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SETD6 is a negative regulator of oxidative stress response

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ABSTRACT

The protein methyltransferase SETD6 is a key regulator of proliferation and inflammatory processes. However, the role of SETD6 in the regulation of additional cell signaling pathways has not been well studied. Here we show that SETD6 is a negative regulator of the oxidative stress response. Depletion of SETD6 from cells results in elevated Nrf2 levels and a significant increase in Nrf2 antioxidant target gene expression. Using proteomic tools, we uncovered a novel interaction between SETD6 and the oxidative stress sensor DJ1, a protein required for Nrf2-dependent transcription of antioxidant target genes. We show that SETD6 binds DJ1 both *in-vitro* and in cells but does not methylate DJ1. Under basal conditions, SETD6 and DJ1 are associated at chromatin. Through this interaction, SETD6 inhibits DJ1 activity, which in turn leads to the repression of Nrf2-dependent transcription. In response to oxidative stress, the transcription of Nrf2 antioxidant genes increases. We here show that under this condition, SETD6 mRNA and protein levels are reduced, leading to elevation in Nrf2 expression level and to a weaken interaction between SETD6 and DJ1 at chromatin.
Taken together, these findings demonstrate that SETD6 negatively regulates the Nrf2-mediated oxidative stress response through a physical and catalytically independent interaction with DJ1 at chromatin.
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1. Introduction

Protein methylation like other well studied post-translational modifications (PTMs)—including phosphorylation, acetylation and others, is a key regulator of the biochemical and physiological properties, functions and interactions of proteins in eukaryotic cells [1]. A lysine residue in a given protein can be mono-, di- or tri-methylated by protein lysine (K) methyltransferases (PKMTs) [2,3]. The PKMT SET domain containing protein 6 (SETD6) mono-methylates the major NFKB subunit RelA on K310 and suppresses RelA-regulated target genes through GLP-dependent H3K9 methylation [4,5]. This lysine methylation signaling cascade has been shown to play a fundamental role in the regulation of proliferative and inflammatory processes. SETD6 depletion from several breast cancer cell lines was shown to reduce proliferation, up-regulate the cell cycle inhibitor CDKN1A and induce apoptosis [6]. However, the protein network and the cellular signaling pathways in which SETD6 is involved remain largely unknown.

DJ1, a product of the DJ1/PARK7 gene and belongs to the peptidase C56 family, is ubiquitously expressed and is involved in the regulation of diverse cellular processes [7–11], including its function as an oxidative stress sensor [12–14]. As part of the cellular response to oxidative stress, DJ1 regulates the expression and activity of transcription factors such as p53 [15,16], NFKB [17], SREBP2 [18], androgen receptor (AR) [19,20] and Nrf2 (Nuclear factor erythroid-derived 2) [21,22]. DJ1 has been shown to be required for Nrf2-mediated transcription of antioxidant stress target genes such as GCLC, HO-1 and others [23]. However, the exact mechanism by which DJ1 modulates the activity of Nrf2 is yet unknown.

In this study, we identified a novel physical interaction between SETD6 and DJ1. Surprisingly, SETD6 binds to but does not methylate DJ1. Under basal conditions, SETD6 and DJ1 associate with chromatin which inhibits DJ1 to activate Nrf2 transcription activity. In response to oxidative stress, SETD6 mRNA and protein levels are dramatically reduced. These findings correlate with a significant decrease in the physical interaction between SETD6 and DJ1 and elevated levels of Nrf2 leading to de-repression of Nrf2 target genes.

2. Materials and methods

2.1. Cell lines, transfection and treatments

Human erythromyeloblastoid leukemia K562 cells, human embryonic kidney 293T cells and human breast adenocarcinoma MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma), supplemented with 10% fetal bovine serum (GIBCO). Cells were cultured in a 37 °C humidified incubator with 5% CO_2. Cells were transfected with the TransIT-LT1 or TransIT-X2 transfection reagent (Mirus) according to the manufacturer's protocol. For stable transductions, HEK-293T cells were co-transfected with lentiviral packaging constructs as described [24] and target cells were incubated with viral

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supernatant in the presence of 8 μg/ml polybrene (Sigma). 48 h after transduction, cells were selected with puromycin (3 μg/ml) for 24 h. For oxidative stress experiments, cells were stimulated with Arsenite (5–10 μM; Sigma) or H_2O_2 (100–200 μM; Bio-Lab). For CRISPR/Cas9-mediated gene disruption, lentiCRISPR was purchased from Addgene (#49535) and four different gRNAs for SETD6 were cloned to the lentiCRISPR plasmid. Following transduction and puromycin selection, single clones were isolated and expanded.

2.2. Plasmids

Plasmids used for over-expression in cells were: pcDNA-HA-SETD6, pcDNA-HA-SETD6 Y285A pcDNA-FLAG-DJ1. For *in-vitro* assays, SETD6 and DJ1 were subcloned into pGEX-6P-1 and pETDuet-1. For MBT pull-downs, 3xMBT was expressed from pGEX-3xMBT (kindly provided by the Gozani lab) [25]. For luciferase assays, we used pGL2-4xARE-luc [26] (kindly provided by M. Hannink).

2.3. Luciferase assays

Cells were seeded in 24-well plates and transiently transfected with 0.2 μg firefly luciferase plasmid and 0.02 μg Renilla luciferase plasmid. Total amount of transfected DNA in each dish was kept constant by the addition of an empty vector as necessary. Cell extracts were prepared 30 h after transfection, and firefly luciferase activity was measured with the Dual-Glo Luciferase Assay system (Promega) and normalized to that of Renilla luciferase.

