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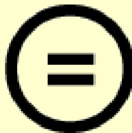

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2024년 2월

박사학위 논문

Rif1 methylation by EHMT2 maintains replication fork stability

조선대학교 대학원

의과학과

김 민 지

조선대학교
CHOSUN UNIVERSITY

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Rif1 메틸화에 따른 복제 포크 안정화 조절 연구

2024 년 2 월 23 일

조선대학교 대학원

의과학과

김 민 지

조선대학교
CHOSUN UNIVERSITY

Rif1 methylation by EHMT2 maintains replication fork stability

지도교수 이 정 희

이 논문을 이학 박사학위신청 논문으로 제출함

2023년 10월

조선대학교 대학원

의과학과

김 민 지

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위　원　　유 호 진 (인)

위　원　　온 탁 범 (인)

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위　원　　이 정 희 (인)

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국문초록

Rif1 메틸화에 따른 복제 포크 안정화 조절 연구

김 민 지

지도교수 : 이 정 희

조선대학교 일반대학원

의과학과

Rif1은 복제 포크 안정화, DNA 이중가닥 끊김(DSB) 복구 조절, 유전자 발현, 텔로미어 유지 등 다양한 기능을 수행하는 것으로 알려진 단백질입니다. 이번 연구는 지금까지 알려지지 않았던 Rif1의 DNA 복제에 대한 다각적인 역할을 새롭게 조명하는 것을 목표로 합니다. 정지된 복제 포크의 뉴클레아제 매개로 한 분해가 유전체 불안정성으로 이어지는 메커니즘은 잘 알려져 있지 않습니다. 본 연구는 Rif1과 EHMT2와의 상호작용 및 EHMT2에 의한 Rif1 메틸화를 통해 정지된 복제 포크의 분해를 방지하는 중요한 메커니즘을 밝혀냈습니다. 먼저

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Rif1 의 복제 포크 안정화 조절 기전 규명을 위해 Yeast two hybrids 분석을 통해 Rif1 에 결합하는 새로운 단백질 EHMT2 를 동정하였고, Rif1 과 EHMT2 의 상호작용 연구를 통해 유전체 안정성 조절에 있어 Rif1 의 새로운 조절 기전을 확인하였습니다. 또한 이 결과는 DNA 복제 과정에서 Rif1 의 중요한 역할을 이해하는 데 도움이 됩니다. EHMT2 는 단백질의 Lysin 잔기 (K) 에 메틸기를 붙이는 대표적인 메틸화 효소로 Rif1 의 메틸화에도 관여된다는 흥미로운 결과를 얻었습니다. Hydroxyurea (HU)는 DNA 복제를 일시적으로 중단시키는 물질로 HU 처리 조건에서 Rif1 의 메틸화 수준이 증가하였고 이는 복제 포크의 안정성과 상관관계가 있음을 확인하였습니다. 단백질 mass spec 분석 결과 Rif1 의 K1160 위치에서의 메틸화가 일어남을 보았고, 새롭게 생성된 DNA 복제 포크에 메틸화 된 Rif1 단백질이 위치함을 단일 세포 수준에서 관찰하는 SIRF (in situ quantification of proteins interactions at DNA replication forks) 분석 실험을 통해 밝혀냈습니다. 이것은 메틸화 된 Rif1 이 복제 손상으로 인해 멈춰진 복제 포크 초기 DNA 를 보호하는 데 중요한 역할을 한다는 것을 강조했습니다. 유전체 안정성 유지에 필수적인 복제 포크의 분해를 방지하고, 정지된 복제 포크를 효과적으로 재시작하는데 있어 Rif1 메틸화가 중요함을 밝힌 연구결과입니다. 복제 스트레스 상황에서 Rif1 메틸화를 통해 복제 포크를 효과적으로 재개함으로써 유전체 안정성을 유지한다는 연구입니다. 다시 한번 요약하면, 이

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연구는 유전체 안정성 유지에 필수적인 부분인 DNA 복제 포크를 보존하는 데 있어 EHMT2 에 의한 Rif1 K1160 메틸화가 중요하며 나아가 게놈 안정성 유지에 기여하는 분자 메커니즘에 대한 새로운 통찰력을 제공하고 메틸화와 관련된 Rif1 의 다양한 조절 시스템에 대한 새로운 관점 및 향후 연구의 잠재적 방향을 제시합니다.

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INTRODUCTION

DNA replication is a fundamental process in the life of a cell, serving as the cornerstone of genetic inheritance and the preservation of vital biological information. It ensures not only the accurate transmission of genetic material from generation to generation but also plays a significant role in maintaining the general health and stability of living organisms. At the core of genetic replication is the replication fork, a dynamic and pivotal structure responsible for the accurate duplication of our genetic information[1] However, this extraordinary biological feat does not transpire without its own set of challenges. Replication stress, a term used to describe circumstances that hinder or perturb the usual process of DNA replication, poses a constant threat to the genetic integrity of an organism. This stress arises from various factors, including DNA damage, clashes with transcription machinery, and the limited supply of nucleotides. When replication stress occurs, it endangers the entire genetic orchestra, pushing the replication fork into a vulnerable state[2].

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Crucially, protection of stalled replication forks is essential to respond to replication stress and minimize its impact on chromosome instability[3, 4]. An interesting mechanism for protecting stalled replication forks involves the concept of replication fork reversal[3, 5−9]. Reversal forks, initiated by enzymes such as Rad51, are formed in response to challenges to by DNA replication, such as DNA damage or obstacles to replication fork progression[10]. These structures play a crucial role in protecting stalled replication forks from potential degradation by nucleases. Key proteins such as BRCA1, BRCA2, and RAD51 are actively involved in protecting reversed replication forks from extensive nucleolytic degradation[8, 9]. This protection is essential to prevent the collapse and rearrangement of stalled forks, ensuring the integrity of the genomic material in the face of DNA damage or obstacles during replication[3, 6, 8, 9].

The initiation of reversal fork formation involves the unwinding of the leading arm, facilitating the transition of the replication fork into a protective four−way junction[10]. This complex process, which is essential for genome stability, becomes particularly important when replication encounters

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obstacles. Reversal fork formation allows stalled forks to be repaired and restarted, ensuring the integrity of the genome in the face of DNA damage or replication obstacles. Failure to form reversal forks in the face of such threats can lead to genomic instability, underscoring the importance of this mechanism in maintaining the integrity of the genetic material[3, 7]. In this context, Rap1-interacting factor 1 (RIF1), a protein known to protect reversed forks from degradation, plays a crucial role in the response to replication stress. By protecting the reversal replication forks, Rif1 contributes significantly to the overall stability of the genomic material, particularly under conditions where replication encounters obstacles or potential threats to its progress[11-13]. However, the mechanism by which Rif1 plays such an important role in protecting stalled replication forks is not well understood, and the aim of this study is to investigate the mechanism of replication stress-induced changes in genome stability.

EHMT2, also known as G9a, is a histone methyltransferase that belongs to the euchromatic histone lysine N-methyltransferase (EHMT) family. Its primary function is to catalyze the methylation of histone H3 at lysine 9 (H3K9).

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This enzymatic activity contributes to the modification of chromatin structure, specifically leading to the establishment of transcriptionally repressive chromatin regions. The addition of methyl groups to histones by EHMT2 is a critical epigenetic mechanism that influences gene expression by regulating the accessibility of DNA to the transcriptional machinery [14−24]. In addition to its role in chromatin modification, EHMT2 has been implicated in several cellular processes, expanding its functional range beyond epigenetic regulation. In particular, it has been implicated in DNA damage response and repair mechanisms. This suggests that the influence of EHMT2 extends to maintaining genomic stability and ensuring the integrity of the genetic material in the face of cellular stress, such as DNA damage.

