLa cells to ultraviolet C (UVC) eliminates the protective effect of cell migration against DNA damage. A, Schematic diagram of the experimental protocol to examine the effect of UVC irradiation on migrating cells. Monolayers of confluent HeLa cells on glass-based dishes (left, top) were scratched to initiate sheet migration into the wound (right, top), incubated for 16 h, exposed to UVC irradiation, and incubated for 3 h (right, bottom). After fixation and immunofluorescent (IF) staining, the leading cells in the front line of migrating cell group (L) and cells included in zones 1 and 2 were examined for cleaved poly(ADP-ribose) polymerase 1 (PARP1) expression. WB, western blot. B, Monolayers of control and Girdin overexpression (OE) HeLa cells were scratched to induce migration for 16 h, and the areas of wounds were measured by ImageJ software and quantified. The percentages of wound closure in 24 images taken from four independent experiments were measured and quantified. C, A monolayer of HeLa cells was scratched to induce migration, followed by UVC exposure and IF staining, showing an uneven distribution of cleaved PARP1-positive cells across the cell groups. D, E, Monolayers of the indicated HeLa cells were subjected to UVC irradiation after cell migration for 16 h (migration (+)), or just immediately after scratching (migration (–)), followed by IF staining for cleaved PARP1 and quantification

as often observed for other cancer cell markers (Figure 1D). We speculated that the intratumor heterogeneity of Girdin expression and its involvement in cancer cell sensitivity to anticancer therapeutics could confound the analysis based on simple measurement of Girdin mRNA and protein expression levels in whole tumors.

3.2 | High Girdin expression is associated with a high sensitivity to UVC irradiation

Given the heterogeneous expression of Girdin between tumor cells and its unclear prognostic significance in esophageal cancer, we speculated that Girdin might be involved in tumor cell sensitivity to anticancer therapeutics. A previous study had reported that Girdin expression regulates the sensitivity of colon cancer cells to the chemotherapeutic drug oxaliplatin.[35] In this study, we examined the significance of Girdin expression in cancer cells' sensitivity to DNA damage induced by radiotherapy. As a model of radiotherapy, we subjected cancer cells to high-dose UVC irradiation (20-100 J/m^2) that produces pyrimidine dimers and double-stranded DNA breaks that contribute to the induction of apoptotic cell death.[36] We first investigated two esophageal cancer cell lines, KYSE140 and KYSE150,[32] that showed high and low levels of endogenous Girdin, respectively, and found that Girdin expression was not affected by UVC irradiation in either cell line (Figure 2A). We found that the number of cells that survived after UVC exposure, which was evaluated by colony-forming capacity, was higher in KYSE150 cells than KYSE140 cells (Figure 2B,C). The data implied that Girdin expression could be associated with cancer cell sensitivity to UVC exposure.

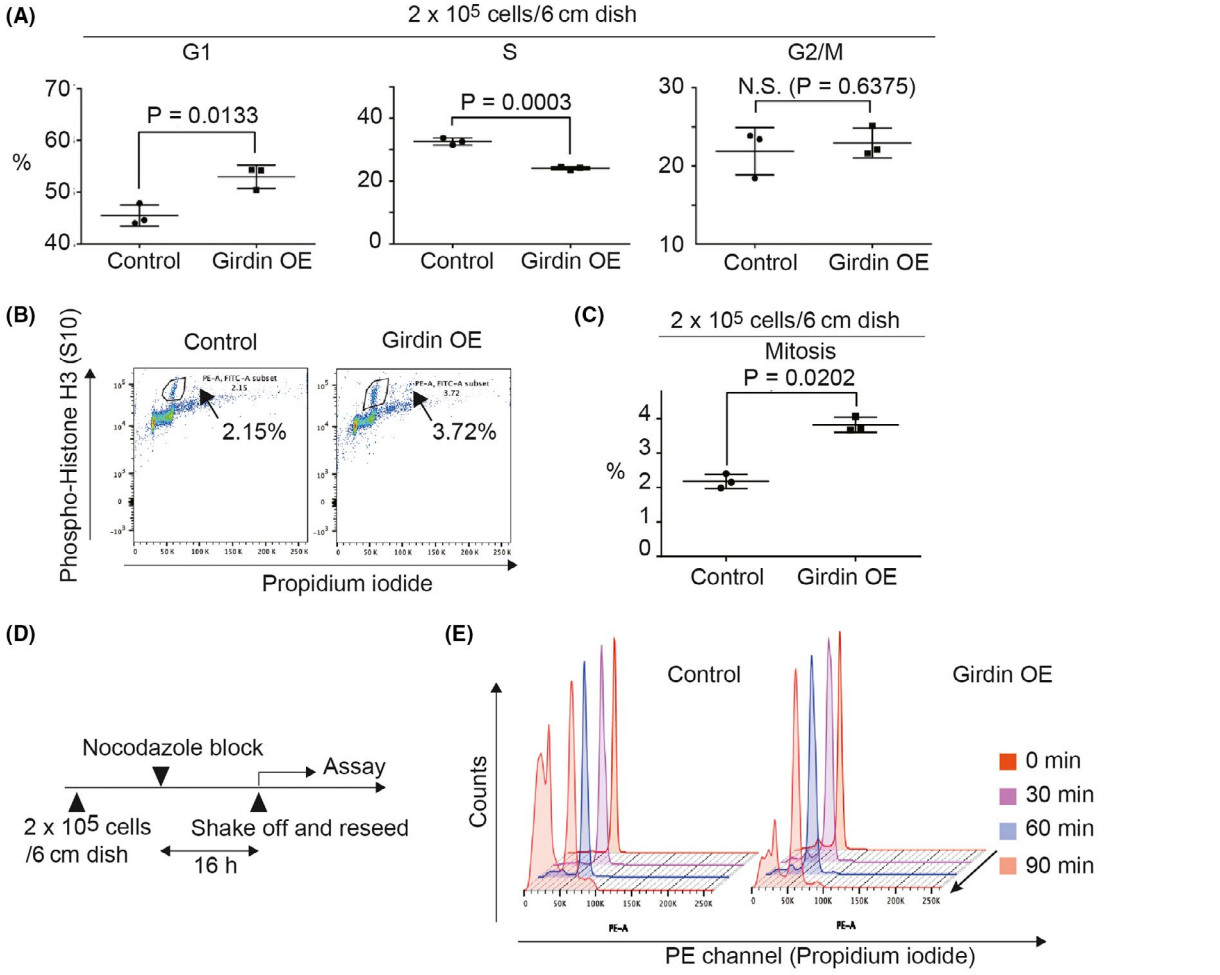
To investigate whether Girdin expression conferred sensitivity to UVC exposure, we attempted to exogenously overexpress Girdin by using lentiviral and retroviral expression systems. However, the large size of Girdin cDNA (5500 bp) made it difficult to achieve high viral packaging efficiency. We therefore adopted the SAM system[33] that permitted us to express an engineered CRISPR/Cas9 complex to augment endogenous Girdin expression by transcriptional activation of the Girdin gene (*CCDC88A*) locus in HeLa human cervical cancer cells (Figure 2D). Western blot analysis showed successful OE of endogenous Girdin in HeLa cells (Figure 2E). Although H2AX phosphorylation was comparable between control and Girdin OE cells, Girdin OE cells showed high sensitivity to DNA damage as shown by a decreased capacity in colony formation after UVC irradiation (Figure 2E-G).

