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Dehydroepiandrosterone Induces Temozolomide Resistance Through Modulating Phosphorylation and Acetylation of Sp1 in Glioblastoma

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Abstract

Glioblastoma is the most malignant type of brain tumor for which there are currently no effective treatments. Patient prognosis is improved by radiation combined with temozolomide (TMZ) therapy but only for a short period of time due to the high prevalence of recurrence. Although O^6-methylguanine-DNA methyltransferase (MGMT)-mediated DNA repair is a well-defined characteristic of TMZ resistance, the mechanism by which MGMT-deficient glioblastoma counteracts TMZ-induced DNA damage, leading to apoptosis, still remains unclear. Previously, we determined that aberrantly activated cytochrome P450 17A1 causes TMZ resistance in MGMT-deficient glioblastoma by increasing the secretion of dehydroepiandrosterone (DHEA), a neurosteroid that maintains the health of neurons and astrocytes. However, the precise mechanism by which DHEA alters the response of glioblastoma to TMZ has not been studied. In the present study, we found that DHEA prevents TMZ-induced apoptosis by attenuating DNA damage in MGMT-deficient glioblastoma. In addition, DHEA activated the LYN-AKT cascade to induce Sp1 phosphorylation. Phospho-Sp1 localized in TMZ-damaged DNA, prevented further DNA damage, and was deacetylated through the recruitment of HDAC1/2. Deacetylated Sp1 recruited proliferating cell nuclear antigen (PCNA) to attenuate DNA damage. To confirm whether the DHEA-induced cellular process contributes to TMZ resistance, we established a TMZ-resistant glioblastoma cell line, A172R, and isolated primary resistant tumor cells, PtR#1, from a glioblastoma patient exhibiting chemotherapeutic resistance. Sp1 exhibited phosphorylated and deacetylated status, and associated with HDAC1/2 and PCNA in TMZ-resistant cells. Based on these findings, we conclude that DHEA induces TMZ resistance in glioblastoma via the induction of phospho-Sp1-mediated DNA repair.

Keywords Glioblastoma · Temozolomide resistance · Dehydroepiandrosterone · Phospho-Sp1

Introduction

Glioblastoma is the most lethal brain tumor that accounts for 80% of patients with malignant glioma; its incidence is 5.26/100000 per year, and 17,000 new diagnoses are made each year [1]. It is still difficult to treat glioblastoma despite the focus of research on therapeutic options over the past decade. The standard treatment protocol is surgery, followed by radiotherapy combined with temozolomide (TMZ)-mediated chemotherapy [2]. TMZ potently induces apoptosis via the methylation of guanine, triggering DNA damage [3], which extends the median survival from 12 to 15 months. However, the efficacy of TMZ is always restricted to a short time period because glioblastoma quickly develops resistance to the treatment [4]. O^6-Methylguanine-DNA methyl-transferase (MGMT) removes the methyl group from guanine, counteracting the effects of TMZ [3]. This capacity to modulate DNA repair highly attenuates TMZ-mediated tumor-suppressive effect, leading to the occurrence of chemotherapeutic resistance [5]. However, MGMT-deficient glioblastoma still develops the resistance through enhancing DNA repair capacity, including homologous recombination

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(HR) and BER [5]. Therefore, the development of new therapeutic strategies for glioblastoma depends upon the characterization of the mechanism underlying DNA repair in response to TMZ.

Specificity protein (Sp) 1 is a transcription factor that recognizes GC-rich promoter sequences and regulates several cellular functions, which have been well-studied in mammalian cells, including proliferation, invasion, apoptosis, and angiogenesis [6]. Furthermore, Sp1 has been shown to regulate DNA repair by transcription-dependent and -independent mechanisms. In addition to MGMT [7], both X-ray repair cross-complementing protein 1 (XRCC1), involved in base excision repair (BER) [8], and DNA-dependent protein kinase [9], involved in non-homologous end-joining (NHEJ), are targets of Sp1-mediated transcription. Sp1 phosphorylation by ataxia-telangiectasia mutated (ATM), leading to protein degradation, sensitizes cells to DNA damage by reducing the efficiency of BER [8], which is aberrantly enhanced in TMZ-resistant glioblastoma [10]. In addition, Sp1 phosphorylation is also an important characteristic of daunorubicin-resistant leukemia [9], supporting the theory that Sp1 phosphorylation is a suitable marker for chemotherapeutic sensitivity. Interestingly, even without a DNA-binding domain, phospho-Sp1 still rescues DNA damage [11], implying that Sp1 may also induce DNA repair through a transcription-independent pathway. In contrast to phosphorylation, acetylation has been reported to suppress the function of Sp1 [12, 13], although its effects on DNA damage have never been determined. Therefore, it remains unknown whether Sp1 modified translationally affects cellular sensitivity to TMZ in glioblastoma, and it will be helpful to investigate how Sp1 is phosphorylated in order to evaluate the chemotherapeutic response of patients.

Dehydroepiandrosterone (DHEA), a neurosteroid, protects neuronal cells from stress-induced apoptosis and acts as a circulating steroid hormone [14, 15]. Multiple DHEA-activated signaling pathways related to cell survival have been reported to mediate cellular protection, such as activating Sigma-1 receptor, SRC/PKA, MEK/MAPK, and PI3K/AKT cascades [16, 17]. Importantly, we previously clarified that DHEA is increased in the serum of patients with glioblastoma due to the overexpression of cytochrome P450 (CYP) 17A1 and significantly decreases cellular sensitivity to TMZ in glioblastoma [18]. We were particularly curious about how DHEA affects the cellular response to TMZ, and we investigated this in the present study.

By extending our previous report that CYP17A1 upregulation induces TMZ resistance by increasing DHEA secretion [18], we found that DHEA suppresses the cytotoxic effect of TMZ by enhancing Sp1-modulated DNA repair in MGMT-deficient glioblastoma. In particular, DHEA increased the efficiency of DNA repair by inducing post-translational modifications of Sp1, including phosphorylation and deacetylation. The activation of the LYN/AKT cascade was required for DHEA-induced Sp1 phosphorylation and DHEA-attenuated DNA damage. In addition, phospho-Sp1 was deacetylated by HDAC1/2 and associated with proliferating cell nuclear antigen (PCNA) in the presence of DHEA. Based on these findings, we conclude that DHEA-induced Sp1 modifications are essential for promoting TMZ resistance via the control of DNA repair.

Materials and Methods

Isolation and Culture of Primary Glioblastoma Cells

The use of human specimens was approved by the Institute Review Board (IRB)/Ethics Committee from the office of human research in Taipei Medical University (Taipei, Taiwan). The consent of each patient was obtained and approved by Taipei Medical University IRB protocols, Nos. 201006011 and 201402018. Pt#3 glioblastoma cells were isolated from the glioblastoma tissue of a male patient, and PtR#1 TMZ-resistant glioblastoma cells were purified from a female recurrent glioblastoma patient exhibiting radio- and chemotherapeutic resistance. Both patients were treated and cared in the Taipei Medical University Hospital (Taipei, Taiwan). The freshly resected tissues were digested by 0.05% collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) and 5 units/ml DNase I (Sigma-Aldrich) at 37 °C for 2 h. After removing undigested tissues by centrifugation, the supernatant was mixed with DMEM supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, South Logan, UT, USA) and transferred to 6-well culture plates. Cells were maintained in DMEM-10% FBS.