2.4. Antibodies

Primary antibodies used were as follows: SETD6 (GTX629891; GeneTex), DJ1 (ab76008; Abcam), Nrf2 (ab62352; Abcam), FLAG (F1804; Sigma), HA (05-904; Millipore), Actin (ab3280; Abcam), GST (ab9085; Abcam), H3 (ab10799; Abcam), Tubulin (ab44928; Abcam) and Pan-methyl (ab23366; Abcam). Secondary HRP-conjugated antibodies (goat anti-mouse and goat anti-rabbit) were from Jackson ImmunoResearch (115-035-062, 111-035-144 respectively). Coomassie was purchased from Expendon (ISB1L).

2.5. Immunoprecipitation

Cells were lysed in RIPA lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS (*v*/*v*), 1 mM dithiothreitol (DTT) and Sigma protease inhibitor cocktail (P8340, diluted 1:100)). Lysates were incubated for 1 h at 4 °C with 10 μl protein A/G beads (Santa Cruz Biotechnology) as a pre-clear step. Pre-cleared lysates were incubated overnight at 4 °C with SETD6 antibody (1 μg) or DJ1 antibody (4 μg) conjugated to beads or beads only as control. For over-expression experiments, cells were lysed as described above and incubated with FLAG-M2-affinity gel beads (A2220; Sigma). After incubation, beads were washed 4 times with lysis buffer, heated at 95 °C for 5 min in Laemmli sample buffer, and resolved by SDS-PAGE. MBT pull-downs were performed as previously described [25,27].

2.6. Mass-spectrometry

After SETD6 immunoprecipitation, SETD6-bound proteins were separated by SDS-PAGE. Gels were sliced to 1 mm squares and subjected to mass-spectrometry as previously described in Elharar et al. [28].

2.7. In-vitro methylation assays

Recombinant GST–DJ1 (1 μg) and/or GST–SETD6 (4 μg) were incubated overnight at 30 °C with 2 mCi ^{3}H-labeled S-adenosylmethionine (AdoMet; Perkin-Elmer) in methylation buffer (50 mM Tris–HCl pH 8, 10% glycerol (*v*/*v*), 20 mM KCl, 5 mM $MgCl_2$ and 1 mM phenylmethyl sulfonyl fluoride). Reaction mixtures were resolved by SDS-PAGE, followed by autoradiography for the detection of methylation events and Coomassie staining to validate the presence of all proteins in the reaction. GST–RelA 1–431 served as a positive control [5].

2.8. Enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Greiner Microlon 96 W) were incubated with 2 μg His–DJ1 (or BSA as a control) for 1 h at room temperature. The plates were then washed with phosphate buffered saline supplemented with 0.1% Tween® 20 (PBST) and blocked with 3% BSA in PBST for 1 h. Following blocking, the plates were washed and covered with 0.5 μg GST–SETD6 or GST protein (negative control) for 1 h. Plates were then washed and incubated with primary antibody (anti-GST, 1:4000 dilution) followed by incubation with secondary HRP-conjugated antibody (goat anti-rabbit, 1:2000 dilution). After addition of TMB reagent and 1N H_2SO_4, absorbance at 450 nm was detected using a Tecan Infinite M200 plate reader.

2.9. RNA extraction, reverse transcription and gene expression analysis

Total RNA was extracted with NucleoSpin RNA (Macherey-Nagel) according to the manufacturer's instructions. Extracted RNA (200 ng) was reverse-transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions. Real-time qPCR was carried out using the UPL probe library system (Roche). All samples were amplified in triplicates in a 384-well plate LightCycler 480 System (Roche). The expression levels were normalized with GAPDH using the 2-DDCt method [29]. The real-time qPCR primers were the following; SETD6; forward, 5′-ggatgaaaaggagcccaact-3′, reverse, 5′-ctaccatccgaagacaattcg-3′, GCLC; forward, 5′-atgccatgggatttggaat-3′, reverse, 5′-gatcataaaggtatctggcctca-3′, GCLM; forward, 5′-gttggaacagct gtatcagtgg-3′, reverse 5′-cagtcaaatctggtggcatc-3′, GSTP1; forward, 5′-tctccctcatctacaccaactatg-3′, reverse 5′-aggtcttgcctccctggt-3′, HO-1; forward 5′-cccttcagcatcctcagttc-3′, reverse 5′-gacagctgccacattaggg-3′, Nrf2; forward, 5′-gagacaggtgaatttctcccaat-3′, reverse 5′-tttgggaatgtgggcaac-3′ and GAPDH; forward, 5′-agccacatcgctcagacac-3′, reverse 5′-aatacgacca aatccgttgact-3′.

2.10. Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed according to the protocol of Ainbinder et al. [30]. Briefly, formaldehyde cross-linked protein-DNA complexes were immunoprecipitated by overnight incubation with the indicated antibodies. Precipitated DNA fragments were extracted with Chelex 100 resin (Bio-Rad) as described in [31, 32] and amplified by real-time qPCR with specific primers. The sequences of the primers used were 5′-cgcgggatgagtaacggt-3′ and 5′-gggagagctgattccaaactga-3′ for the GCLM promoter; 5′-atcgactgcggc aatcctag-3′ and 5′-cgtgactcagcgctttgtg-3′ for the GCLC promoter; 5′-ccctgctgagtaatcctttcccga-3′ and 5′-atgtcccgactccagactcca-3′ for human HO-1 promoter.

2.11. Protein–protein chromatin immunoprecipitation (ChIP)

Protein–protein ChIP was modified from a published protocol [31]. After cross-linking, cells were harvested and washed twice with phosphate buffered saline (PBS) and then lysed in 1 ml lysis buffer (20 mM Tris–HCl pH 8, 85 mM KCl, 0.5% NP-40 and 1% protease inhibitor cocktail; 10 min on ice). Nuclear pellets were re-suspended in 200 μl nuclei lysis buffer (50 mM Tris–HCl pH 8, 10 mM EDTA, 1% SDS and 1% protease inhibitor cocktail; 10 min on ice) and then sonicated (Bioruptor, Diagenode) with high power settings for 3 cycles, 6 min each cycle (30 s on/off). Samples were centrifuged (17 min, 13,000 rpm, 4 °C) and the soluble chromatin fraction was collected. The soluble chromatin was

immunoprecipitated as described above, washed according to the published protocol, resolved by SDS-PAGE gel and analyzed by immunoblot.