3.3 | HeLa cells overexpressing Girdin are vulnerable to UVC irradiation

Using western blot analysis of UVC-irradiated HeLa cells that expressed high levels of endogenous Girdin, we found that they expressed higher levels of cleaved PARP1, a marker of apoptosis, than did control cells (Figure 3A,B). This was confirmed by IF staining of control and Girdin OE HeLa cells with an Ab specific for cleaved PARP1 (Figure 3C,D). Conversely, the knockdown of Girdin by siRNA-mediated RNAi resulted in a significant decrease in cleaved PARP1 levels compared to control cells after UVC irradiation (Figure 3E-H). Exogenous overexpression of Girdin in KYSE150 cells also resulted in an increase in cleaved PARP1 levels after UVC irradiation (Figure S1A,B). Furthermore, KYSE140 cells, which express a higher level of Girdin, showed a higher sensitivity to UV exposure than KYSE150 cells with a low Girdin expression (Figure S1C). These data suggested that Girdin expression is associated with the sensitivity of cancer cells to UVC-mediated DNA damage and subsequent cell apoptosis.

3.4 | Girdin-mediated increase in UVC sensitivity eliminates the DNA damage-protective effect of cell migration

In contrast to proliferating cells, migrating cells are significantly resistant to DNA damage-induced apoptosis.[24,25] Given the well-established roles of Girdin in cell migration,[9,11,15,16,26] it was plausible to speculate that the observed effect of Girdin expression on UVC-induced apoptosis was attributed to an altered cell migratory response. To address this question, we scratched confluent monolayers of HeLa cells to induce directional cell migration, followed by UVC irradiation, western blot analysis, and IF staining (Figure 4A). In a control experiment, the cells were fixed immediately after the scratch and UVC irradiation without inducing cell migration (Figure 4A). Throughout the experiments, the cells were cultured with a low concentration of FBS (0.5%) in order to minimize their proliferation.


(A)
2 x 10^5 cells/6 cm dish
G1
S
G2/M
P = 0.0133
P = 0.0003
N.S. (P = 0.6375)
%
Control
Girdin OE
(B)
Control
Girdin OE
2.15%
3.72%
Phospho-Histone H3 (S10)
Propidium iodide
(C)
2 x 10^5 cells/6 cm dish
Mitosis
P = 0.0202
%
Control
Girdin OE
(D)
Nocodazole block
Assay
2 x 10^5 cells /6 cm dish
Shake off and reseed
16 h
(E)
Control
Girdin OE
Counts
0 min
30 min
60 min
90 min
PE channel (Propidium iodide)