Cell Culture, Treatment, and Transfection

Both of A172 and U87MG cells were purchased from ATCC (Manassas, VA, USA). A172, Pt#3, U87MG, and PtR#1 cells were maintained in DMEM supplemented with 10% FBS. Both TMZ and DHEA were purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) according to the supplier's instructions (Sigma-Aldrich). The photography of cell morphology was provided in Supplementary Fig. S1. For transfection, cells were maintained at 80% confluency, and PolyJet reagent (SignaGen Laboratories, Rockville, MD, USA) was used according to the manufacturer's instructions.

Establishment of TMZ-Resistant Cells

A172 and Pt#3 cells, both of which exhibit resistance to TMZ, were established according to a previous report [18]. TMZ-resistant U87MG (U87MGR) was established in the media containing 100 μM TMZ in our previous report [18].

Briefly, cells were treated with 50 μM TMZ for 1 day and divided among 96-well plates (1 cell per well). Cells were subsequently incubated with 50 μM TMZ for 21 days, and surviving cells were cultured in the media containing 100 μM TMZ for additional 39 days. These cells were maintained in DMEM-10% FBS containing 100 μM TMZ for at least 60 days. TMZ resistance was confirmed by colony formation assay.

Comet Assay

The reagent kit for the comet assay was purchased from Trevigen, Inc. (#4251-050-K; Gaithersburg, MD, USA) and used according to the manufacturer's instructions. The signal was detected by silver staining and quantified using ImageJ software.

Apurinic/Apyrimidinic (AP) Site Detection

A DNA damage quantification kit was purchased from Biovision Inc. (#K253-25; Milpitas, CA, USA), and genomic DNA was analyzed according to the manufacturer's instructions. The signal was measured using an absorbance reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 650 nm.

MTT Assay

The experiment was performed according to the protocol described in the previous report [19].

Immunoprecipitation

Cell lysates prepared using RIPA buffer containing protease and phosphatase inhibitors (Biotools Inc., New Taipei City, Taiwan) and 500-μg proteins were incubated with 2-μg anti-Sp1 antibody at 4 °C for 1 h. After mixing with 50-μL protein A agarose (Merck Millipore, Bedford, MA, USA) for 1 h and washing four times, the immunocomplex was analyzed by western blotting.

Western Blotting

Western blotting was performed as described in a previous report [18], and primary antibodies are listed in Supplementary Table S1.

RTK Phosphorylation Array

Human RTK phosphorylation array was purchased from RayBiotech, Inc. (Norcross, GA, USA), and used according to the instruction.

Immunofluorescent Staining

After staining using antibodies against Sp1, p-ATM, and γH2Ax, cells on coverslips were incubated with Alexa Fluor 488 (1:200; Thermo Fisher Scientific, Waltham, MA, USA) or 568 (1:200; Thermo Fisher Scientific) at room temperature for 2 h. Subsequently, stained cells were mounted with mounting media containing 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific) and photographed using an immunofluorescent microscope.

Preparation of Chromatin-Bound Fraction

The protocol described in a previous report [20] was followed. After washing with PBS and incubating with buffer A containing 10 mM HEPES (pH 7.9), 1 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol, 0.1% Triton X-100, and a protease inhibitor cocktail for 5 min on ice, the nuclear fraction was pelleted by centrifugation at 1500×*g* for 5 min at 4 °C. After mixing with buffer B containing 3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, and a protease inhibitor cocktail for 10 min on ice, soluble nuclear protein was separated by centrifugation at 2000×*g* for 5 min and discarded. The chromatin-bound fraction was further pelleted by centrifugation at 13000×*g* for 1 min and analyzed by western blotting. In total, 200 μg of chromatin-bound fraction was subjected to immunoprecipitation assay.

Colony Formation Assay

The experimental procedure was performed as described in a previous report [18]. Briefly, A172 (1000 cells/dish), A172R (1000 cells/dish), Pt#3 (1200 cells/dish), Pt#3R (1200 cells/dish), or PtR#1 (1200 cells/dish) cells were seeded into 6-cm culture dishes and incubated for 16 days in the presence or absence of TMZ in the final 4 days. The colony formation assay for U87MG and U87MGR was performed in our previous report [18].

DNA Repair Assay: Homologous Recombination (HR) and NHEJ

Protocols from previous reports were followed [21, 22]. For the HR assay, cells stably expressing CMV-driven GFP interrupted by a stop codon ($GFP–^{HR}$) flanked by two I-SceI recognition sites were established. In addition, $GFP–^{HR}$ constructs contain a segment that possesses homology with the stop codon. After transfection with I-SceI, double-strand breaks within $GFP–^{HR}$ were induced, and stop codons were removed. Simultaneously, HR repaired the damaged site using the homological sequence, generating wild-type GFP ($GFP+^{HR}$). For the NHEJ assay, cells expressing the GFP gene ($GFP–^{NHEJ}$) disrupted by an intron flanked by two I-SceI sites

were established. The GFP–^{NHEJ} construct for the NHEJ assay, which lacks a GFP homological sequence, was different than that used for the HR assay. When these cells were transfected with I-SceI, the intron within the GFP gene was removed and repaired by NHEJ to generate a GFP+^{NHEJ} signal. In both HR and NHEJ assays, the green fluorescence of GFP was measured by flow cytometry.

Statistical Analysis

Differences between groups were analyzed using the Student's *t* test, and *P* values < 0.05 were considered significant. The comparison of sensitivity to TMZ in Fig. 6 was performed using two-way analysis of variance.

Results

DHEA Prevents TMZ-Induced DNA Damage

Previously, we reported that DHEA decreases the sensitivity of MGMT-deficient glioblastoma cells to TMZ [18], leading to resistance. Here, we attempted to uncover the mechanism underlying DHEA-induced resistance in the absence of MGMT expression. We aimed to determine whether DHEA affects the occurrence of DNA damage in the presence of TMZ in an astrocytoma cell line, A172, and in primary glioblastoma cells, Pt#3. Figure 1a shows the results of the comet assay, in which TMZ induced the formation of the tail and DHEA significantly attenuated it. Following DNA damage, AP sites formed before DNA repair [23], and DHEA blocked TMZ-induced AP sites (Fig. 1b). In addition, TMZ-induced γH2Ax, an indicator of DNA damage, was not observed in the presence of 5 μM DHEA (Fig. 1c). These results indicate that DHEA effectively prevents TMZ-induced DNA damage in glioblastoma cells.

We then investigated whether DHEA blocks the effect of TMZ by the regulation of DNA repair. According to fluorescence-based quantitation by flow cytometry, TMZ-treated cells exhibited 13.12% HR compared with the DMSO-treated group, which exhibited 18.12%; TMZ-treated cells in the presence of DHEA exhibited 16.2% HR (Fig. 1d, left panel). In the normalized DMSO-treated group, TMZ treatment reduced HR efficacy from 100 to 72.4%, whereas DHEA raised the efficacy from 72.4 to 89.4%, indicating that DHEA attenuates TMZ-inhibited HR. NHEJ was increased from 100 to 111.9% by TMZ; the addition of DHEA nearly returned this to 100% (Fig. 1d, right panel). Based on this evidence, we conclude that DHEA prevents TMZ-induced DNA damage by the regulation of DNA repair.