2.12. Biochemical fractionation

Biochemical fractionation was performed as previously described [33], with a final step added. Briefly, the chromatin pellet was re-suspended for 30 min in buffer C (RIPA buffer, 1 mM $MgCl_2$ and benzonase nuclease enzyme (Sigma)) and collected by low-speed centrifugation (5 min, 1700 × *g*, 4 °C).

3. Results

3.1. SETD6 interacts with DJ1 at the chromatin

The PKMT SETD6, a mono-methyltransferase, was recently identified as a key regulator of proliferation and inflammatory processes [5]. However, the role of SETD6 in cell signaling pathways, cancer and other diseases remains mostly unknown. In order to deepen our knowledge of SETD6, we used IP/MS to identify proteins that interact with endogenous SETD6 in K562 cells (Fig. 1A). Approximately 115 SETD6 bound proteins were identified (Supplementary Table T1), including DJ1, a sensor for oxidative stress that has been shown to protect neurons from oxidative stress and cell death. We validated the SETD6–DJ1 interaction by over-expressing tagged versions of the two proteins in HEK-293T cells (Fig. 1B). In addition, the interaction between endogenous SETD6 and DJ1 was confirmed in MDA-MB-231 cells (Fig. 1C), which express high levels of both DJ1 and SETD6 [6,34]. In biochemical fractionation experiments, that separate the cytosol from the nucleus, we observed that DJ1 and SETD6 are localized to all fractions including the chromatin (Fig. 1D). These were surprising results, due to the fact that DJ1 was never detected before to be present at the chromatin (Fig. 1D). In accordance with these findings, protein–protein ChIP experiments revealed that the interaction between SETD6 and DJ1 takes place at chromatin (Fig. 1E).

3.2. SETD6 directly interacts with but does not methylate DJ1

A direct physical interaction between purified SETD6 and DJ1 was observed by ELISA (Fig. 2A). This result suggested that DJ1 may serve as a direct target for methylation by SETD6. To test this hypothesis, we performed an *in-vitro* methylation assay (Fig. 2B). Surprisingly, DJ1 was not methylated by SETD6 (Fig. 2B). This result was confirmed by mass-spectrometry (data not shown). To determine if SETD6 methylation of DJ1 is mediated by other proteins, we performed MBT pull-down experiments and asked if over-expression of SETD6 resulted in altered DJ1 methylation levels (Fig. 2C). HEK-293T cells were transfected with either HA–SETD6 or an empty vector, and methylated proteins were isolated using GST-tagged MBT domain, which binds methylated proteins in a sequence non-specific manner [25,27]. Endogenous DJ1 was co-purified with GST–MBT, indicating that it is methylated in cells. However, no increase in DJ1 methylation was observed in the presence of HA–SETD6 (Fig. 2C). Consistent with these results, we could not identify a further methylation increase in Flag–DJ1 that was immunoprecipitated from MDA-MB-231 cells in the presence of HA–SETD6, using a Pan-methyl antibody (Fig. 2D). Furthermore, the catalytically dead SETD6 mutant still binds to DJ, suggesting that the interaction does not depend on SETD6 enzymatic activity (Supplementary Figure S1). Taken together, these data demonstrate that SETD6 physically interacts with but does not methylate DJ1.

3.3. SETD6 is down-regulated following oxidative stress

DJ1 plays a critical role in the cellular response to oxidative stress [10,35]. Having demonstrated that SETD6 interacts with DJ1, we investigated a potential role for SETD6 in the oxidative stress response. To this end, we measured SETD6 protein and mRNA levels in MDA-MB-231 cells after treatment with either H_2O_2 or Arsenite (ASN). SETD6 protein and mRNA levels were both reduced after 4 h of treatment (Fig. 3A–C). To further probe these changes in SETD6 protein levels, we performed biochemical fractionation experiments and found that in