Such as Post−translational modifications of proteins can affect a wide variety of biological processes, including protein activity, cellular signaling, stability, degradation, cellular structure and mobility, gene expression, and cell cycle regulation. These modifications are intricately linked to normal cellular function, development, and overall cell survival [25, 26]. Investigation of Rif1 modifications, particularly methylation, is crucial to understanding its role in

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maintaining genomic stability and responding to replication stress. This study is the first investigation of Rif1 methylation modifications, which is a critical step in understanding its regulatory processes.

In the specific context of Rif1 methylation, EHMT2 plays an important role as a key methyltrasferase. This protein is responsible for adding methyl groups to specific residues of Rif1, a protein involved in the protection of replication forks during DNA replication. The role of methylated Rif1 by EHMT2, especially under conditions of replication stress, is a major focus of our investigation, with significant implications for understanding how Rif1 functions to protect and stabilize replication forks. We will investigate the interaction between EHMT2 and Rif1 using IP experiments. In addition, we will use DNA fiber assays and SIRF (in situ quantification of proteins interactions at DNA replication forks) experiments to investigate the multiple effects of methylation on stalled replication forks. In addition, our study aims to elucidate the effect of Rif1 methylation on the DNA2 nuclease, a crucial enzyme responsible for reverse fork degradation. DNA2, a conserved helicase/nuclease involved in DNA end resection and replication stress response, plays a pivotal role in

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processing stalled replication forks and ensuring genomic stability[4, 27]. Understanding the regulatory mechanisms between Rif1 methylation and DNA2 activity is essential to unravel the intricate mechanisms of replication fork protection in the context of DNA replication stress[11−13]. We anticipate that our research will contribute to the maintenance of replication fork stability and genomic stability through Rif1 methylation. With the expectation that these findings will significantly contribute to maintaining replication fork stability and ensuring genomic integrity, this research seeks to expand the frontiers of knowledge in the field of molecular biology.

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RESULTS

1. Rif1 interacts with EHMT2

Mammalian Rif1 has multiple functions, including mediating non-homologous end joining (NHEJ) at double-strand breaks (DSBs), regulating replication origin timing and catenane resolution, and playing a critical role in ensuring replication fork stability and regulating DNA replication processes [13, 28]. The aim of our study was to identify novel binding partners of Rif1. To this end, we performed yeast two-hybrid experiments (Figure 1A). Among the candidate proteins (Figure 1B), EHMT2 stood out due to its reported strong homologous recombination repair activity [23]. EHMT2 functions as a histone methyltransferase [15]. The absence of methylation in the post-translational modifications of Rif1 increased our interest in EHMT2. Therefore, we first performed an immunoprecipitation (IP) experiment to investigate whether Rif1 and EHMT2 interact. Endogenous Rif1 was observed to co-immunoprecipitate with endogenous EHMT2 under conditions with or without treatment with ionizing radiation (IR) and hydroxyurea (HU) and reciprocally (Figure 2A).

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Furthermore, interactions between GFP−Rif1 and HA−EHMT2 were detected by ectopic expression and co−immunoprecipitation of GFP−Rif1 and HA−EHMT2 in HEK293T cells (Figure 2B). To determine which specific EHMT2 domain is involved in this interaction, we generated truncated constructs containing only the SET domain (EHMT2−SET) and without the SET domain (EHMT2−ΔSET) and performed an additional IP experiment (Figure 2C). GFP−Rif1 co−immunoprecipitated with both EHMT2−WT and EHMT2−ΔSET, suggesting that the EHMT2 SET domain, which has histone methyltransferase (HMTase) activity [14, 15, 19, 20] is not important for interaction with Rif1 (Figure 2D). Instead, it suggests that another region of EHMT2, possibly the ankyrin (ANK) domain, plays a critical role in mediating this interaction. This finding helps to elucidate the specific domain of EHMT2 involved in the interaction with Rif1.

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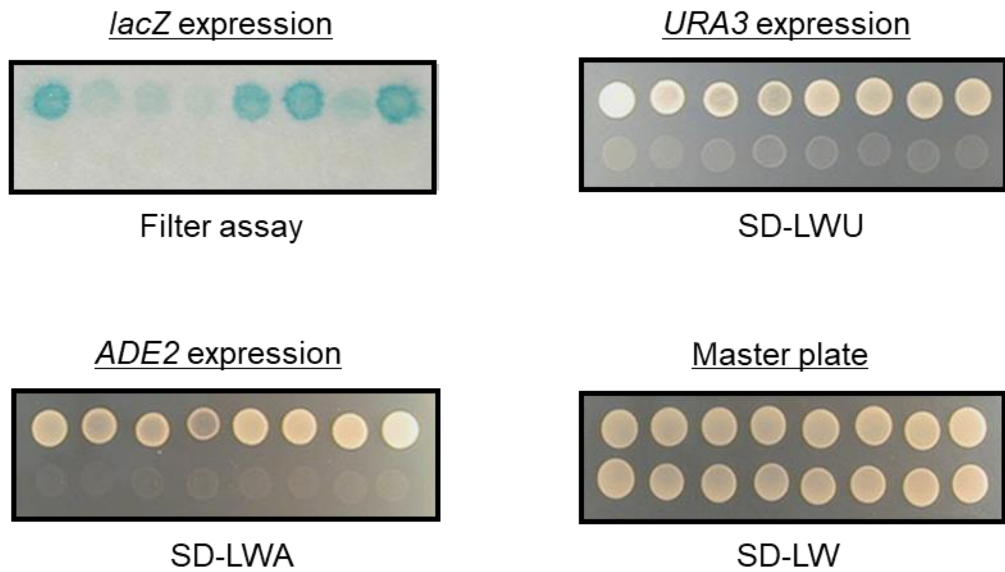
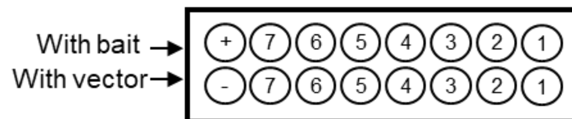
Figure 1

A



Number: each of AD-hybrid preys
+: positive control of protein-protein interaction
-: negative control of protein-protein interaction



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B

Prey ID	Description	Reporter expression		
		lacZ	*URA3*	*ADE2*
AD-Hybrid - 1	The activation domain (AD) is fused to 5' UTR (untranslated region) of RNA binding protein with multiple splicing (**RBPMS**), transcript variant 3 mRNA at -308nt (NM_001008712).	+	+	+
AD-Hybrid - 2	The activation domain (AD) is fused in frame to the 41st aa of cDNA FLJ26957 fis, clone SLV00486 (AK130467).	+	+	+
AD-Hybrid - 3	The activation domain (AD) is fused in frame to the 1196th aa of caspase 8 associated protein 2 (**CASP8AP2**), transcript variant 1 (NM_012115).	+	+	+
AD-Hybrid - 4	The activation domain (AD) is fused in frame to the 65th aa of E2F transcription factor 5 (**E2F5**), transcript variant 1 (NM_001951).	+	+	+
AD-Hybrid - 5	The activation domain (AD) is fused in frame to the 19th aa of euchromatic histone lysine methyltransferase 2 (**EHMT2**), transcript variant 4 (NM_001318833).	+	+	+
AD hybrid - 6	The activation domain (AD) is fused in frame to the 321st aa of coiled-coil alpha-helical rod protein 1 (**CCHCR1**), transcript variant 1 (NM_001105564).	+	+	+
AD hybrid - 7	The activation domain (AD) is fused to progesterone immunomodulatory binding factor 1 (PIBF1) (NM_006346).	+	+	+

Figure 1. Search for Rif1−binding proteins using Yeast Two−Hybrid Screening assay.