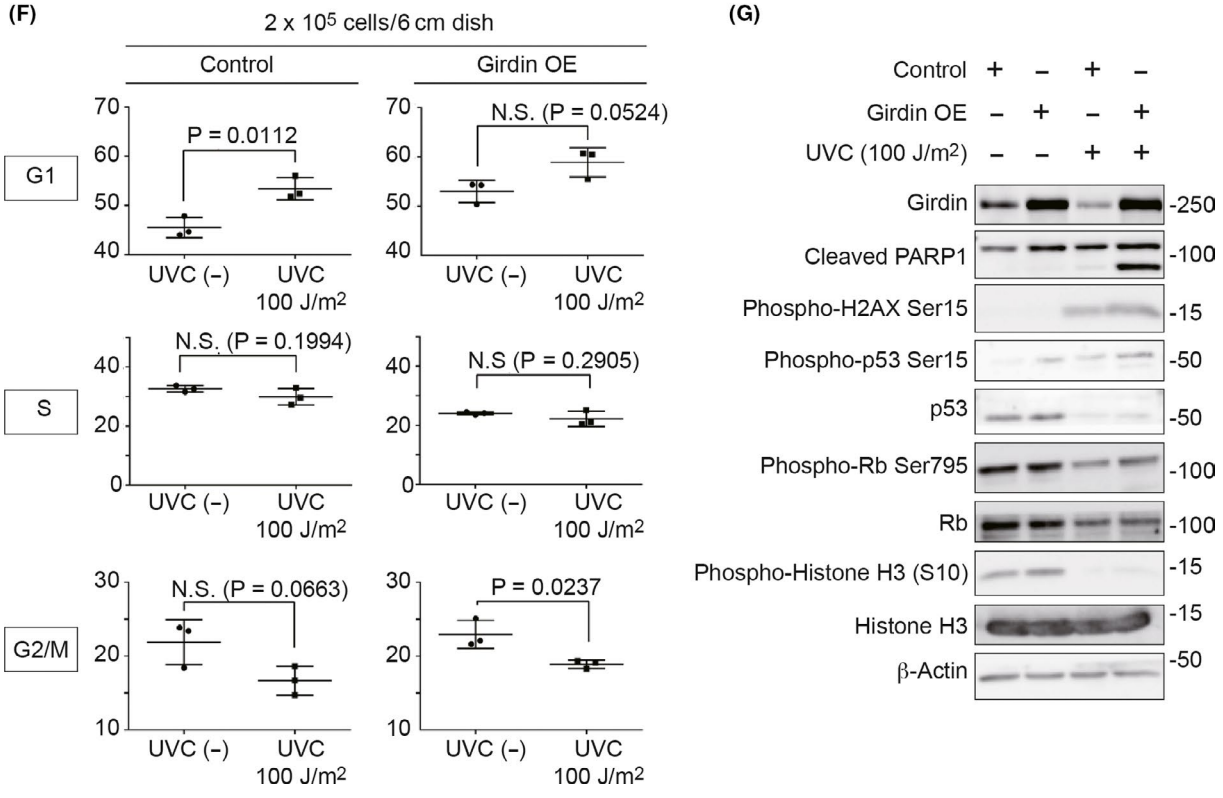

(F)
2 x 10^5 cells/6 cm dish
Control
Girdin OE
G1
S
G2/M
P = 0.0112
N.S. (P = 0.0524)
N.S. (P = 0.1994)
N.S (P = 0.2905)
N.S. (P = 0.0663)
P = 0.0237
UVC (–)
UVC 100 J/m2
(G)
Control
Girdin OE
UVC (100 J/m2)
Girdin
Cleaved PARP1
Phospho-H2AX Ser15
Phospho-p53 Ser15
p53
Phospho-Rb Ser795
Rb
Phospho-Histone H3 (S10)
Histone H3
β-Actin
-250
-100
-15
-50
-50
-100
-100
-15
-15
-50

FIGURE 5 Girdin overexpression (OE) HeLa cells show dysregulated cell cycle progression with prolonged G_1 and M phases. A, Flow cytometric analysis of nonsynchronized control and Girdin OE HeLa cells. Cell cycle phase is shown at top. B, C, Nonsynchronized control and Girdin OE HeLa cells were stained for phospho-histone H3 and then propidium iodide (PI), followed by flow cytometric analysis. Representative flow histograms depicting mitotic fraction defined by arrows are shown in (B), and the percentages of mitotic cells were quantified in (C). Results are expressed as the means ± SD of three independent experiments. D, E, HeLa cells were synchronized at M phase by incubating the cells with nocodazole at 60 ng/mL for 16 h, collecting mitotic cells by mitotic shaking and replating (D). Temporal changes in cell cycle distribution after replating were examined by flow cytometric analysis for PI stained cells (E). F, G, Nonsynchronized control and Girdin OE HeLa cells were exposed to ultraviolet C UVC irradiation (100 J/m^2) and incubated for 3 h, followed by cell cycle analysis by flow cytometric analysis of PI stained cells (F) and western blot analysis with the indicated Abs (G). N.S., not significant; Rb, retinoblastoma

Consistent with the known promigratory role of Girdin, Girdin OE cells showed more rapid migration as assessed by the closure of the wounds made by scratching of cell monolayers (Figure 4B). Interestingly, IF staining of migrating HeLa cells showed an uneven distribution of cleaved PARP1-positive cells in the scratched monolayers after UVC irradiation (Figure 4C). We therefore counted and quantified cleaved PARP1-positive apoptotic cells in three groups of migrating cells: leading cells in the front line of migrating cell groups (the first row of cells; L), the most anterior cells including the leading cells (300 μm in distance from the front line; zone 1), and cells behind the zone 1 cells (300-600 μm from the front line; zone 2) (Figure 4A,C). In migrating cells, but not nonmigrating cells, the frequency of apoptotic cells in the L group was significantly lower than those in zones 1 and 2, further confirming that migrating cells are resistant to DNA damage-induced apoptosis (Figure 4D, left and middle panels). Interestingly, Girdin OE cells showed similar proportions of apoptotic cells in L, zone 1, and zone 2 groups, and an increase in the numbers of apoptotic cells across all the groups (Figure 4D, right panel). Conversely, the knockdown of Girdin equalized the distribution of apoptotic cells without increasing the numbers of those cells across all the groups (Figure 4E). These data suggested that Girdin OE increases the sensitivity of cancer cells to UVC-induced DNA damage even when they migrate, and eliminates the DNA damage-protective effect of cell migration.