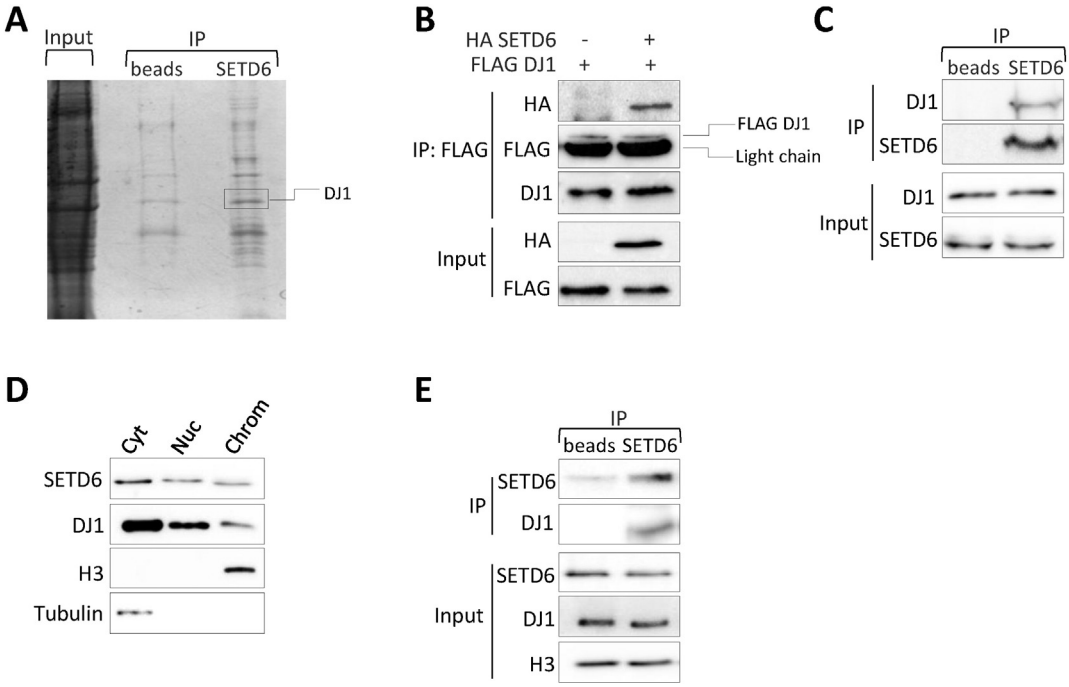

Fig. 1. SETD6 interacts with DJ1 in cells: (A) Coomassie staining of endogenous SETD6 immunoprecipitated from K562 cells. (B) HEK-293T cells were transfected with Flag–DJ1 alone or together with HA–SETD6. Cells were subjected to FLAG IP and analyzed by Western blot, using the indicated antibodies. (C) Immunoblot analysis of immunoprecipitated SETD6 (IP) or empty beads as control from MDA-MB-231 cells. (D) Immunoblot analysis of MDA-MB-231 cells biochemically separated into cytoplasmic (Cyt), nucleoplasmic (Nuc) or chromatin-enriched (Chrom) fractions; tubulin and H3 signals serve as controls for fractionation integrity. (E) Immunoblot analysis of SETD6 or control beads protein–protein ChIP, probed with the indicated antibodies.

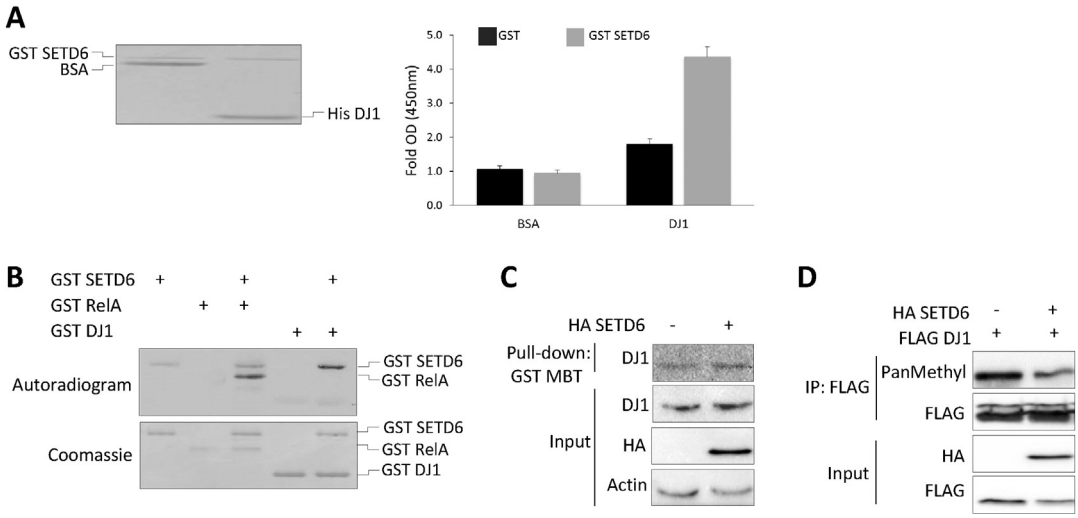

Fig. 2. SETD6 directly interacts with but does not methylate DJ1: (A) ELISA-based analysis of the interaction between recombinant GST–SETD6 and His–DJ1. 96-wells plate coated with 2 μg His–DJ1 or BSA as a control, covered with 0.5 μg GST–SETD6 and detected by GST antibody. Coomassie stain of the recombinant proteins used in the reactions is shown on the left. Data are from at least three experiments (error bars, S.E.M.). (B) *In-vitro* methylation assay in the presence of ^{3}H-labeled S-adenosyl methionine (SAM) with recombinant GST–DJ1 as substrate and GST–SETD6. Coomassie stain of the recombinant proteins used in the reactions is shown on the bottom. GST–RelA serves as a positive control for the reaction [5]. (C) HEK-293T cells transfected with empty vector or HA–SETD6 were subjected to MBT pull-down followed by Western blot with the indicated antibodies. (D) MDA-MB-231 cells were transfected with Flag–DJ1 alone or with HA–SETD6. Cells were subjected to FLAG IP and analyzed by Western blot, using the indicated antibodies.

response to H_2O_2, cytoplasmic SETD6 levels were slightly reduced. Strikingly, SETD6 levels at chromatin were dramatically reduced (Fig. 3D and Fig. 3E). Together, these data indicate that in response to oxidative stress, SETD6 levels at chromatin are reduced, and suggesting that SETD6 might modulate the transcription of antioxidant genes.

3.4. SETD6 is a negative regulator of Nrf2 transcriptional activity under basal conditions

An important mechanism in the cellular defense against oxidative stress is the activation of the Nrf2-antioxidant response element signaling pathway. It is known that DJ1 mediates Nrf2-dependent transcription of antioxidant target genes [22]. To evaluate the relationship between SETD6 and Nrf2-mediated transcription, we generated SETD6 knockdown MDA-MB-231 cells using the CRISPR/Cas9 system (Fig. 4A). Under basal conditions, Nrf2 protein and mRNA levels were dramatically increased after depletion of SETD6 with two independent guide RNAs (Fig. 4A and Fig. 4B, respectively), which correlate with the up-regulation of the two Nrf2 target genes HO-1 and GCLM (Fig. 4C and Fig. 4D) [23]. These data suggest that under basal conditions SETD6 negatively regulates the expression of Nrf2 and Nrf2 target genes.