(A) Yeast cells were co−transformed with a bait plasmid expressing GAL4−DNA binding domain (BD)−fused Rif1 N−terminal domain (2−980 aa) and a prey plasmid expressing GAL4 transcription activation domain−fused HeLa cDNA library. Interactions between prey and bait proteins were monitored using visible blue color (filter assay) on SD−LWU and SD−LWA plates. Out of the transformants that were screened, 7 independent positive clones were

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isolated. (B) Table summarizing the identified clones resulting from the screening process using Rif1 as bait, were providing relevant information about their interactions.

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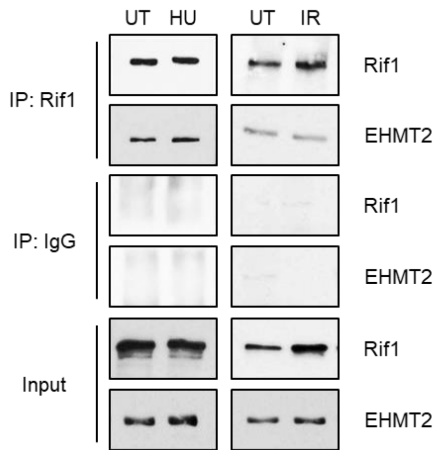
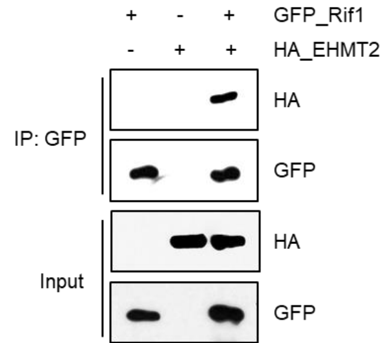
Figure 2

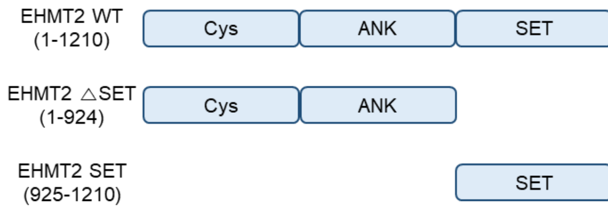
A



B



C



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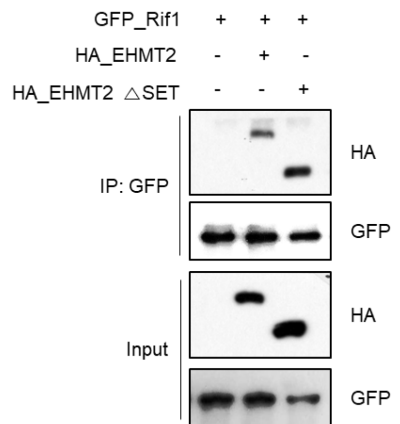

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Figure 2. Rif1 binds to EHMT2.

(A) HeLa cells were lysed and subjected to immunoprecipitation followed by immunoblotting with the indicated antibodies either 3 hours after ionizing radiation (IR) or 3 hours after HU treatment. (B) HEK293T cells were transfected with GFP−RIF1, with or without HA−EHMT2. Immunoprecipitation was performed on total cell lysates using an anti−GFP antibody, followed by detection with the indicated antibodies. (C) The mutant EHMT2 constructs used in this study are shown schematically. (D) HEK293T cells were co−transfected with GFP−RIF1 and the indicated HA−EHMT2 constructs. Subsequent immunoprecipitation and immunoblotting procedures were performed with the indicated antibodies.

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2. EHMT2 induces methylation of Rif1

Histone methyltransferases methylate a variety of nuclear and cytoplasmic proteins, as well as histones[19]. Lysine methylation of proteins, such as EHMT2, plays a crucial role in the transcriptional regulation and protein stability of transcription factors [19]. Therefore, we hypothesized that EHMT2 methylates Rif1 and lysine methylation may also be significant for the function of Rif1. First, to investigate whether EHMT2 methylates Rif1, we examined the level of methylation of Rif1. HeLa cells treated with or without ionizing radiation (IR) and hydroxyurea (HU), and performed co-immunoprecipitation (IP) using anti-Rif1 and methylated lysine antibodies. The results showed an increase of Rif1 methylation levels in HU-treated cells, but not in IR-treated cells (Figure 3A). To confirm the findings, HeLa cells were transfected with EHMT2 siRNA and co- immunoprecipitated with anti-Rif1 and methylated lysine antibodies. A decrease in methylation of Rif1 was observed in the EHMT2 depleted cells (Figure 3B). Similarly, when HEK293T cells were transfected with GFP-Rif1 WT either with or without HA-EHMT2, we observed a corresponding increase in methylated GFP-Rif1 levels after co- immunoprecipitation with anti-

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methylated lysine and GFP antibodies (Figure 3C). To investigate endogenous Rif1 methylation mediated by EHMT2, HeLa cells were treated with BIX-01294, an EHMT2 inhibitor[29], and Rif1 methylation levels were measured. The results confirmed the role of EHMT2 in catalyzing Rif1 methylation, as Rif1 methylation levels decreased in BIX-01294-treated HeLa cells (Figure 3D). EHMT2 is characterized by its Su(var)3-9, Enhancer-of-zeste, Trithorax (SET) domain and plays a critical role in histone methyltransferase (HMTase) activity. Overexpression of HA-EHMT2 WT significantly increased Rif1 methylation levels, whereas deletion of the SET domain in HA-EHMT2 △SET failed to induce such an increase (Figure 3E). Taken together, these results support the notion that EHMT2 methylates Rif1.

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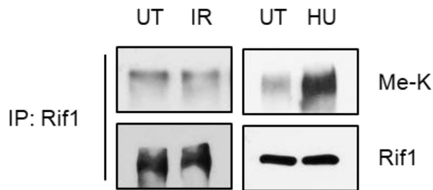
Figure 3

A



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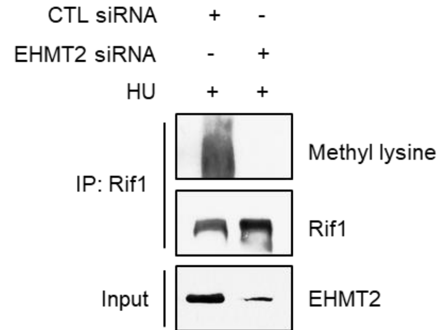

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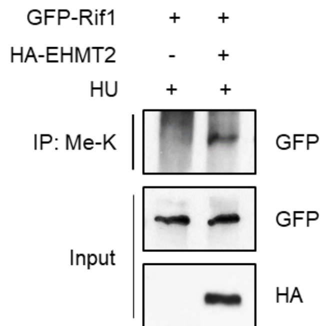

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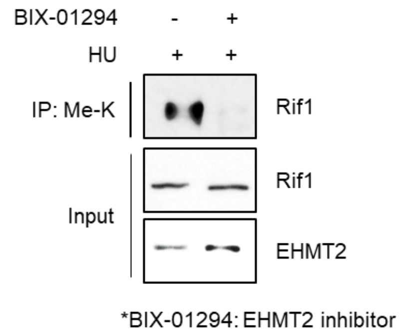