Sp1 Localizes to the Site of DNA Damage and Is Responsible for Preventing DNA Damage

Immunofluorescence revealed that Sp1 localizes to the site of DNA damage (Fig. 2a–c) and associates with γH2Ax and phospho-ATM upon TMZ treatment in Pt#3 cells (Fig. 2d). Sp1 overexpression prevented TMZ-induced γH2Ax expression (Fig. 2e), and Sp1 knockdown significantly decreased HR efficiency from 100 to 55.24% ± 10.87% (Supplementary Fig. S2 and Fig. 2f). Importantly, DHEA-mediated protection from DNA damage, which was indicated by phospho-Rad50 and γH2Ax, was counteracted by Sp1 knockdown (Fig. 2g), suggesting that Sp1 mediates the effect of DHEA on DNA repair in glioblastoma.

DHEA Induces Sp1 Phosphorylation and Deacetylation and Promotes Subsequent Recruitment by Damaged DNA

Based on the evidence that DHEA prevents DNA damage through Sp1, we attempted to investigate whether DHEA affects Sp1 expression in glioblastoma. However, Sp1 expression was unchanged upon treatment with 50 nM to 5 μM of DHEA in Pt#3 cells for 24 h. Previous studies have indicated that phosphorylation and deacetylation greatly enhance Sp1 function [9, 12]. Therefore, we evaluated the effect of DHEA on these two types of post-translational modifications. As shown in Fig. 3a, DHEA induced Sp1 phosphorylation in a dose-dependent manner at residue T739, which is an important modification that enhances the function of Sp1 [24], and also decreased the acetylation level of Sp1 in Pt#3 cells (Fig. 3b). This indicates that DHEA in glioblastoma regulates Sp1 function by inducing phosphorylation and decreasing acetylation. In response to TMZ treatment (100 μM) for 8 h in the presence of 5 μM DHEA, the levels of chromatin-bound phospho-Sp1-T739 and PCNA were increased, whereas the levels of Apex1 and XRCC1 involved in BER were similar to those in TMZ-treated cells in the absence of DHEA (Fig. 3c). To further confirm the status of chromatin-bound Sp1 in Pt#3 cells, immunoprecipitation assay revealed that Sp1 is phosphorylated and deacetylated by DHEA in the chromatin fraction (Supplementary Fig. S3). In addition, we found that in the presence of TMZ, DHEA-induced phospho-Sp1 co-localized with PCNA (Fig. 3d), providing further evidence that DHEA regulates DNA repair by inducing Sp1 phosphorylation.

DHEA Induces Sp1 Phosphorylation by Activating the LYN/AKT Cascade

Several reports have indicated that DHEA induces multiple signaling pathways to regulate cellular functions, including PI3K/AKT, MEK/MAPK, Src/STAT3, and

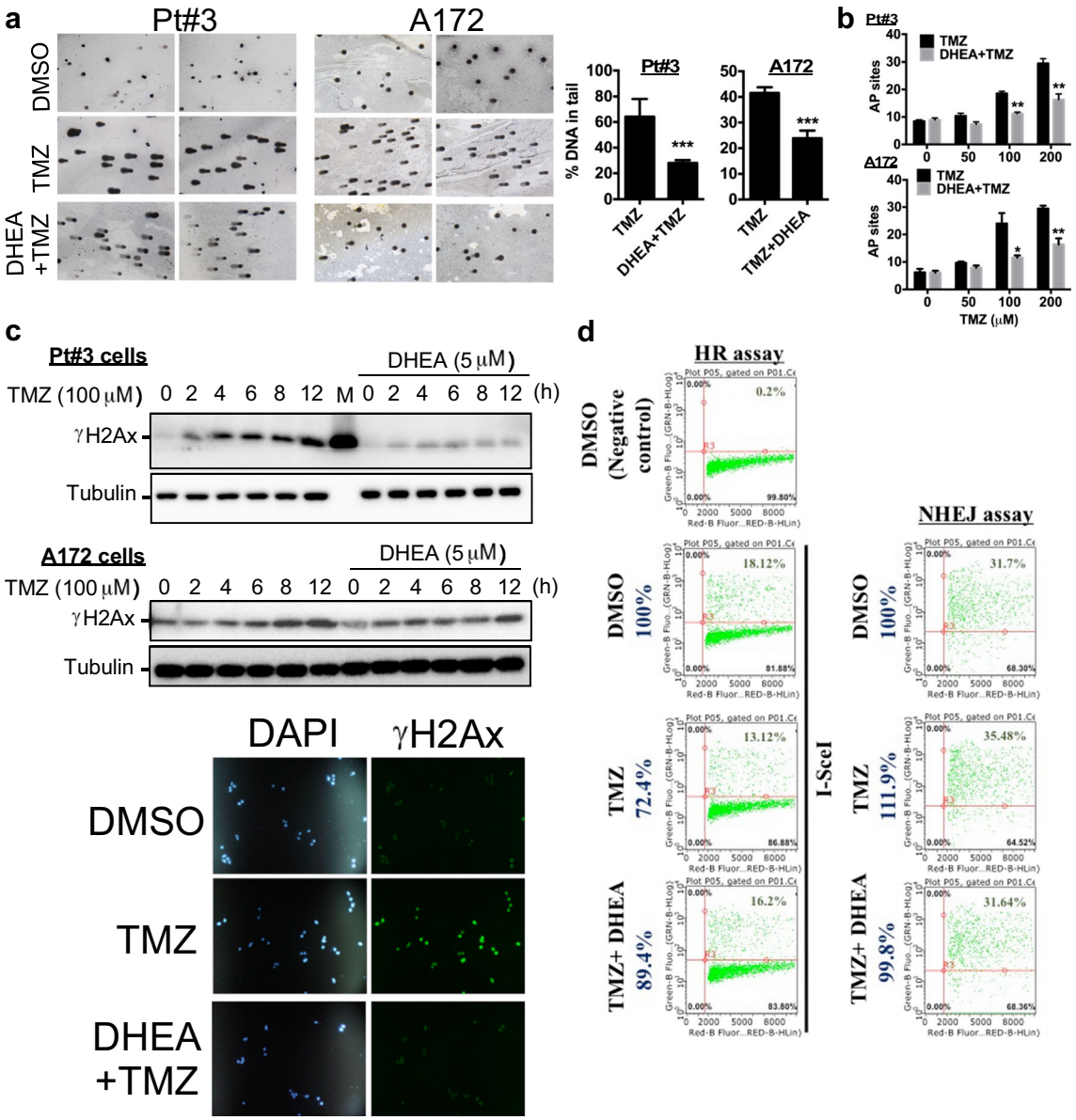

Fig. 1 Effect of DHEA on TMZ-induced DNA damage in glioblastoma. **a** A172 and Pt#3 cells pre-treated with 5 μM DHEA for 48 h were treated with 100 μM TMZ for 4 h and examined by the comet assay. The left panel shows representative images; the right panel shows the quantitative result. Experiments were performed three times, and data are expressed as the mean ± s.e.m. (***$P < 0.001$). **b** After treatment, genomic DNA of A172 and Pt#3 cells was isolated and analyzed using the DNA Damage Quantification Colorimetric Kit. Experiments were performed three times in triplicate, and data are expressed as the mean ± s.e.m. (**$P < 0.01$). **c** Upper panel: Cells were harvested for protein collection and subjected to western blotting using the anti-γH2Ax antibody. Lower panel: Pt#3 cells seeded onto the coverslip were treated as indicated and subjected to immunofluorescent staining using the anti-γH2Ax antibody. M: The marker of molecular weight. **d** The left panel shows the HR assay: After pre-treatment with DHEA for 48 h, cells expressing DR-GFP were transfected with the I-SceI plasmid and incubated with TMZ for 12 h. Cellular fluorescence was quantitated by flow cytometry. The percentage of alteration was calculated as the ratio of treatment/DMSO. The right panel shows the NHEJ assay: After treatment, cells were transfected with NheI-cut GFP and incubated with TMZ for 12 h. The fluorescent signal was measured by flow cytometry