Given that Nrf2 was up-regulated in the SETD6 depleted cells together with the fact we did not observe a physical interaction between SETD6 and Nrf2 (Supplementary Figure S2), we posited that SETD6's effect on Nrf2-dependent transcription is mediated by DJ1. To address this hypothesis we measured the endogenous expression of GCLM, GCLC and HO-1 genes by real-time qPCR in the presence of over-expressed DJ1 (Fig. 5A). These data suggest that SETD6 inhibits DJ1 activity which is required for Nrf2-dependent transcription. Having demonstrated that DJ1 and SETD6 interact at the chromatin (Fig. 1E), we next examined whether SETD6/DJ1 are enriched at the promotors of Nrf2 target genes. Chromatin immunoprecipitation (ChIP) analysis in MDA-MB-231 cells indeed revealed that the two proteins are enriched at Nrf2-regulated promoters together with Nrf2 (Fig. 5B).

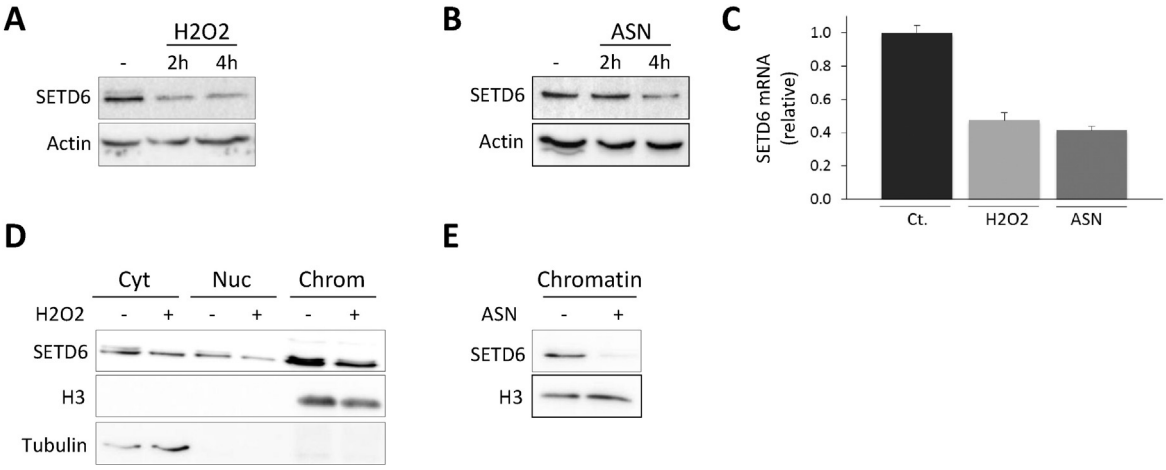

Fig. 3. SETD6 is down-regulated following oxidative stress: (A and B) MDA-MB-231 cells were treated with 200 μM H_2O_2 (A) or 5 μM Arsenite (ASN) (B) for the indicated time points followed by Western blot with the indicated antibodies. (C) Real-time qPCR analysis of SETD6 mRNA levels following treatments with 200 μM H_2O_2 or 10 μM ASN for 4 h or for 3 h, respectively. Data are from at least three experiments (error bars, S.E.M.). (D) Immunoblot analysis of SETD6 protein levels at the cytosol, nuclei and chromatin-enriched fractions upon H_2O_2 treatment. H3 and tubulin signals serve as controls for fractionation integrity. (E) Immunoblot analysis of SETD6 protein levels at the chromatin-enriched fraction upon ASN treatment.

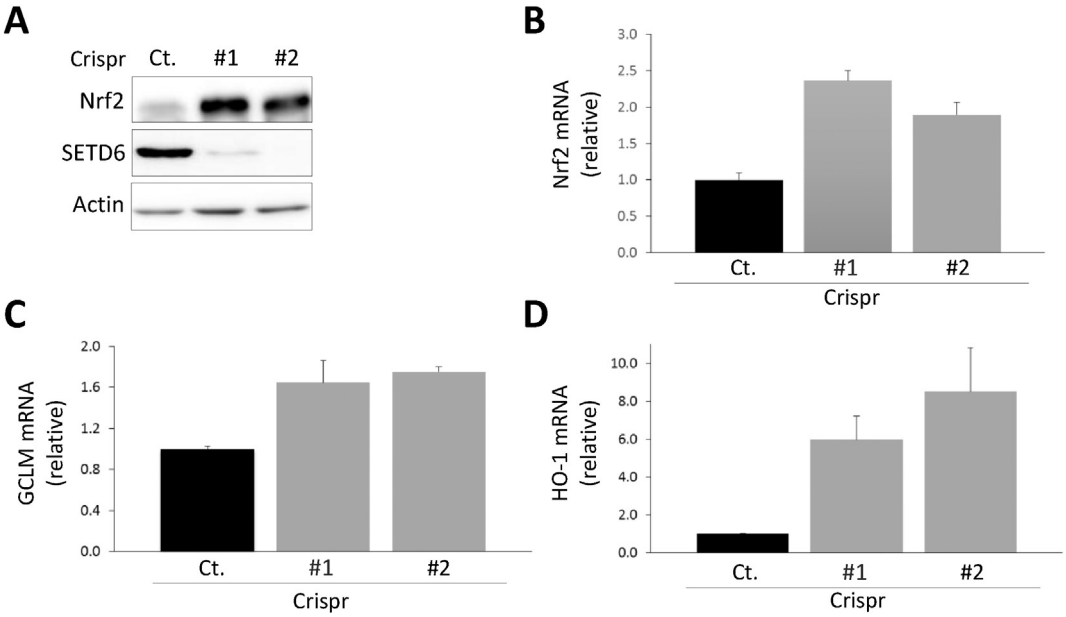

Fig. 4. SETD6 is a negative regulator of Nrf2 transcriptional activity: (A) Immunoblot analysis of SETD6 knock-down MDA-MB-231 cells targeted with two independent gRNAs. (B–D) Real-time qPCR analysis of Nrf2 and Nrf2 target genes in wild-type and SETD6 knock-down MDA-MB-231 cells. Results are normalized to GAPDH mRNA and are presented relative to wild-type cells (error bars, S.E.M.).