3.5 | Altered cell cycle distribution in Girdin OE cells

We next explored the mechanism by which high Girdin expression was linked to high sensitivity to DNA damage and subsequent apoptosis. To that end, we examined the cell cycle distribution of Girdin OE HeLa cells. Flow cytometric analysis of nonsynchronized cells stained with PI showed that Girdin OE cells accumulated in G_1 phase with a lower fraction in the S phase in both HeLa and the esophageal cancer cell line KYSE150 (Figures 5A and S1D,E). This was confirmed by another set of experiments, in which we analyzed the cell cycle distribution using EdU incorporation and PI staining (Figure S2A,B). WST-1 assay showed statistically significant but marginal differences in cell proliferation between control and Girdin OE cells (Figure S2C), suggesting that Girdin OE perturbs cell cycle distribution without affecting the length of the cell cycle.

Further flow cytometric analysis showed that the number of mitotic cells identified based on PI staining and their reactivity with anti-phospho-histone H3 (Ser10) Ab was increased in Girdin OE cells compared to control cells (Figure 5B,C). This was confirmed in synchronized HeLa cells in which cells were treated with nocodazole (60 ng/mL) for 16 hours to generate a mitotic block, followed by shaking off to select for mitotic cells and replating them to release them from the block and induce progression to G_1 phase[34] (Figure 5D). Flow cytometric analysis showed that the percentage of cells that remained arrested in M phase 90 minutes after the release was higher in Girdin OE cells than in control cells (Figure 5E). The mitotic delay in Girdin OE cells compared to control cells was also manifest when the cells were arrested by double thymidine block at the G_1/S boundary and then released to reach a peak at mitosis (Figure S3A,B). These data showed that high expression of Girdin was associated with the dysregulation of the cell cycle distribution, with longer G_1 and M phases.

3.6 | Basal dysregulation of the cell cycle and p53 activation could increase apoptosis in Girdin OE cells after UVC irradiation

We next found that the exposure of control cells to UVC irradiation resulted in the accumulation of cells in the G_1 phase of the cell cycle (Figure 5F), consistent with previous studies.[37] Although this effect was also observed in Girdin OE cells, the most remarkable change found in Girdin OE cells after UVC irradiation was a decrease in the number of cells in the G_2/M phase (Figure 5F). The data suggested that the longer M phase in Girdin OE cells contributed to their vulnerability to DNA damage and apoptosis. Previous studies have shown that p53 becomes stabilized and activated after prolonged mitosis and mitotic arrest to inhibit cell growth.[38] Consistent with this, western blot analysis showed the activation of p53, but not that of another tumor suppressor, Rb, in Girdin OE cells before UVC irradiation, which became more apparent after the irradiation (Figure 5G). Given the established roles of p53 in apoptosis following DNA damage, it is plausible that prolonged mitosis and concomitant p53 activation found in Girdin OE cells could sensitize cells to subsequent UVC-induced DNA damage. The activation of p53 was also found in the esophageal cancer cell lines KYSE140 and KYSE150 exposed to UVC (Figure S1C). However, it was not clear whether p53 activation levels correlated with Girdin expression in those cells, suggesting that Girdin-mediated sensitization of cancer cells to UVC irradiation might involve multiple mechanisms and not be simply explained by p53.