Src/PKA cascades [17, 25]. Therefore, we estimated the effect of DHEA on the phosphorylation of AKT, ERK, and STAT3 and found that AKT phosphorylation is induced by DHEA in a dose-dependent manner in Pt#3 cells (Fig. 4a). To determine what kind of upstream signaling is required for DHEA-activated AKT to trigger Sp1 phosphorylation, we evaluated the phosphorylation of 71 receptor tyrosine kinases (RTK) with or without DHEA using the RTK phosphorylation array. We found that only LYN kinase is phosphorylated by DHEA-treated cells (Fig. 4b). We also confirmed this in Pt#3 and A172 cells. After DHEA treatment for 24 h, the phosphorylation of Sp1, AKT, and LYN was induced (Fig. 4c). Subsequently, DHEA-induced Sp1

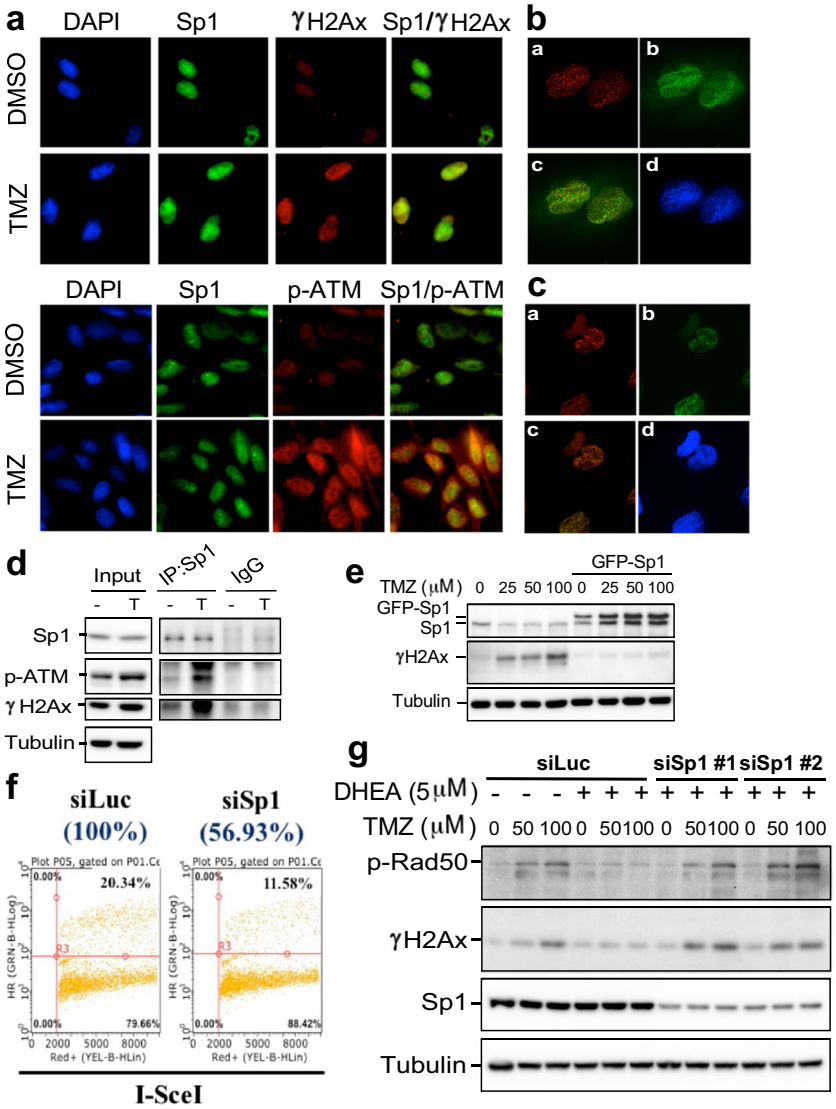

Fig. 2 Sp1 localizes to DNA damage sites and is essential for DHEA to attenuate DNA damage. **a** Upper panel: After treatment with 100 μM TMZ for 8 h, Pt#3 cells were co-stained using anti-Sp1 and anti-γH2Ax antibodies. Lower panel: Immunostaining using anti-Sp1 and anti-p-ATM antibodies. Fluorescent images were acquired under ×40 magnification. **b** TMZ-treated cells were stained immunofluorescently and photographed under ×100 magnification. (a) γH2Ax, (b) Sp1, (c) γH2Ax/Sp1, and (d) DAPI. **c** TMZ-treated cells were stained and photographed under ×100 magnification. (a) p-ATM, (b) Sp1, (c) p-ATM/Sp1, and (d) DAPI. **d** Cells were subjected to immunoprecipitation using the anti-Sp1 antibody and analyzed by western blotting using indicated antibodies. **e** After transfection with the GFP or GFP-Sp1 plasmid for 24 h, Pt#3 cells were treated with TMZ for 8 h, and protein lysates were collected for western blotting. **f** After knockdown for 24 h, cells were transfected with the I-SceI plasmid and subjected to the HR assay. The efficiency of HR was determined by flow cytometry. The percentage of alteration was calculated as the ratio of siSp1/siLuc. **g** After transfection with indicated siRNA for 24 h, cells were treated with DHEA for 48 h and incubated with TMZ for an additional 8 h. Cell lysates were collected for western blotting using indicated antibodies

phosphorylation at residue T739 was blocked by LYN and AKT inhibitors. In addition, the LYN inhibitor also blocked AKT phosphorylation, whereas the AKT inhibitor failed to block LYN phosphorylation (Fig. 4d). This evidence suggests that DHEA induces a phosphorylation cascade from LYN and AKT to Sp1 in glioblastoma. Importantly, the overexpression of mutant Sp1 (T278/739A) failed to prevent TMZ-induced phospho-Rad50 and γH2Ax (Fig. 4e), suggesting that Sp1 phosphorylation at residues T278/739 is essential for attenuating DNA damage.