3.5. SETD6 regulates the expression of antioxidant genes under oxidative stress

To examine a potential role for SETD6 in regulating the expression of Nrf2 target genes in response to oxidative stress, we utilized a luciferase-based system containing an antioxidant response element (ARE-luc, [36]) (Fig. 6A). As expected, exposure of MDA-MB-231 cells to H_2O_2 resulted in an increase in luciferase activity (Fig. 6B). In SETD6 knock-down cells, luciferase activity was two-fold higher than in control cells (Fig. 6B). Consistent with these results, SETD6 depletion in the presence or absence of ASN treatment resulted in a significant increase in the endogenous expression of the anti-oxidative genes GCLC, GCLM and HO-1. No increase was observed for the Nrf2 target gene GSTP1, suggesting that not all of the known antioxidant target genes are regulated by the same mechanism (Fig. 6C). Based on these data, we conclude that under H_2O_2 and ASN treatment, SETD6 negatively regulates the transcription of several Nrf2 target genes.

We next measured ARE-luc activity in cells after DJ1 over-expression in the presence or absence of H_2O_2 treatment (Fig. 7A). As expected [21], DJ1 over-expression resulted in an increase in luciferase activity. However, an additional increase was observed in cells lacking SETD6. SETD6 level at chromatin is dramatically reduced in response to oxidative stress (Fig. 3D and 3E). Thus, we assumed that the physical interaction between SETD6 and DJ1 at chromatin might determine the inhibitory effect of SETD6 on Nrf2 transcriptional activity. To this end, we examined the interaction between SETD6 and DJ1 in the presence or absence of oxidative stress using protein–protein chromatin immunoprecipitation. The results revealed a significant decrease in the interaction between the proteins at chromatin after ASN or H_2O_2 treatment (Fig. 7B).

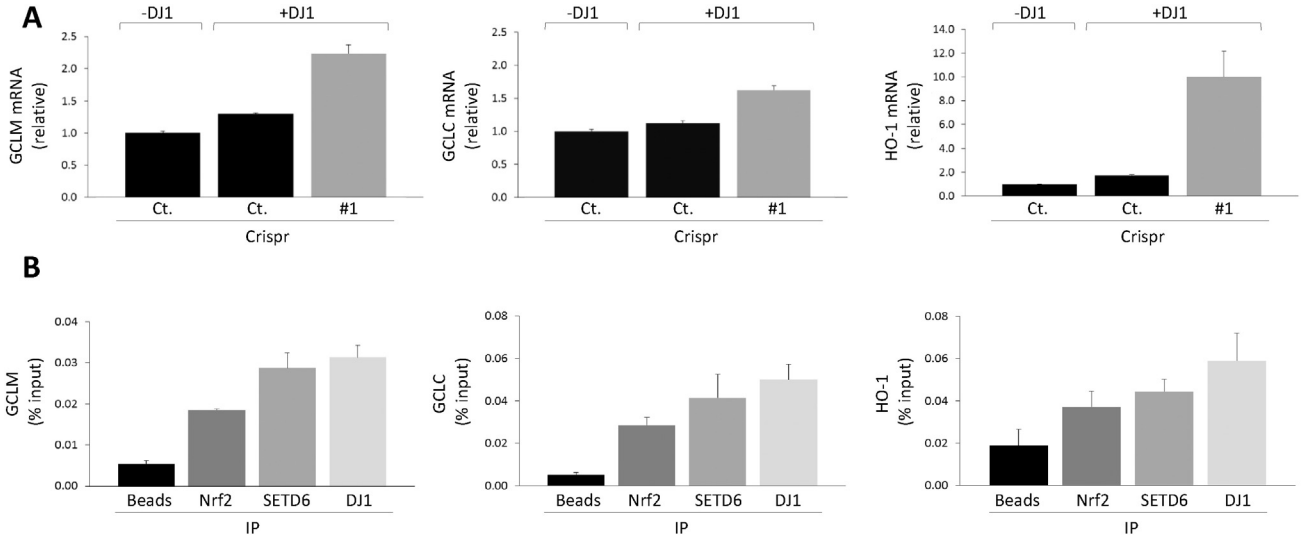

Fig. 5. DJ1 and SETD6 are enriched at Nrf2 target genes under basal conditions: (A) Real-time qPCR analysis for GCLM, GCLC and HO-1 mRNA with or without over-expression of Flag–DJ1 in wild-type or SETD6 knock-down MDA-MB-231 cells (error bars, S.E.M.). (B) ChIP real-time qPCR analysis of the occupancy of SETD6, DJ1 and Nrf2 (as positive control) at the promoters of GCLM, GCLC and HO-1 in MDA-MB-231 cells. Enrichment is presented as (ChIP/input) × 100.