*BIX-01294: EHMT2 inhibitor

E

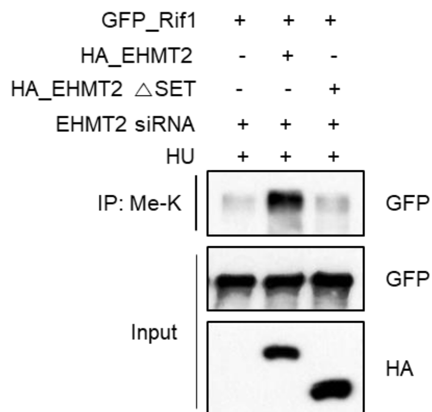

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Figure 3. The methylation of Rif1 by EHMT2

(A) Cell lysates from HeLa cells were obtained either three hours after ionizing radiation (IR) treatment or three hours after HU treatment. Subsequent procedures included immunoprecipitation with a Rif1 antibody followed by immunoblotting with a methyl-lysine antibody. (B) HeLa cells were transfected with siRNA and immunoprecipitated with anti-Rif1. The associated proteins were eluted, resolved by SDS-PAGE, and immunoblotted with anti-methyl-lysine antibody. (C) HEK293T cells were transfected with GFP-Rif1 WT either with or without HA-EHMT2. Immunoprecipitation was performed using anti-methyl lysine antibodies, and the eluted proteins were analyzed by Western blotting with anti-GFP antibodies. (D) HeLa cells were exposed to BIX-01294 at a concentration of 5 μM for a duration of 24 hours, immunoprecipitated with anti-methyl lysine antibodies, and the associated proteins were eluted. These proteins were then resolved by SDS-PAGE and immunoblotted with anti-Rif1. (E) Cell lysates from HEK293T cells were transfected with GFP-Rif1 WT and the indicated plasmids or siRNA. Immunoprecipitation was performed using anti-methyl-lysine antibodies, and

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the associated proteins were subsequently eluted. These eluted proteins were then separated by SDS−PAGE and immunoblotted with anti−GFP antibody. (B−E) Lysed after treatment with HU at 5mM for 3 hours.

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3. Rif1 is methylated at K1160 in response to HU.

Methylation of proteins is a crucial post−translational modification that can have a significant impact on their functions. To precisely localize the methylation site(s) on Rif1, we used a multi−step approach that included Coomassie Blue staining for protein visualization and comprehensive protein pattern analysis under different treatment conditions. In this experimental design, we used GFP−tagged Rif1 WT transfected cells and exposed them to different conditions, including untreated samples, exposure IR at 5 Gy, and treatment with HU at 5 mM. These different conditions allowed us to study how Rif1 methylation might be affected by different cellular stressors. We then performed SDS−PAGE separation of the protein samples, followed by detailed analysis using ion trap mass spectrometry (Figure 4A). Our analysis included extensive enzymatic digestion using trypsin and chymotrypsin, which allowed us to identify the specific coverage regions of the Rif1 protein. Interestingly, our results revealed that methylation occurred exclusively at a specific site, specifically the lysine 1160 residue within the Rif1 fragment (Figure 4B).

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Methylation sites were exclusively detected in HU−treated cells, as revealed by mass spectrometry analysis to identify methylation sites, and were significantly absent in ionizing radiation (IR)−treated cells (Figure 4B). These findings are consistent with our previous research and have led us to shift our focus to HU−induced signaling pathways (Figure 4C). To validate and further confirm this intriguing discovery, we performed a Lysine−to−Arginine mutation, converting Lysine (K) to Arginine (R) at K1160 site, and performed a comparative analysis of methylation levels between GFP−Rif1 WT and GFP−Rif1 K1160R using an co−IP assay (Figure 4D). The result of this experiment provided compelling evidence, clearly demonstrating lower methylation levels in GFP−Rif1 K1160R compared to wild−type GFP−Rif1.

Taken together, these results highlight the critical role of EHMT2 in directing Rif1 methylation at the K1160 site and shed light on the precise regulatory mechanisms underlying this important post−translational modification.

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Figure 4

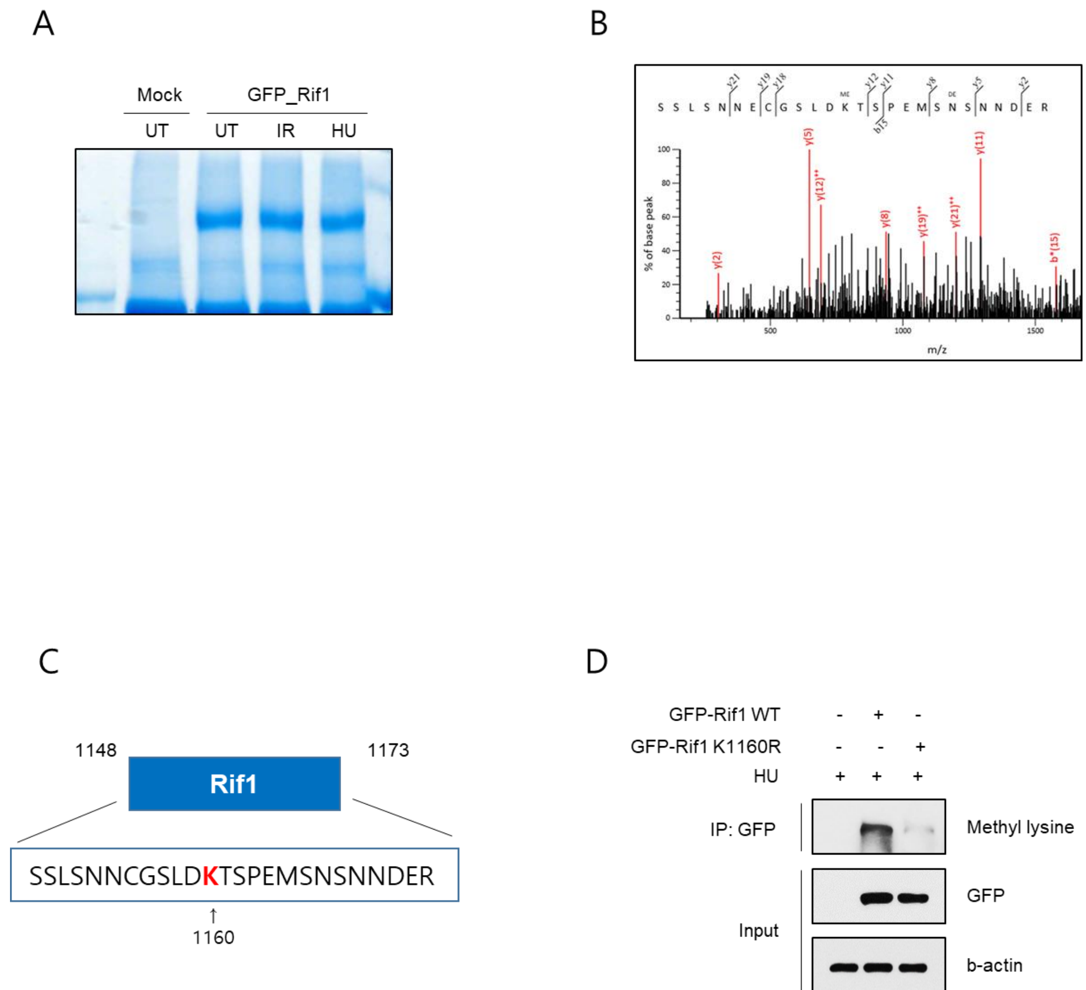

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Figure 4. EHMT2 is mediated methylation of Rif1 at K1160.