Given that Sp1 induced both phosphorylation and deacetylation, we then attempted to clarify the correlation between these two post-translational modifications. As shown in Fig. 4f, the inhibition of Sp1 phosphorylation by both LYN and AKT inhibitors rescued DHEA-induced deacetylation,

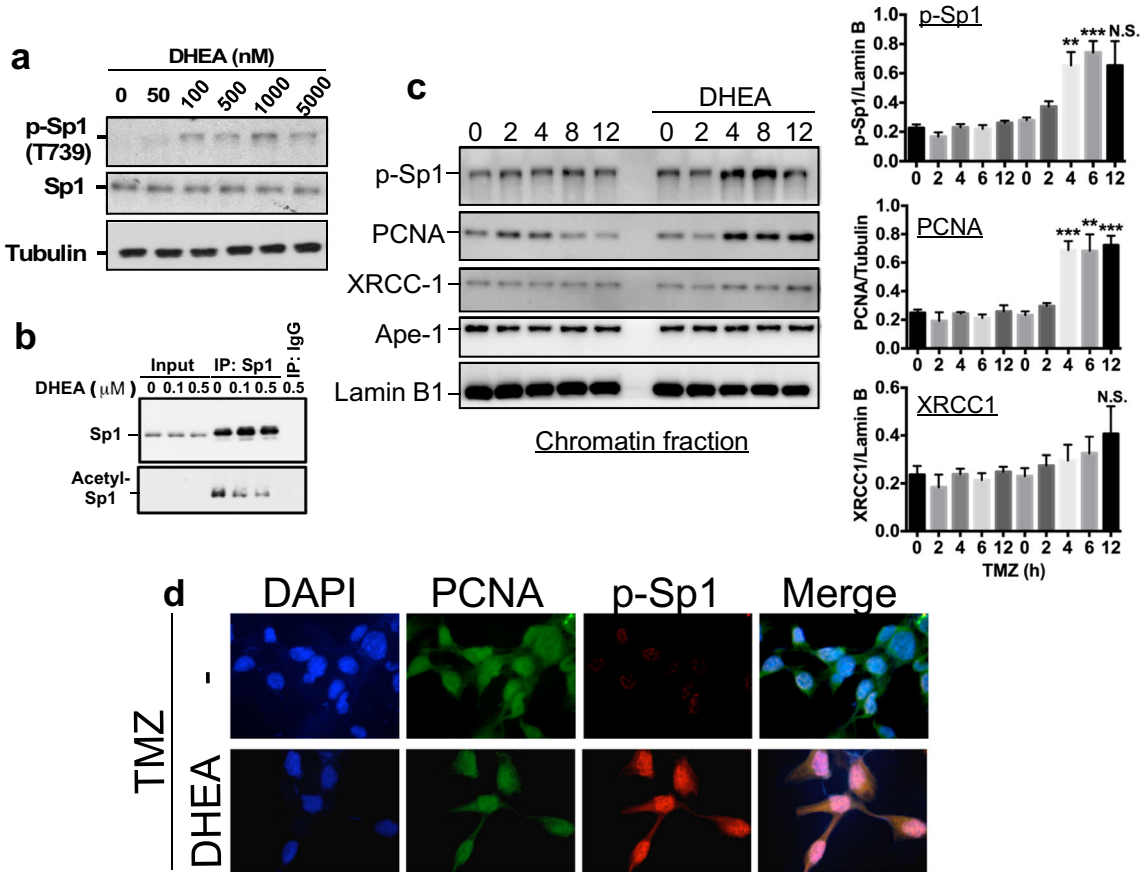

Fig. 3 DHEA induces the accumulation of PCNA and Sp1 modified by both phosphorylation and deacetylation in the nucleus. **a** After treatment with DHEA for 24 h, Pt#3 cell lysates were prepared and subjected to western blotting using anti-Sp1 and anti-p-Sp1 (T739) antibodies. **b** Immunoprecipitation assay was performed with the anti-Sp1 antibody, and the immunocomplex was analyzed with the antibodies indicated. **c** Left panel: Cells pre-treated with DHEA for 48 h were treated with 100 μM TMZ for indicated times. After preparing the chromatin fraction, proteins were analyzed by western blotting using indicated antibodies. Right panel: Experiments were performed three times, and data are expressed as the mean ± s.e.m. (**$P < 0.01$; ***$P < 0.001$). **d** Cells pre-treated with DHEA for 48 h were treated with 100 μM TMZ for 8 h. Immunofluorescence staining was performed using antibodies against p-Sp1 (T739) and PCNA, and images were acquired under ×20 magnification

suggesting that phosphorylation is required for the deacetylation of Sp1.

Phosphorylation Is Required for the Subsequent HDAC1/2-Mediated Deacetylation of Sp1

In addition to phosphorylation, DHEA reduced the acetylated level of Sp1 in a dose-dependent manner and increased the association of Sp1 with HDAC1/2 (Fig. 5a). In particular, the association of Sp1 with PCNA was also increased (Fig. 5a), suggesting that HDAC1/2-induced deacetylated Sp1 associates with PCNA to regulate DNA repair in the presence of DHEA. Moreover, upon DHEA treatment, mutant Sp1 (T278/739A) failed to associate with HDAC1/2 and PCNA (Fig. 5b), suggesting that Sp1 phosphorylation is essential for following deacetylation and protein associations. To elucidate the role of Sp1 deacetylation in the prevention of DNA damage characterized by phospho-Rad50 and γH2Ax, we knocked down HDAC1/2 to block deacetylation in Pt#3 cells. We found that Sp1 significantly attenuates TMZ-induced DNA damage, whereas HDAC1/2 knockdown significantly reduced the effect of Sp1 (Fig. 5c). In addition, HDAC1/2 knockdown also significantly reduced DHEA-mediated protection from TMZ-induced DNA damage (Fig. 5d). These results suggest that DHEA attenuates DNA damage in glioblastoma by inducing the association of phosphorylated and deacetylated Sp1 with PCNA.

Both Phosphorylation and Deacetylation of Sp1 Are Significant in TMZ-Resistant Glioblastoma

To confirm whether these DHEA-induced cellular processes occur in TMZ resistance, in addition to U87MGR which was established in our previous report [18, 19], we established TMZ-resistant glioblastoma cell lines, including A172R and Pt#3R, and purified TMZ-resistant glioblastoma cells, PtR#1, from a patient exhibiting therapeutic resistance. As shown in Fig. 6a, the colony formation assay was performed to confirm