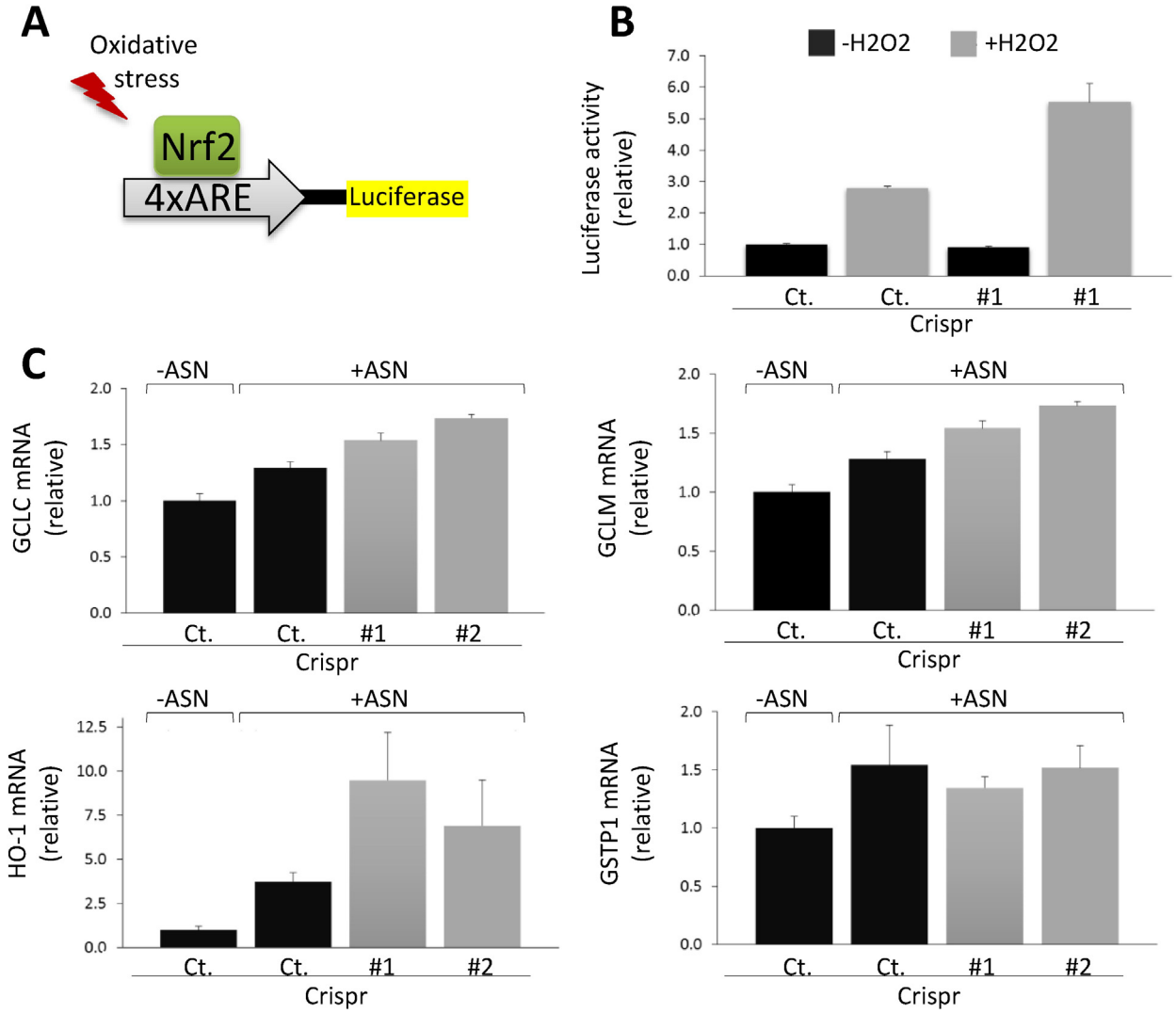

Fig. 6. SETD6 regulates the transcription of anti-oxidative genes in response to oxidative stress: (A) Schematic representation of luciferase ARE element induction following oxidative stress. (B) Wild-type or SETD6 knock-down MDA-MB-231 cells were transfected with 4xARE luciferase plasmid and Renilla plasmid and treated with 200 μM H_2O_2 for 4 h. Results are normalized based on Renilla luciferase expression. (C) Real-time qPCR analysis of Nrf2 target genes in wild-type or SETD6 knock-down MDA-MB-231 cells following 10 μM ASN treatment for 3 h. Results are normalized to GAPDH mRNA and are presented relative to wild-type cells without treatment. Data in B and C are from at least three experiments (error bars, S.E.M.).

Collectively, our data support a model (Fig. 7C) in which under basal conditions, SETD6 inhibits DJ1 to activate Nrf2. In response to oxidative stress, SETD6 level is reduced, leading to an increase in the cellular level of Nrf2. Simultaneously, a decrease in the interaction between SETD6 and DJ1, releases DJ1 to activate Nrf2-dependent transcription.

4. Discussion

While DJ1 has been linked to the regulation of Nrf2 mediated cellular oxidative stress response [10,22], the molecular mechanism by which this process is mediated is not clear. Here, we have identified the PKMT SETD6 as a new regulator of this signaling regulatory pathway. We show that SETD6 represses Nrf2 transcriptional activity under normal conditions by inhibiting DJ1. Under these basal conditions, SETD6 binds to DJ1 on chromatin but does not methylate it. In response to oxidative stress the transcription of Nrf2 antioxidant genes increases. We show that under this condition, SETD6 mRNA and protein levels are reduced which in-turn leads to elevation in Nrf2 expression level and to a reduced interaction between SETD6 and DJ1 on chromatin.

DJ1 has been shown to stabilize the Nrf2 protein in the cytosol by preventing its association with Keap1 [37,38]. In the present study we show that DJ1 is also functional in nucleus to mediate Nrf2 transcriptional activity. This suggests that DJ1 regulates Nrf2 activity by two different mechanisms at distinct cellular locations. It is unclear whether these mechanisms work simultaneously or are mutually exclusive; however it seems that both are important for maintaining cellular homeostasis.