(A) HEK293T cells were transfected with GFP Rif1 WT and were either untreated or treated with IR (5Gy) or HU (5mM), respectively. They were then separated by SDS-PAGE and subjected to mass spectrometry analysis. **(B)** GFP-Rif1 modified with methylation at K1160 in response to HU. The peptides containing Lys 1160 methylation are indicated. **(C)** Schematic diagrams of the methylation of Rif1 at Lysine 1160 residues are shown. **(D)** HEK293T cells were transfected with either the control GFP vector, GFP-Rif1 WT, or GFP-Rif1 K1160R constructs followed by treatment with 5mM HU. Cell lysates were subjected to immunoprecipitation with an anti-GFP antibody followed by immunoblot analysis with an anti-methyl lysine antibody.

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4. Rif1 and EHMT2 contribute to the protection of replication forks

RIF1 promotes replication fork protection and efficient restart to maintain genome stability[13]. To investigate whether EHMT2, which is known to interact with Rif1, also contributes to replication fork protection, we performed experiments using a DNA fiber assay focused on replication fork protection under stress conditions. For this assay, cells were sequentially labeled with IdU (green) and CIdU (red) and HU was used to arrest ongoing replication forks. To measure fork degradation, we used the length of CIdU, which was shorter after HU treatment (Figure 5A). We analyzed the extent of degradation of individual replication forks in Rif1-depleted HeLa cells, EHMT2-depleted cells, and cells depleted of both Rif1 and EHMT2 (Figure 5B). Notably, both Rif1-depleted and EHMT2-depleted cells showed a significant reduction in CIdU tract length. However, when Rif1 and EHMT2 were co-depleted, no additional decrease in CIdU tract length was observed (Figure 5C). Taken together, these data suggest a regulatory role for EHMT2 in Rif1-mediated replication fork protection.

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Figure 5

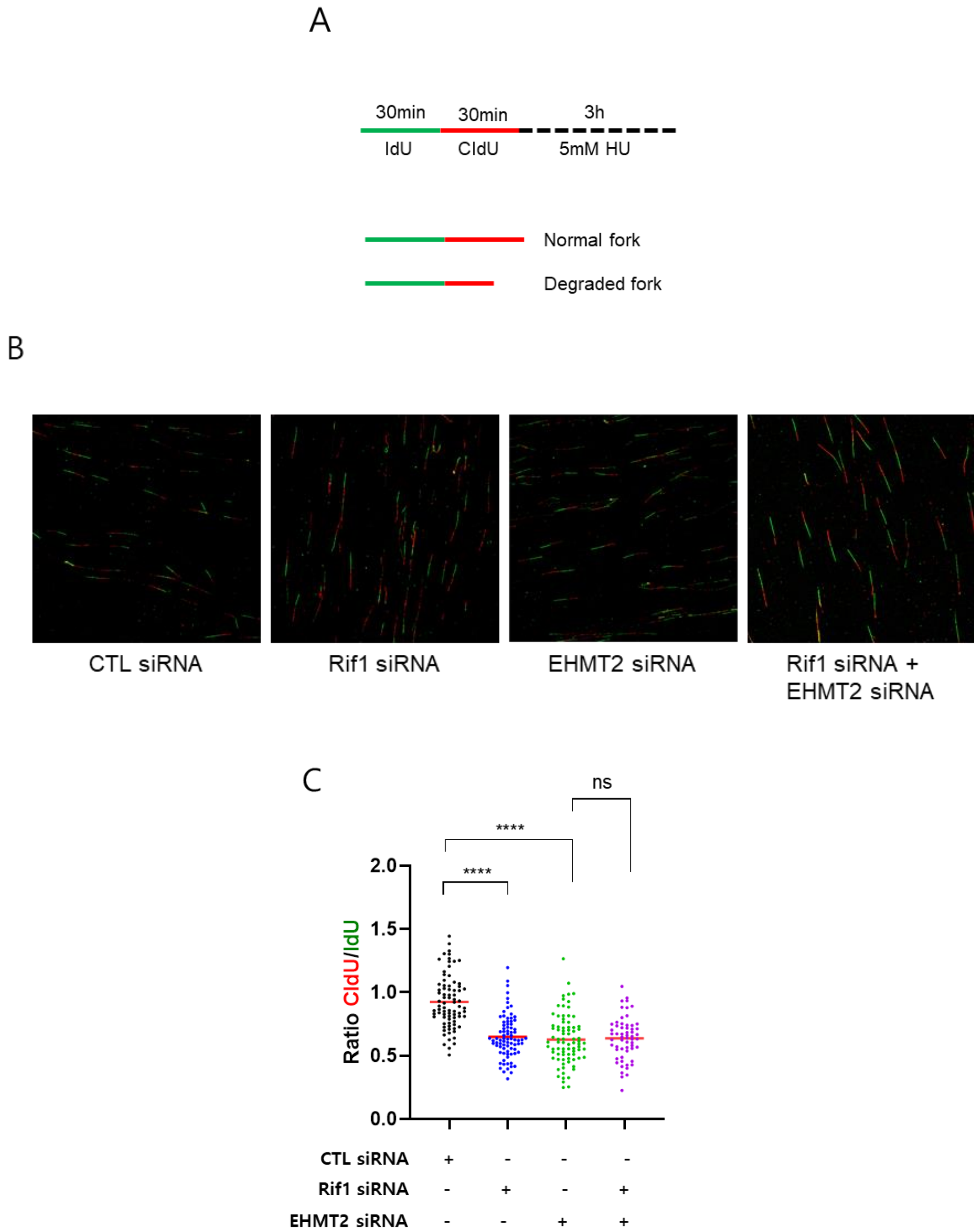

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Figure 5. Replication fork protection by Rif1 and EHMT2

(A) Schematic illustrating the labeling of cells in the fork degradation assay. Representative images of normal and degraded forks are presented below the schematic. **(B)** Replication fork degradation rates were assessed in control, Rif1-depleted, EHMT2-depleted, and Rif1- and EHMT2-depleted HeLa cells. Cells were labeled with IdU (green) and CIdU (red) for 30 minutes each, followed by measurement of IdU and CIdU track lengths after 3 hours of HU treatment. **(C)** The ratio of CIdU to IdU tract length was plotted as a readout for fork degradation. The results are the (SD) of triplicates of biological repeats. Student's t-test was done. ****P < 0.0001.

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5. EHMT2 mediated Rif1 methylation protect replication fork.

We investigated the effect of EHMT2-mediated methylation of Rif1 on replication fork protection under stress conditions using a DNA fiber assay. To validate our findings, we initiated Rif1 knockdown by siRNA targeting the UTR and reconstituted it with ectopically expressed GFP-Rif1 WT or GFP-Rif1 K1160R mutant, followed by HU treatment. We then observed replication fork degradation (Figure 6A). Rif1-depleted cells showed a higher level of fork degradation, and reconstitution of GFP-Rif1 WT in Rif1-depleted cells, but not GFP-Rif1 K1160R, rescued fork degradation (Figure 6B). This suggest that methylation of Rif1 at K1160 plays a critical role in replication fork protection.

Consistently, EHMT2 was depleted using siRNA targeting the UTR region, and subsequent reconstitution involved the introduction of ectopically expressed HA-EHMT2 WT or HA-EHMT2 △SET, representing the methyltransferase activity domain, followed by HU treatment (Figure 7A). Our observations revealed that reconstitution with HA-EHMT2 WT effectively reduced fork degradation, whereas reconstitution with HA-EHMT2 △SET did not, suggesting that the methylation activity of EHMT2 is indispensable for

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replication fork protection (Figure 7B). Taken together, our results suggest that K1160 methylation of Rif1 by EHMT2 interact synergistically and play a critical role in maintaining replication fork stability.