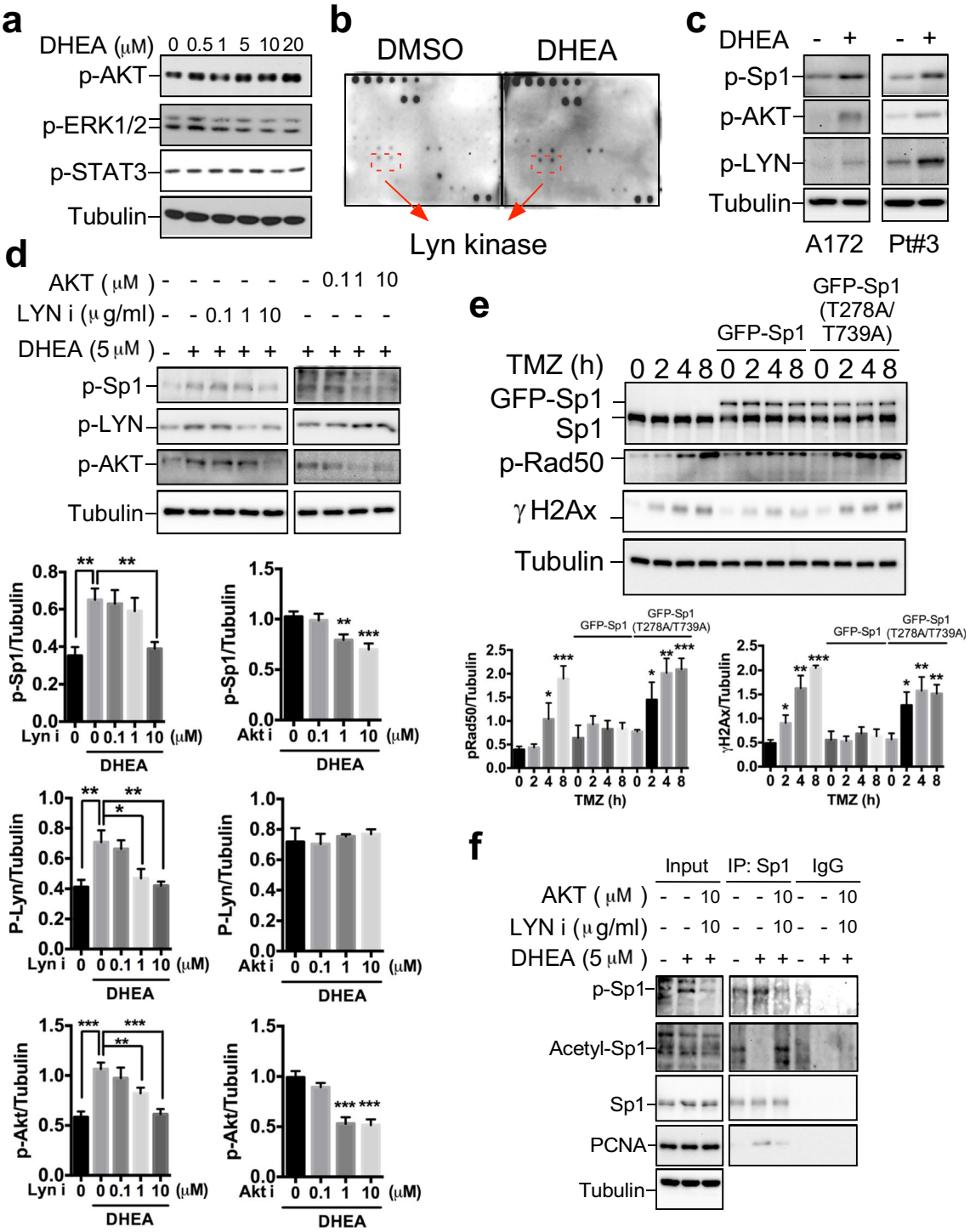

Fig. 4 Effect of DHEA on the LYN-AKT cascade toward Sp1 phosphorylation. **a** After treatment with DHEA for 24 h, Pt#3 cell lysates were prepared and subjected to western blotting. **b** After isolating from DMSO-treated and DHEA-treated Pt#3 cells, cell lysates were analyzed by the RTK phosphorylation array. **c** Cell lysates were analyzed by western blotting. **d** Upper panel: After treatment with DHEA in the presence of AKT or LYN inhibitor, cell lysates were collected for western blotting. Lower panel: Experiments were performed three times, and data are expressed as the mean ± s.e.m. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). **e** Upper panel: After transfection with GFP-Sp1 or mutant GFP-Sp1 (T278/739A), Pt#3 cells were treated with 100 μM TMZ for indicated times, and cell lysates were subjected to western blotting. Lower panel: Experiments were performed three times, and data are expressed as the mean ± s.e.m. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the GFP group without treatment). **f** After treatment with DHEA for 24 h in the presence of LYN or AKT inhibitor, cell lysates were prepared for immunoprecipitation analyzed by western blotting