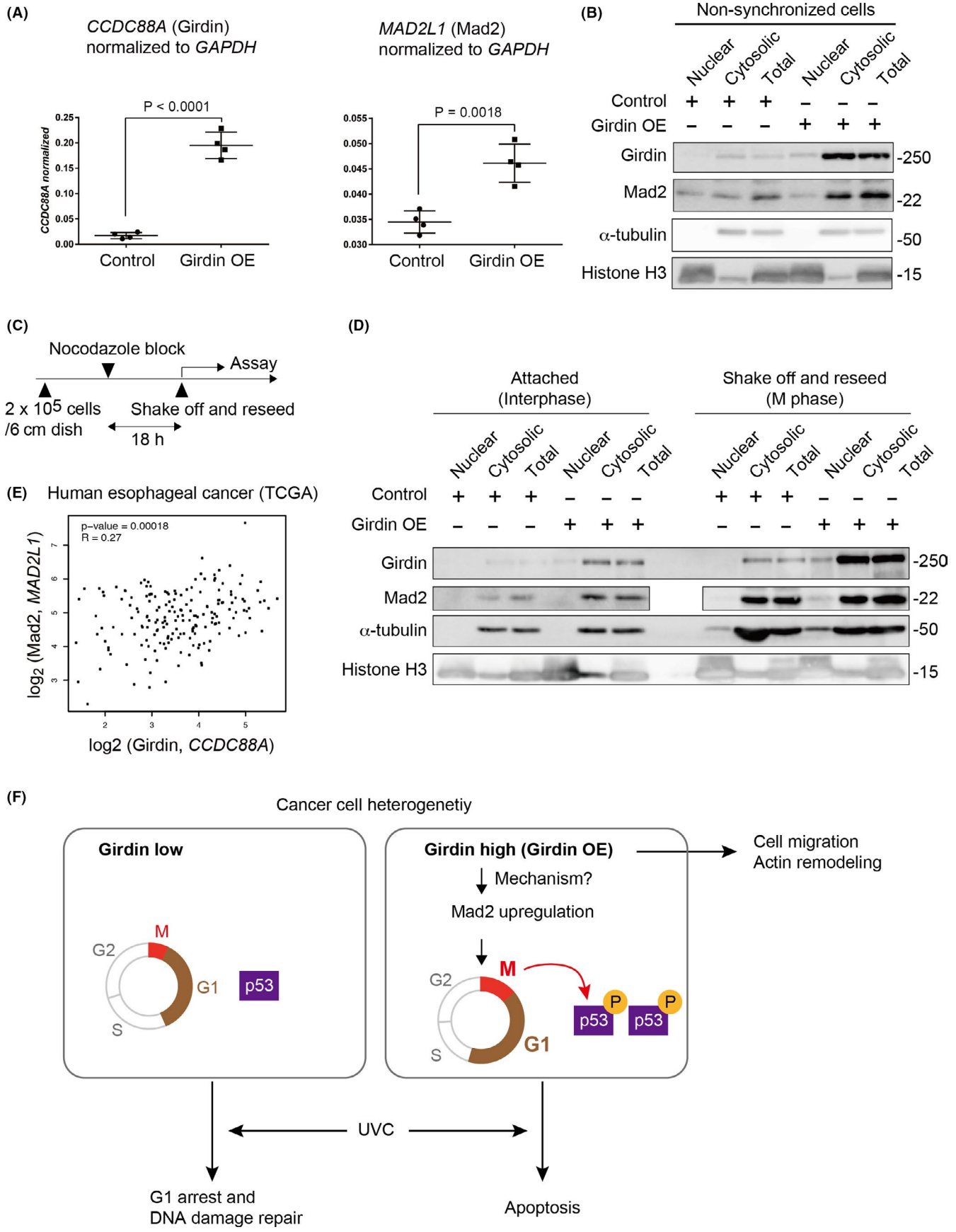

(A)
CCDC88A (Girdin) normalized to GAPDH
P < 0.0001
CCDC88A normalized
Control
Girdin OE
MAD2L1 (Mad2) normalized to GAPDH
P = 0.0018
Control
Girdin OE
(B)
Non-synchronized cells
Nuclear
Cytosolic
Total
Control
Girdin OE
Girdin
Mad2
α-tubulin
Histone H3
-250
-22
-50
-15
(C)
Nocodazole block
Assay
2 x 10^5 cells /6 cm dish
Shake off and reseed
18 h
(D)
Attached (Interphase)
Shake off and reseed (M phase)
(E) Human esophageal cancer (TCGA)
p–value = 0.00018
R = 0.27
log2 (Mad2, MAD2L1)
log2 (Girdin, CCDC88A)
(F)
Cancer cell heterogenetiy
Girdin low
G2
M
G1
S
p53
Girdin high (Girdin OE)
Mechanism?
Mad2 upregulation
P
Cell migration
Actin remodeling
UVC
G1 arrest and DNA damage repair
Apoptosis

FIGURE 6 Upregulation of Mad2 in Girdin overexpression (OE) cells and expression correlation between Girdin and Mad2. A, mRNAs for Girdin (left) and Mad2 (right) isolated from control and Girdin OE cells were measured and quantified by quantitative PCR. B, Total cell lysates (Total) of control and Girdin OE HeLa cells were fractionated into nuclear and cytosolic fractions, followed by western blot analysis with the indicated Abs. C, D, HeLa cells were synchronized at M phase by incubating the cells with nocodazole at 60 ng/mL for 16 h, collecting mitotic cells by mitotic shake-off, and replating (C). Expression of Girdin and Mad2 was examined by western blot analysis after the fractionation of the attached cells and those in M phase (shake-off and reseed) (D). E, Correlation of Girdin and Mad2 expression in esophageal cancer samples (N = 180) available in The Cancer Genome Atlas (TCGA) database. F, Schematic model of the increased sensitivity to ultraviolet C (UVC) in cancer cells with high Girdin expression. Data shown here suggest that the OE of Girdin perturbs cell cycle distribution with prolonged G_1 and M phases and aberrant p53 activation but without affecting the length of the cell cycle, which leads to an increase in sensitivity to DNA damage. The present study showed that the upregulation of the spindle checkpoint protein Mad2 in Girdin OE cells could be involved in dysregulated cell cycle progression. However, the detailed mechanism behind the observed vulnerability of Girdin OE cells to UVC is not clear at present. Given the known function of Girdin in promoting cell migration and the theory of migration-proliferation dichotomy, the present data reveals the complexity of cellular responses to high Girdin expression and its role in cancer progression

3.7 | Girdin OE increases the expression level of Mad2, a mitotic spindle checkpoint protein, in HeLa cells