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Figure 6

A

CTL siRNA

Rif1 siRNA

Mock

Mock

GFP Rif1 WT

GFP Rif1 K1160R

B

Ratio CldU/IdU

2.0
1.5
1.0
0.5
0.0

****

****

ns

GFP-Rif1 WT	-	-	+	-
GFP-Rif1 K1160R	-	-	-	+
Rif1 siRNA	-	+	+	+

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Figure 6. Replication fork protection through methylated Rif1

(A) HEK293T cells were transfected with either control or Rif1−depleted cells reconstituted with GFP−Mock, GFP−Rif1 WT, or GFP−Rif1 K1160R constructs. After transfection, the cells were labeled with IdU and CIdU, followed by exposure to 5 mM HU for 3 hours to induce replication stress. Replication fork degradation rates were then measured. **(B)** The ratio of CIdU to IdU tract length was plotted as a readout for fork degradation. The results are shown as means ± SD (n=3), . ****P < 0.0001.

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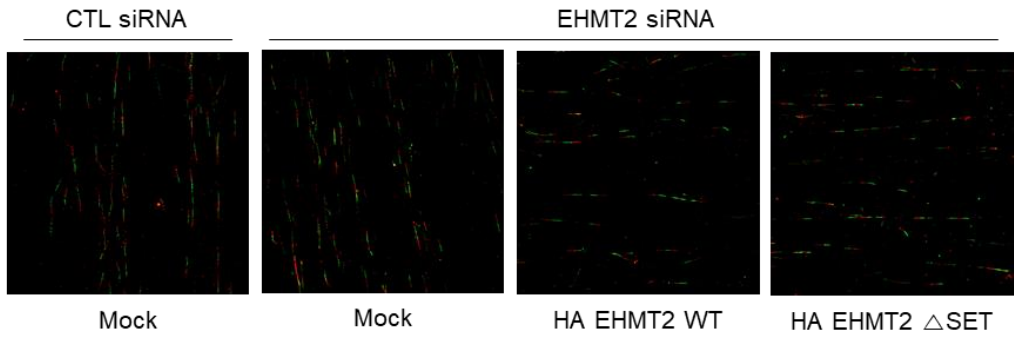
Figure 7



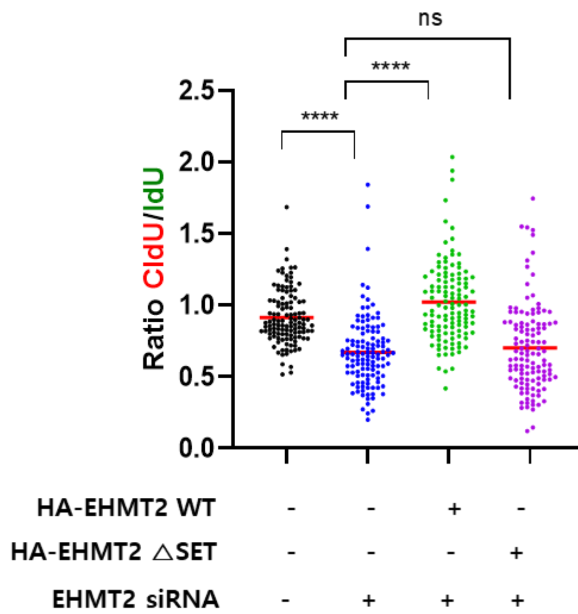


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Figure 7. EHMT2 Methyltransferase regulates Replication Fork Protection.

(A) HEK293T cells were transfected with either control or EHMT2 depleted cells reconstituted with HA-Mock, HA-EHMT2 WT, or HA-EHMT2 △SET DNA. After transfection, the cells were labelled with IdU and CIdU and exposed to 5 mM HU for 3 hours to induce replication stress. Replication fork degradation rates were measured. **(B)** The ratio of CIdU to IdU tract length was plotted as a readout for fork degradation. The results are shown as means ±SD (n=3), . ****P < 0.0001.

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6. Rif1 and EHMT2 recruitment to stalled replication forks.

RIF1 localization to DNA double−strand breaks (DSBs) is dependent on 53BP1, but its enrichment at stalled replication forks occurs independently of 53BP1[13]. However, the mechanism of RIF1 recruitment to stalled replication forks remains elusive. To investigate whether EHMT2 mediates RIF1 recruitment to stalled replication forks, we first examined the recruitment of EHMT2 to stalled replication forks in HU−treated HeLa cells after EdU labeling. As a positive control, we performed a parallel experiment to confirm that RIF1 was indeed recruited to stalled replication forks. In contrast to the co−localization of EdU and RIF1 at DNA stalled forks (Figure 8A), in experiments where EHMT2 cells were depleted, the EHMT2 antibody had no effect and failed to co−localization with EdU (Figure 8B), raising concerns about the specificity and reliability of the EHMT2 antibody in immunofluorescence experiments.

To address these issues and gain further understanding, we performed a SIRF experiment, a technique designed to study the interaction of specific proteins with Nascent DNA replication forks[30]. This experiment was

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designed to elucidate the recruitments EHMT2 and Rif1 at the stalled replication fork. HEK293T cells were transfected with GFP-Rif1 WT as a positive control, left untreated or treated with EdU for 10 min as a negative control. Cells were then incubated with 5 mM HU for 3 hours and the SIRF experiment was performed (Figure 9A). The results showed that GFP-Rif1 recruits to the stalled replication fork (Figure 9B-E) as previous results (Figure 8A). We also performed similar experiments in HEK293T cells transfected with HA-EHMT2 WT and found that HA-EHMT2 recruits to the stalled replication fork (Figure 10A-D). These results support the role of EHMT2 in recruiting RIF1 to stalled replication forks, suggesting its crucial function in maintaining replication fork stability.

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Figure 8

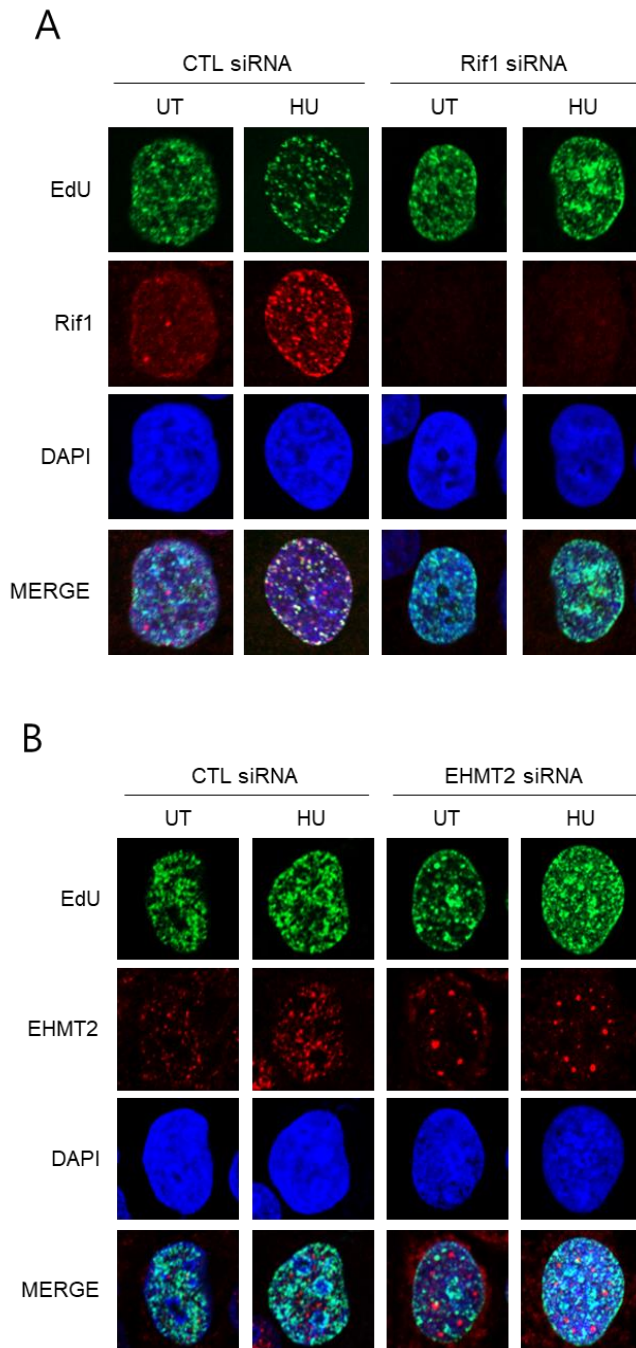

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Figure 8. Localization of Rif1 and EHMT2 at stalled replication forks