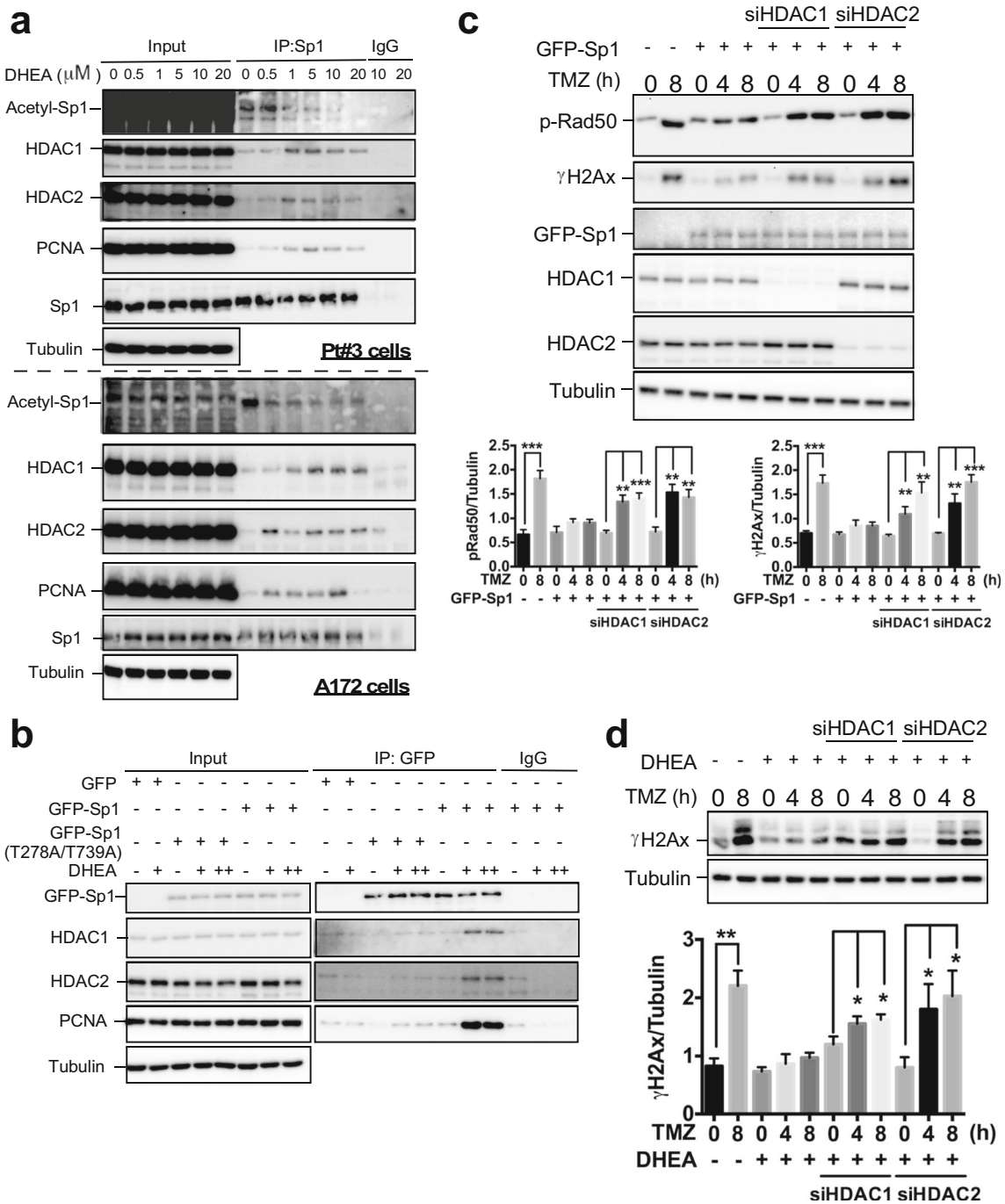

Fig. 5 Sp1 phosphorylation triggers its deacetylation and induces the association with PCNA in the presence of DHEA. **a** After treatment for 24 h and subsequent immunoprecipitation with the anti-Sp1 antibody, the Pt#3 cell immunocomplex was analyzed by western blotting using indicated antibodies. **b** After transfection for 24 h and subsequent treatment with DHEA for an additional 24 h, an immunoprecipitation assay was performed and analyzed by western blotting. **c** After transfection with GFP-Sp1 in the presence or absence of siRNA, Pt#3 cells were treated with 100 μM TMZ, and cell lysates were analyzed by western blotting. Lower panel: Experiments were performed three times, and data are expressed as the mean ± s.e.m. (**$P < 0.01$; ***$P < 0.001$). **d** Cells transfected with siLuc or siRNA targeting HDAC1/2 were treated with 5 μM DHEA for 24 h, and lysates were analyzed by western blotting using the anti-γH2Ax antibody. Lower panel: Experiments were performed three times, and data are expressed as the mean ± s.e.m. (*$P < 0.05$; **$P < 0.01$)

the TMZ-resistant phenotype and revealed that, compared with wild-type cells, A172R, Pt#3R, and U87MGR [18] cells exhibited stronger tolerance in response to TMZ treatment for 4 days. Moreover, compared with wild-type Pt#3, chemo-resistant PtR#1 exhibited the resistance in response to TMZ. In addition, EC50 of TMZ treatment for 96 h on suppressing survival of Pt#3 and PtR#1 was determined as approximate 75 and 160 μM, respectively (Supplementary Fig. S4B),

indicating that PtR#1 belongs to intrinsically TMZ-resistant glioblastoma cells. Importantly, both Sp1 phosphorylation and deacetylation were induced in the presence of LYN and AKT phosphorylation in TMZ-resistant cells in which Sp1 strongly associated with HDAC1/2 and PCNA (Fig. 6b). Consistently, intrinsically TMZ-resistant PtR#1 cells, not TMZ-sensitive Pt#3, also exhibited the similar characteristics with that of acquired TMZ-resistant cells, including A172R, Pt#3R, and U87MGR (Supplementary Fig. S4 and Fig. 6b). These results suggest that DHEA-induced cellular process is developed in TMZ-resistant glioblastoma. Moreover, we confirmed that DHEA decreases TMZ-induced cell death by regulating Sp1 function. MTT assay revealed that DHEA increases the cellular tolerance to TMZ, and Sp1 knockdown abolished it (Fig. 6c). In particular, Sp1 phosphorylation at residues T278/739 was important for Sp1-induced TMZ resistance (Fig. 6d). The effect of Sp1 was also abolished by knocking down HDAC1/2 and PCNA (Fig. 6e), suggesting that the interaction of Sp1

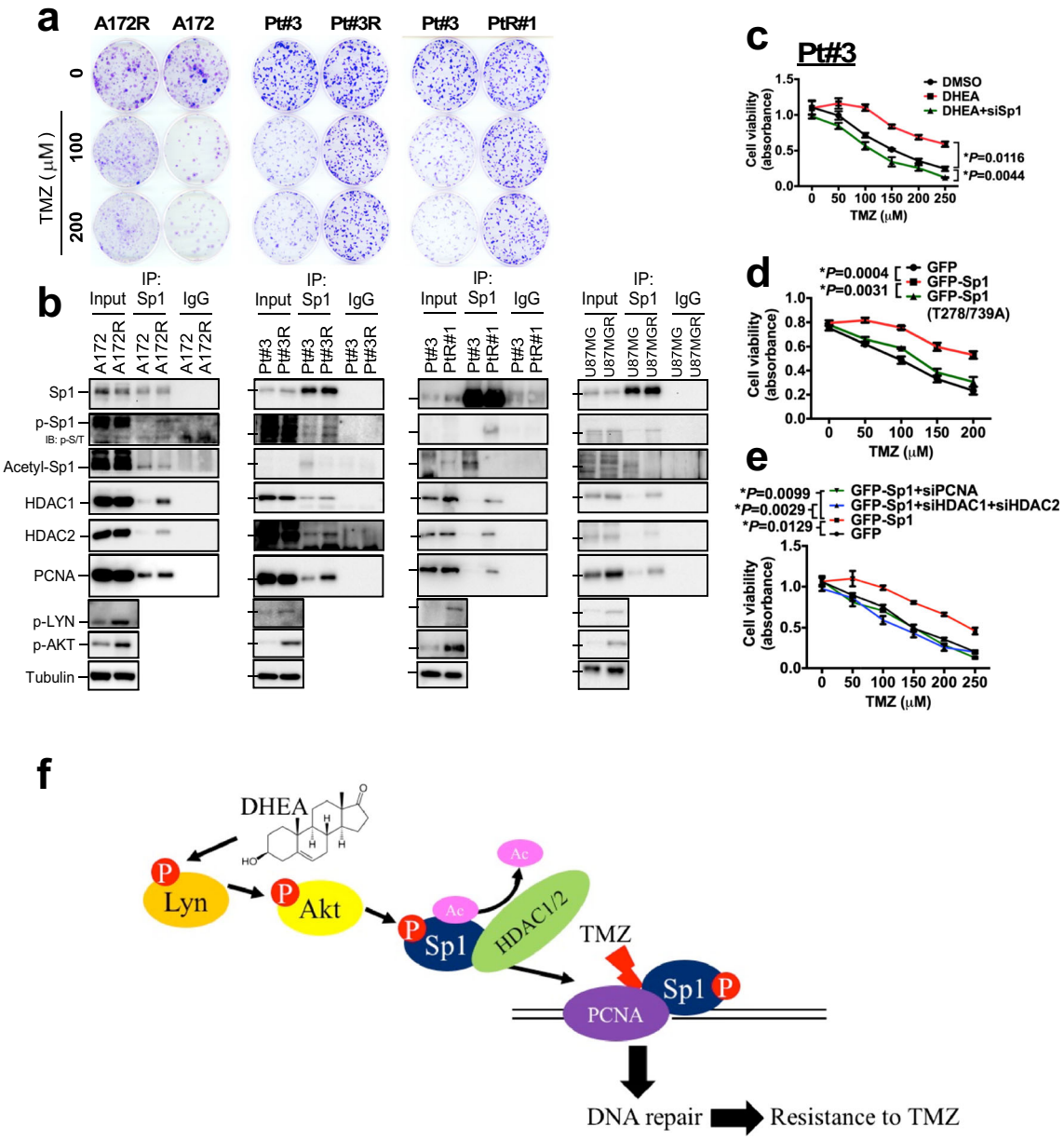

Fig. 6 DHEA-induced Sp1 phosphorylation and deacetylation confer TMZ resistance to glioblastoma. **a** In total, cells were cultured in 6-cm dishes for 16 days, and incubated with TMZ for the last 4 days. Cell colonies were stained by 0.5% crystal violet overnight. **b** After immunoprecipitation assay by using the anti-Sp1 antibody, western blotting was used to analyze precipitated complexes using indicated antibodies. **c** After transfection and pre-treatment with DHEA, cells were treated with TMZ, and the surviving proportion was estimated by the MTT assay. **d**, **e** After transfection and treatment with TMZ, the MTT assay was performed. Experiments were performed three times in replicate, and data are expressed as the mean ± s.e.m. (*P* values as indicated). **f** DHEA induces TMZ resistance by enhancing Sp1 (phospho- and deacetylated status)-regulated DNA repair

with HDAC1/2 and PCNA determines the role of Sp1 in regulating cellular sensitivity to TMZ. Based on our findings, we conclude that DHEA attenuates TMZ-induced DNA damage by enhancing DNA repair in order to induce cellular resistance to TMZ. The underlying mechanism involves the phosphorylation of Sp1 through the LYN-AKT cascade and the deacetylation of p-Sp1 by HDAC1/2, followed by association with PCNA for DNA repair (Fig. 6f).