We have previously shown that SETD6 catalytic activity negatively regulates the transcriptional activity of RelA [5]. We could not however, detect any enzymatic activity for SETD6 on nucleosomes [5]. This suggests that SETD6 might have a direct or indirect role in the regulation of active transcription by recruiting transcription factors and other chromatin associated proteins. Future genomic studies are needed to shed new light on this phenomenon.

Depletion of SETD6 leads to induction of Nrf2 level (Fig. 4B). Increased Nrf2 level has a direct effect on the activation and up-regulation of antioxidant target genes. How SETD6 regulates Nrf2 expression levels and the precise molecular mechanism that governs this effect were not tested here but open an important avenue for future

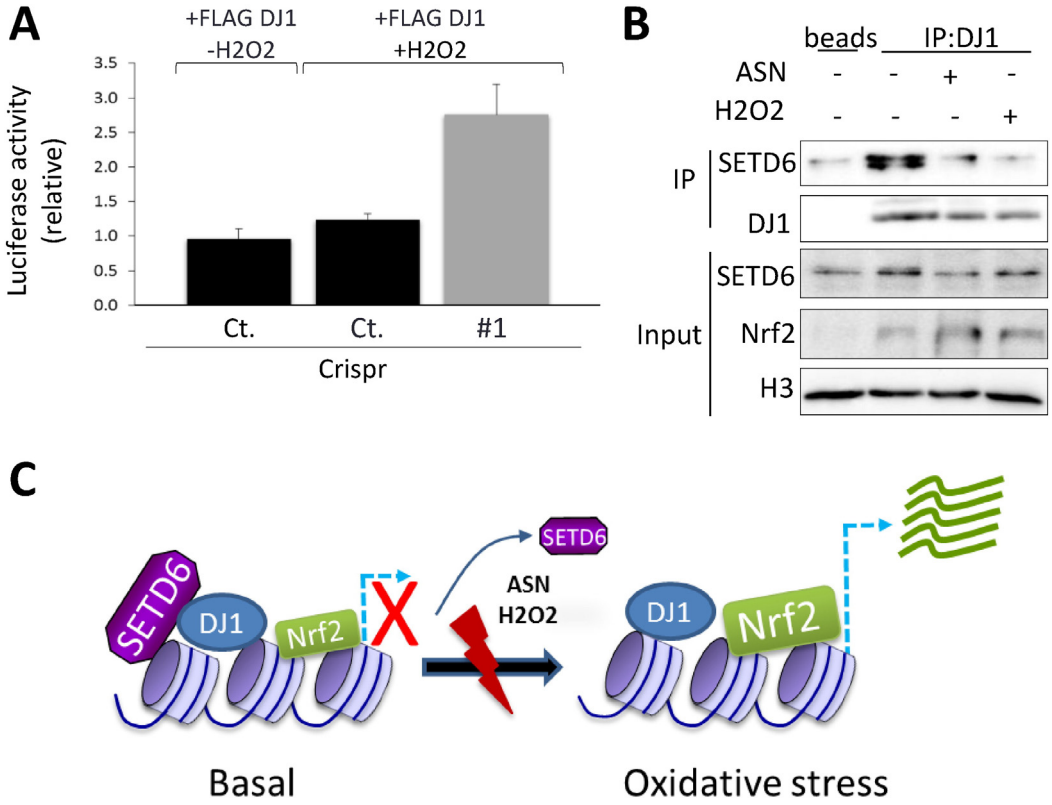

Fig. 7. SETD6 and DJ1 interaction is reduced upon oxidative stress: (A) Activity of ARE-luciferase reporter after transfection of wild-type or SETD6 knock-down MDA-MB-231 cells with Flag–DJ1 with or without H_2O_2 treatment for 4 h. Results are normalized based on Renilla luciferase expression. (B) MDA-MB-231 cells were treated with 10 μM ASN for 3 h or 200 μM H_2O_2 for 4 h after which they were subjected to protein–protein ChIP analysis with control beads or DJ1 antibody-coupled beads, followed by Western blot with the indicated antibodies. (C) Suggested model for the interplay between SETD6 and DJ1 on chromatin in response to oxidative stress (see text).

study. We do believe that SETD6 regulation of Nrf2 level and SETD6–DJ1 cross talk at chromatin are complement mechanisms to obtain an efficient cellular response to oxidative stress.

This is the first study linking SETD6 to oxidative stress. However, SETD6 is not the first methyltransferase shown to be involved in this process. SETD7/9 methylates FOXO3 directly *in-vitro* and in cells and FOXO3 acts as a transcriptional activator of oxidative stress programs. The methylation of FOXO3 by SETD7/9 down-regulates its transcriptional activity and leads to inhibition of cell death upon oxidative stress. Whether and how other PKMTs mediate this process merits further exploration.

SETD6-mediated regulation of the oxidative stress response through DJ1 is based on interaction only and not through a direct methylation of DJ1. Based on our experiments both *in-vitro* and in cells DJ1 is not methylated by SETD6. These are surprising results as we would predict that a direct physical interaction between the proteins would be coupled to a methylation event. This finding suggests that in addition to its activity as a methyltransferase, SETD6 might have other functions which are not related to its enzymatic activity. We could not however, exclude the possibility that other oxidative response proteins are subjected to methylation. As SETD6 does not methylate DJ1 (Fig. 2B) nor binds and methylates Nrf2 (Supplementary Figure S2 and data not shown) there is a possibility that there is an unknown protein which bridges the interaction within the protein complex and may be a SETD6 substrate for methylation that contribute to this regulation.

Together, our data identify a new regulatory mechanism for the anti-oxidative stress response pathway and link SETD6 for the first time to the regulation of oxidative stress. These findings reveal a new and important layer of regulation to maintain redox homeostasis in cells, with implications for normal physiology and disease.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2016.01.003.

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