The phenotype observed in Girdin OE cells was not likely to be explained by any previously identified Girdin-interacting protein, including actin filaments, the cell polarity regulator Par-3, DISC1, or α subunits of trimeric G proteins,[9-11,39] or cellular processes that Girdin is involved in. It was intriguing to find an increase in Cdk5-dependent phosphorylation of Girdin in UVC-irradiated Girdin OE cells (Figure S4), but it was difficult to interpret the role of the Cdk5-Girdin pathway in the context of UVC-mediated apoptotic cell death and Girdin-mediated mitotic delay. We therefore searched for other mechanisms that involve Girdin to regulate cell cycle progression, and found that Mad2, a key component of the spindle checkpoint machinery that is crucial for anaphase onset in M phase,[40-42] was highly upregulated in Girdin OE cells compared to control cells at both the protein and mRNA levels (Figure 6A,B). It was noted that Mad2 expression was significantly increased in both nonsynchronized and synchronized Girdin OE cells (Figure 6B-D). Fractionation experiments showed that the Girdin OE-mediated increase in Mad2 expression was more prominent in the cytosolic fraction of cells in interphase but not M phase, suggesting a role of the aberrantly expressed Mad2 in dysregulating the progression of the cell cycle in interphase (Figure 6D). However, IF staining for tubulin proteins identified no apparent disorganization or alignment of the microtubules in interphase, but revealed that the number of metaphase or early anaphase cells that undergo multipolar division was increased in Girdin OE cells compared to control cells (Figure S5A,B). Thus, it could be possible that Girdin OE induces mitotic delay by interfering with Mad2-mediated regulation of the spindle checkpoint machinery. A correlation between Girdin and Mad2 expression was also observed in tissue samples of a human esophageal cancer cohort available from the TCGA database (Figure 6E). Although speculative, the data implied that Mad2 overexpression has a role in cell cycle dysregulation found in Girdin OE cells also in human cancer.

3.8 | No significant statistical correlation was found between Girdin expression and response to radiation therapy in esophageal cancer

The findings described above were obtained with cultured cancer cells. To extend those data, we asked whether Girdin expression was correlated with the response to radiotherapy in cancer patients. Given the availability of both pre- and postradiation tissue samples in a cohort of esophageal cancer patients (N = 28) in our institution, we used IHC to examine Girdin expression in the biopsies and surgical samples that were obtained both pre- and postradiation, respectively (Table 1, Figures 1E and S6). We adopted several scoring systems, including one developed in a previous study,[17] to evaluate Girdin expression levels in human esophageal cancer. The data from both pre- or postradiation samples, however, did not show a correlation between Girdin expression levels and histopathological evaluation of the response to radiation therapy (Figure S6). Although not conclusive (given the limited number of samples), it seems that elevated Girdin expression alone does not confer sensitivity to radiotherapy, at least in esophageal cancer patients. That suggests a far more complex mechanism of radiosensitivity or the need to stratify and select patients who will benefit from elevated Girdin expression.

4 | DISCUSSION

The present study showed an unexpected link between Girdin (a regulator of cell migration in development and cancer progression) and the sensitivity of cancer cells to DNA damage. In contrast to the general view that migratory cells are resistant to DNA damage, HeLa cells that expressed high levels of Girdin showed increased sensitivity to UVC-mediated DNA damage and subsequent apoptosis (Figure 6F). Pathological analysis showed extensive intratumor heterogeneity of Girdin expression, supporting the view that the sensitivity to DNA damage is also variable among cancer cells. Together with previous studies that have shown a crucial role of Girdin in

cancer cell invasion and metastasis, the data reveal complex effects of Girdin expression on cancer patients' outcomes.

Previous studies of Girdin function, including those in our laboratory, have shown that Girdin is involved in many cellular processes, including actin reorganization, cell migration, polarization, proliferation, and metabolism.[9,11,12,30] Studies of Girdin-deficient mice indicated that a major in vivo role of Girdin is the regulation of collective migration and proper positioning of newborn neurons in developing and young adult brains.[14,39,43] Given the results obtained from Girdin-deficient mice, the results of Bhandari et al were unexpected. They showed that Girdin functions as a regulator of the migration-proliferation dichotomy.[23] The migration-proliferation dichotomy is a hallmark of normal as well as cancer cells that cell migration and proliferation do not occur simultaneously.[22] This mechanism helps to explain the resistance of migratory cancer cells to various cytotoxic therapeutics that target proliferating cells.[24,25] Girdin promotes migration upon phosphorylation by Cdk5, whereas nonphosphorylated Girdin promotes proliferation.[23] Our present study added a new dimension to Girdin function, ie that high expression of Girdin enhances the vulnerability of cancer cells to DNA damage. These results seem to contradict the previous finding that Girdin promotes cell migration and cancer progression but could provide opportunities for therapeutic intervention if Girdin expression could be manipulated in human malignancies.

An unexpected but intriguing finding of the present study was that overexpression of Girdin delayed G_1 and M phases, both of which are sensitive to UVC (Figure 6F). This might contribute to the increased sensitivity of Girdin OE cells to DNA damage. Our analysis identified that Mad2 expression was transcriptionally upregulated in Girdin OE cells, which could explain the dysregulation of the cell cycle in Girdin OE cells. We have not yet, however, delved into the detailed mechanisms underlying this observation. Given the role of Mad2 in prolonging checkpoint arrest caused by DNA damage,[44] it is plausible that elevated Girdin and Mad2 are both involved in the dysregulation of the cell cycle and subsequent UVC-induced DNA damage and cell death.

Our clinicopathological analysis showed no correlation of Girdin expression levels with the response to radiation therapy in a cohort of esophageal cancer patients. This finding is inconsistent with the hypothesis proposed in the present study. Alternatively, it suggests a complex compensatory mechanism underlying the resistance of cancer cells to radiation therapy. Expression of Girdin in various compartments of the tumor microenvironment, including tumor vessels and CAFs, must be considered.[27] Further studies will be needed to identify the in vivo significance of the present study, and care should be taken in developing new therapeutics that target pathways involving Girdin.