Discussion

The effect of neurosteroidogenesis on drug resistance in cancer had never been determined until we clarified that CYP17A1 overexpression induces TMZ resistance in glioblastoma by increasing DHEA secretion [18]. In the present study, we uncovered the mechanism underlying DHEA-induced resistance to TMZ. By inducing post-translational modifications of Sp1, including phosphorylation and deacetylation, DHEA enhanced DNA repair to attenuate TMZ-induced DNA damage, leading to a reduction in cytotoxicity in glioblastoma cells.

In the brain, DHEA has been shown to exhibit a protective effect on neurons in response to ROS accumulation [15]. Herein, we identified a similar effect of DHEA on glioblastoma; DHEA protected glioblastoma from TMZ-induced cell death. It remains unknown whether DHEA also decreases ROS abundance to protect glioblastoma. However, we found that DHEA activates membrane-bound LYN and subsequently the AKT/Sp1 cascade, a novel mechanism, to mediate its protective effect. In addition to acting as an agonist of the androgen receptor, several cell survival-related kinases were shown to be activated by DHEA in previous reports, such as SRC, STAT3, AKT, and ERK [17, 25]. Only AKT was phosphorylated by DHEA-activated LYN in glioblastoma cells; this presents a discrepancy with other studies and requires further investigation. LYN, a tyrosine kinase belonging to the SRC family, has been shown to promote the development of imatinib resistance in leukemia [26] and correlate with antiestrogen resistance in breast cancer [27]. In the present study, DHEA-activated LYN induced TMZ resistance in glioblastoma, supporting the role of LYN in chemotherapeutic resistance.

Sp1 has been shown to regulate multiple cellular functions, but the role of Sp1 in drug resistance has not been determined, particularly Sp1 modified by post-translational modifications. Phosphorylation has been shown to promote the transcriptional activity of Sp1 [6], and several kinases have been shown to phosphorylate Sp1, such as ERK, AKT, and JNK [6]. In addition, Sp1 phosphorylation is essential to attenuate DNA damage [9, 11] and contributes to the enhancement of XRCC1 expression [8]. However, in the results of our microarray analysis and western blotting, Sp1 knockdown failed to affect the expression of DNA repair-related genes, including XRCC1, APEX1, and Mpg (Supplementary Fig. S5). This suggests that Sp1 regulates DNA repair via a transcription-independent mechanism. Therefore, we studied the association of phospho-Sp1 with PCNA, which is important to DNA repair and replication [28], and found that this association is required to attenuate DNA damage. Moreover, before associating with PCNA, phospho-Sp1 was deacetylated by HDAC1/2, which has been reported to enhance transcriptional activity [12]. After sequential phosphorylation and deacetylation, Sp1 acquired the ability to associate with PCNA to regulate DNA repair. Unfortunately, we are still uncertain about how the Sp1/PCNA complex repairs DNA directly, although a transcription-independent pathway has been mentioned previously [11]. Based on this evidence, the phosphorylation and acetylation of Sp1 may predict the efficacy of TMZ-mediated chemotherapy.

TMZ is a well-known alkylating agent used to trigger the DNA damage response. Aberrant activated DNA repair systems, including HR, BER, NER, and mismatch repair, are important characteristics of TMZ resistance [5, 29]. Understanding how these repair systems are activated in response to TMZ will be helpful for overcoming TMZ resistance. Here, we found that DHEA promotes HR efficiency, and Sp1 positively regulated it for attenuating the DNA damage response. This suggests that neurosteroids interfere with the cytotoxic effect of TMZ to reduce the efficacy of chemotherapy. However, there is a lack of studies that show whether neurosteroids directly regulate DNA repair, and this needs to be elucidated in the future. In Fig. 1, in contrast to decreasing HR, we were surprised that TMZ increased NHEJ efficiency, and DHEA abolished this effect. We speculated that enhanced NHEJ contributes to inducing resistance due to the occurrence of chromosome instability during the NHEJ process [30, 31]. The other typical reason to induce TMZ resistance through DNA repair is MGMT expression. MGMT is also a DNA repair protein through removing the methyl group from DNA and is well established as a hallmarker of TMZ resistance [3, 32]. In the current study, we clarified that DHEA-induced TMZ resistance is mediated by MGMT-independent pathway because the cell lines we used, including A172, Pt#3, and U87MG, exhibited negative MGMT expression (Supplementary Fig. 6A). Despite of that we established TMZ-resistant cells, MGMT was still maintained silence (Supplementary Fig. 6A). In particular, the silence of MGMT in A172 and U87MG has been shown to be caused by promoter methylation [32, 33]. However, we evaluate effect of DHEA on MGMT-positive glioblastoma cells, T98G, and found that T98G was absolutely resistant in response to TMZ treatment for 48 h (Supplementary Fig. 6B). In particular, DHEA slightly promoted the extent of TMZ resistance, but effect of DHEA on T98G did not reach significance. Besides, DHEA failed to affect MGMT expression. We

speculate that DHEA is not important for MGMT-positive T98G exhibiting intrinsic TMZ resistance (Supplementary Fig. 6B, C).

TMZ resistance is still an unsolved issue and an obstacle that must be overcome to improve patient prognosis. According to our results, the DHEA levels may predict the response of patients to TMZ, and blocking the action of DHEA may, synergistically with TMZ, kill glioblastoma cells. However, it remains unknown whether the CYP17A1 inhibitor abiraterone (Zytiga®), which decreases DHEA biosynthesis and is used for treating prostate cancer [34], can be used to treat glioblastoma; we plan to investigate this in the future.

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Author Contributions Wen-Bin Yang: execution of experiments and data organization.

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Chiun-Yung Ko: execution of experiments.

Wen-Chang Chang: manuscript preparation.

Tsung-I Hsu: concept development and integration, execution of experiments, data interpretation and organization, and manuscript preparation.

Compliance with Ethical Standards

The use of human specimens was approved by the Institute Review Board (IRB)/Ethics Committee from the office of human research in Taipei Medical University (Taipei, Taiwan). The consent of each patient was obtained and approved by Taipei Medical University IRB protocols, Nos. 201006011 and 201402018.

Conflict of interest The authors declare that they have no conflicts of interest.

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