(A) HeLa cells, labeled with EdU (10 μM) for 10 minutes, were treated with HU (5 mM) for 3 hours. After fixation, permeabilization, and EdU incorporation using a Click−IT reaction (488 nm), Rif1 was stained (1:200, rabbit) to demonstrate co−localization. **(B)** Following the protocol in (A), HeLa cells labeled with EdU (10 μM) and treated with HU (5 mM) were fixed and permeabilized for co−localization with EdU (488 nm) and EHMT2 staining (1:200, rabbit).

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Figure 9

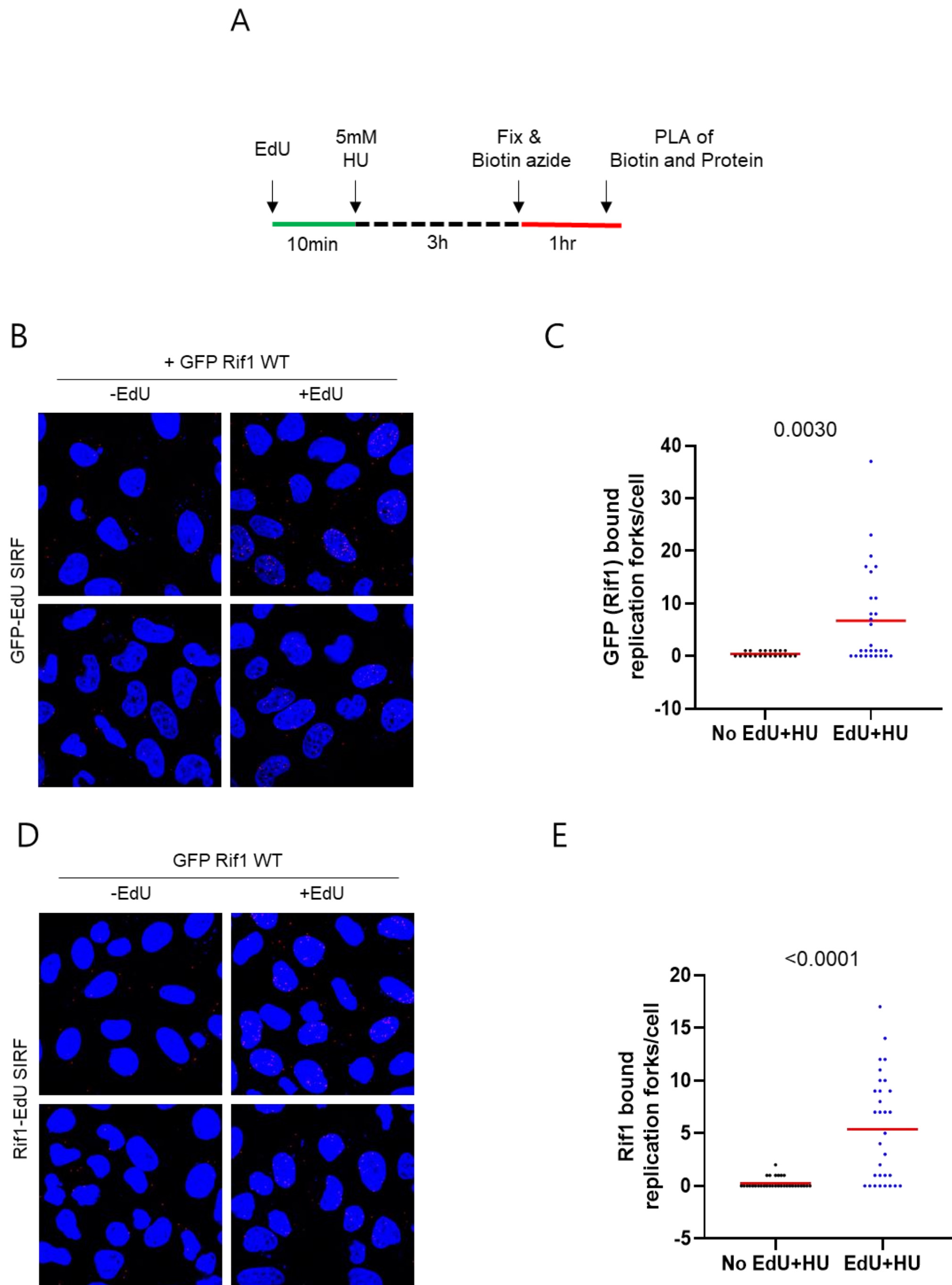

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Figure 9. Rif1 recruitments to stalled replication forks.

(A) Schematic of the experimental design for the SIRF assay to assess recruitment to nascent DNA in single cells. **(B)** HEK293T cells transfected with GFP−Rif1 WT were exposed to EdU for 10 minutes and then treated with 5 mM HU for 3 hours. The SIRF assay using GFP antibody was used to detect the localization of GFP−Rif1 at nascent forks. **(C)** Data shown are mean ± SD (n = 3). P values comparing the indicated samples were computed using a Mann−Whitney test. **(D)** Corresponding to (B), HEK293T cells were transfected with GFP−Rif1 WT and subjected to the same experimental conditions as described in (B). Using the SIRF assay with Rif1, the localization of Rif1 at nascent forks was detected. **(E)** Mean ± SD (n = 3) data with P values calculated by Mann−Whitney test as described in (C).

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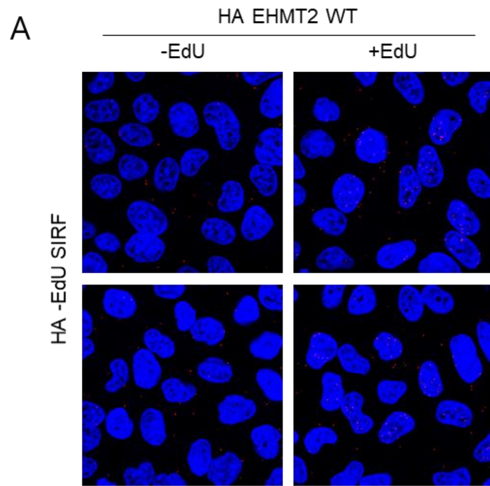
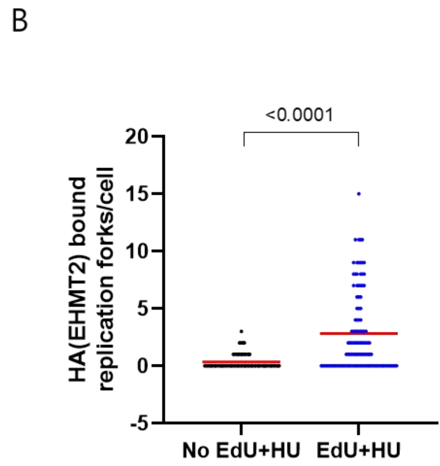
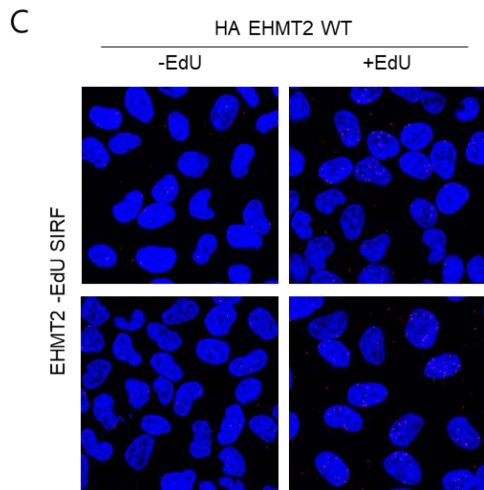
Figure 10