One limitation of this study can be attributed to the sources of cells used in the analyses. Given the availability of pre- and postradiation tissue samples, we examined tissue samples obtained from patients with esophageal cancer. However, we mainly relied on a HeLa cell line for in vitro studies because it had been widely used for cell cycle analysis. It is plausible that the mechanisms and frequencies of the acquisition of resistance to cytotoxic therapies are different across cancer types. The generality of the findings of this study, therefore, must be confirmed by further studies in the future. Another concern regarding the present study is that it was based on the use of UVC and not ionizing radiation such as that used clinically, ie X-ray photon beams. Considering that the high dose of UVC adopted in this study resulted in DSBs similar to the DNA damage caused by ionizing radiation, we believe that the present findings recapitulate the effects of X-ray irradiation therapy and other radiation therapies.[45] Further studies on tumor mouse models with genetically engineered expression of Girdin could provide insights into the biological significance of Girdin expression in cancer cell sensitivity to radiation therapies.

ACKNOWLEDGMENTS

We gratefully thank Kaori Ushida and Maki Takagishi for support in immunostaining and Yasuyuki Mizutani and Minoru Tanaka for support in flow cytometry. This work was supported by a Grant-in-Aid for Scientific Research (S) (26221304 to MT) and a Grant-in-Aid for Scientific Research (B) (18H02638 to AE) commissioned by the Ministry of Education, Culture, Sports, Science and Technology of Japan; AMED-CREST (Japan Agency for Medical Research and Development, Core Research for Evolutional Science and Technology; 19gm0810007h0104 and 19gm1210008s0101 to AE); and the Project for Cancer Research and Therapeutic Evolution (P-CREATE) from AMED (19cm0106332h0002 to AE).

DISCLOSURE

No potential conflicts of interest were disclosed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Atsushi Enomoto https://orcid.org/0000-0002-9206-6116
Tetsuro Taki https://orcid.org/0000-0001-8654-6896
Shinji Mii https://orcid.org/0000-0001-8266-3235
Masahide Takahashi https://orcid.org/0000-0002-2803-2683

REFERENCES

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-674.
2. Barker HE, Paget JTE, Khan AA, et al. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. *Nat Rev Cancer*. 2015;15:409-425.
3. Frosina G. DNA repair and resistance of gliomas to chemotherapy and radiotherapy. *Mol Cancer Res*. 2009;7:989-999.
4. Musgrove E, Sutherland R. Biological determinants of endocrine resistance in breast cancer. *Nat Rev Cancer*. 2009;9:631-643.
5. Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? *Nat Rev Cancer*. 2012;12:323-334.
6. Fisher R, Pusztai L, Swanton C. Cancer heterogeneity: implications for targeted therapeutics. *Br J Cancer*. 2013;108:479-485.
7. Rich JN. Cancer stem cells in radiation resistance. *Cancer Res*. 2007;67:8980-8984.

8. Aguirre-Ghiso J. Models, mechanisms and clinical evidence for cancer dormancy. *Nat Rev Cancer*. 2007;7:834-846.
9. Enomoto A, Murakami H, Asai N, et al. Akt/PKB regulates actin organization and cell motility via Girdin/APE. *Dev Cell*. 2005;9:389-402.
10. Le-Niculescu H, Niesman I, Fischer T, DeVries L, Farquhar MG. Identification and characterization of GIV, a novel Gαi/s-interacting protein found on COPI, endoplasmic reticulum-Golgi transport vesicles. *J Biol Chem*. 2005;280:22012-22020.
11. Ohara K, Enomoto A, Kato T, et al. Involvement of Girdin in the determination of cell polarity during cell migration. *PLoS One*. 2012;7:e36681.
12. Weng L, Enomoto A, Miyoshi H, et al. Regulation of cargo-selective endocytosis by dynamin 2 GTPase-activating protein girdin. *EMBO J*. 2014;33:2098-2112.
13. Garcia-Marcos M, Ghosh P, Farquhar MG. GIV/Girdin transmits signals from multiple receptors by triggering trimeric G protein activation. *J Biol Chem*. 2015;290:6697-6704.
14. Wang Y, Kaneko N, Asai N, et al. Girdin is an intrinsic regulator of neuroblast chain migration in the rostral migratory stream of the postnatal brain. *J Neurosci*. 2011;31:8109-8122.
15. Wang X, Enomoto A, Asai N, et al. Collective invasion of cancer: perspectives from pathology and development. *Pathol Int*. 2016;66:183-192.
16. Wang X, Enomoto A, Weng L, et al. Girdin/GIV regulates collective cancer cell migration by controlling cell adhesion and cytoskeletal organization. *Cancer Sci*. 2018;109:3643-3656.
17. Shibata T, Matsuo Y, Shamoto T, et al. Girdin, a regulator of cell motility, is a potential prognostic marker for esophageal squamous cell carcinoma. *Oncol Rep*. 2013;29:2127-2132.
18. Dunkel Y, Diao K, Aznar N, et al. Prognostic impact of total and tyrosine phosphorylated GIV/Girdin in breast cancers. *FASEB J*. 2016;30:3702-3713.
19. Jiang P, Enomoto A, Jijiwa M, et al. An actin-binding protein Girdin regulates the motility of breast cancer cells. *Cancer Res*. 2008;68:1310-1318.
20. Garcia-Marcos M, Jung BH, Ear J, Cabrera B, Carethers JM, Ghosh P. Expression of GIV/Girdin, a metastasis-related protein, predicts patient survival in colon cancer. *FASEB J*. 2011;25:590-599.
21. Ghosh P, Tie J, Muranyi A, et al. Girdin (GIV) expression as a prognostic marker of recurrence in mismatch repair-proficient stage II colon cancer. *Clin Cancer Res*. 2016;22:3488-3498.
22. Ghosh P, Beas AO, Bornheimer SJ, et al. A Gαi-GIV molecular complex binds epidermal growth factor receptor and determines whether cells migrate or proliferate. *Mol Biol Cell*. 2010;21:2338-2354.
23. Bhandari D, Lopez-Sanchez I, To A, et al. Cyclin-dependent kinase 5 activates guanine nucleotide exchange factor GIV/Girdin to orchestrate migration-proliferation dichotomy. *Proc Natl Acad Sci USA*. 2015;112:E4874-E4883.
24. Joy AM, Beaudry CE, Tran NL, et al. Migrating glioma cells activate the PI3-K pathway and display decreased susceptibility to apoptosis. *J Cell Sci*. 2003;116:4409-4417.
25. Theys J, Jutten B, Habets R, et al. E-Cadherin loss associated with EMT promotes radioresistance in human tumor cells. *Radiother Oncol*. 2011;99:392-397.
26. Kitamura T, Asai N, Enomoto A, et al. Regulation of VEGF-mediated angiogenesis by the Akt/PKB substrate Girdin. *Nat Cell Biol*. 2008;10:329-337.
27. Yamamura Y, Asai N, Enomoto A, et al. Akt-Girdin signaling in cancer-associated fibroblasts contributes to tumor progression. *Cancer Res*. 2015;75:813-823.
28. Choi J-S, Kim KH, Oh E, et al. Girdin protein expression is associated with poor prognosis in patients with invasive breast cancer. *Pathology*. 2017;49:618-626.
29. Natsume A, Kato T, Kinjo S, et al. Girdin maintains the stemness of glioblastoma stem cells. *Oncogene*. 2012;31:2715-2724.
30. Weng L, Han Y-P, Enomoto A, et al. Negative regulation of amino acid signaling by MAPK-regulated 4F2hc/Girdin complex. *PLoS Biol*. 2018;16:e2005090.
31. Han YP, Enomoto A, Shiraki Y, et al. Significance of low mTORC1 activity in defining the characteristics of brain tumor stem cells. *Neuro Oncol*. 2017;19:636-647.
32. Shimada Y, Imamura M, Wagata T, Yamaguchi N, Tobe T. Characterization of 21 newly established esophageal cancer cell lines. *Cancer*. 1992;69:277-284.
33. Konermann S, Brigham MD, Trevino AE, et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature*. 2015;517:583-588.
34. Jackman J, O'Connor PM. Methods for synchronizing cells at specific stages of the cell cycle. *Curr Protoc Cell Biol*. 2001;Chapter 8:Unit 8.3.
35. Zhang YJ, Li AJ, Han Y, Yin L, Lin MB. Inhibition of Girdin enhances chemosensitivity of colorectal cancer cells to oxaliplatin. *World J Gastroenterol*. 2014;20:8229-8236.
36. Takasawa R, Nakamura H, Mori T, Tanuma S. Differential apoptotic pathways in human keratinocyte HaCaT cells exposed to UVB and UVC. *Apoptosis*. 2005;10:1121-1130.
37. Di Leonardo A, Linke SP, Clarkin K, Wahl GM. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev*. 1994;8:2540-2551.
38. Fong CS, Mazo G, Das T, et al. 53BP1 and USP28 mediate p53-dependent cell cycle arrest in response to centrosome loss and prolonged mitosis. *Elife*. 2016;5:e16270.
39. Enomoto A, Asai N, Namba T, et al. Roles of disrupted-in-schizophrenia 1-interacting protein girdin in postnatal development of the dentate gyrus. *Neuron*. 2009;63:774-787.
40. Kallio M, Weinstein J, Daum JR, Burke DJ, Gorbsky GJ. Mammalian p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events. *J Cell Biol*. 1998;141:1393-1406.
41. Fang G, Yu H, Kirschner MW. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev*. 1998;12:1871-1883.
42. Shah JV, Cleveland DW. Waiting for anaphase: Mad2 and the spindle assembly checkpoint. *Cell*. 2000;103:997-1000.
43. Muramatsu A, Enomoto A, Kato T, et al. Potential involvement of kinesin-1 in the regulation of subcellular localization of Girdin. *Biochem Biophys Res Commun*. 2015;463:999-1005.
44. Dotiwala F, Harrison JC, Jain S, Sugawara N, Haber JE. Mad2 prolongs DNA damage checkpoint arrest caused by a double-strand break via a centromere-dependent mechanism. *Curr Biol*. 2010;20:328-332.
45. Staszewski O, Nikolova T, Kaina B. Kinetics of gamma-H2AX focus formation upon treatment of cells with UV light and alkylating agents. *Environ Mol Mutagen*. 2008;49:734-740.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Chen C, Enomoto A, Weng L, et al. Complex roles of the actin-binding protein Girdin/GIV in DNA damage-induced apoptosis of cancer cells. *Cancer Sci*2020;111:4303–4317. https://doi.org/10.1111/cas.14637