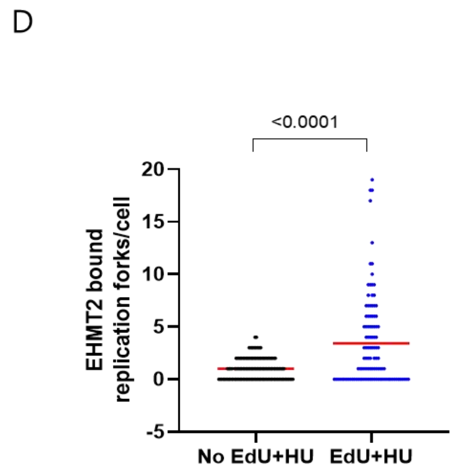


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Figure 10. EHMT2 recruitments to stalled replication forks.

(A) HEK293T cells were transfected with HA-EHMT2 WT and treated with or without EdU for 10 minutes, followed by incubation with 5 mM HU for 3 hours. Localization of HA-EHMT2 at nascent forks was detected by SIRF assay using HA antibody. **(B)** Data are mean ± SD (n = 3). Mann-Whitney test was used to calculate P values between indicated samples. **(C)** Corresponding to (A), HEK293T cells were transfected with HA-EHMT2 WT and subjected to the same experimental conditions as described in (A). EHMT2 antibody was used to detect the localization of EHMT2 at nascent forks using the SIRF assay. **(D)** Mean ± SD (n = 3) data with P values calculated by Mann-Whitney test as described in (B).

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7. EHMT2 mediates recruitment to stalled replication forks of methylated Rif1.

To further explore the mechanism of Rif1-mediated replication fork protection, we focused on investigating recruitments of Rif1 to the stalled replication fork depending on its methylation status. For this purpose, we transfected HEK293T cells with GFP-Rif1 WT or GFP-Rif1 K1160R, and performed SIRF assay (Figure 11A). The results showed that GFP-Rif1 WT was recruited to stalled replication fork, whereas GFP-Rif1 K1160R mutant was not recruited (Figure 11B). Taken together, these data suggest that methylation of Rif1 by EHMT2 is involved in the localization of Rif1 to the stalled replication fork.

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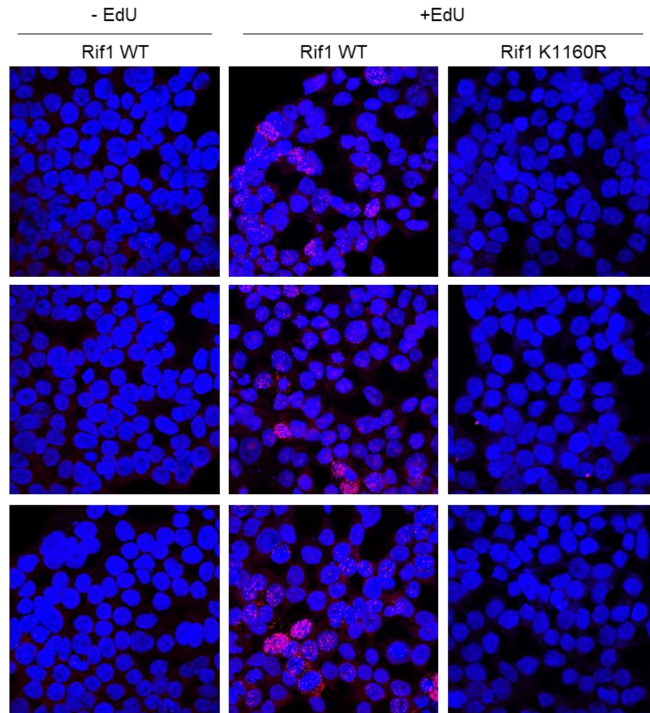
Figure 11



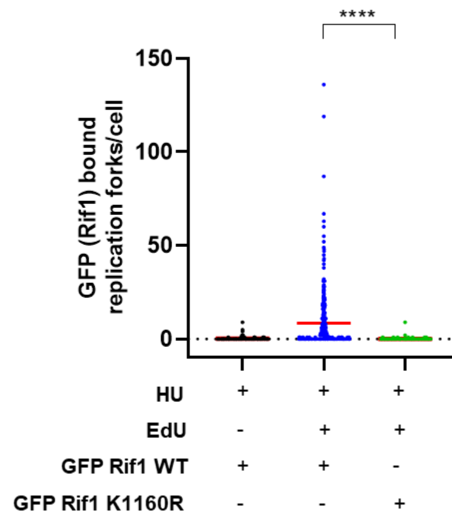


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Figure 11. Methylated Rif1 recruits to stalled replication forks.

(A) HEK293T cells transfected with either GFP−Rif1 WT or GFP−Rif1 K1160R were briefly labeled with EdU or left unlabeled, followed by treatment with 5 mM HU for 3 hours. The SIRF assay was then used to visualize the localization of GFP−Rif1 at the stalled replication forks. Representative images are shown. from SIRF experiments showing EHMT2−mediated Rif1 methylation at stalled replication forks. Samples include EdU untreated and treated cells transfected with either GFP−Rif1 WT or GFP−Rif1 K1160R in HEK293T cells. **(B)** Quantitative analysis of the SIRF data.

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8. EHMT2−mediated Rif1 methylation prevents DNA2−mediated fork degradation.

RIF1 plays a critical role in preserving nascent DNA at stalled replication forks, shielding it from DNA2 nuclease−mediated degradation. This protection is essential to prevent genome instability[11−13]. RIF1 deficiency leads to the hyperphosphorylation of DNA2, resulting in the degradation of reversed replication forks[11−13]. To investigate the phosphorylation status of DNA2 in the absence of EHMT2, we performed immunoprecipitation (IP) experiments. Upon depletion of EHMT2 in HeLa cells, DNA2 phosphorylation levels were similar to those observed in Rif1−depleted cells. Furthermore, depletion of Rif1 and EHMT2 together did not further increase DNA2 phosphorylation (Figure 12A). We next examined the level of DNA2 phosphorylation in relation to the methylation status of Rif1 using co−IP experiments. Interestingly, a decrease in phosphorylation of DNA2 was observed in GFP−Rif1 WT−expressing cells compared to Rif1−depleted cells. On the order hand, we observed increase in DNA2 phosphorylation in GFP−Rif1 K1160R−expressiong cells (Figure 12B).

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Taken together, these results indicate that methylation of Rif1 by EHMT2 plays a crucial role in regulating the phosphorylation state of DNA2, and influencing the stability of stalled replication forks. These interactions between RIF1, EHMT2 and DNA2 provide valuable insights into the complex molecular mechanisms governing genome stability during replication stress.

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Figure 12

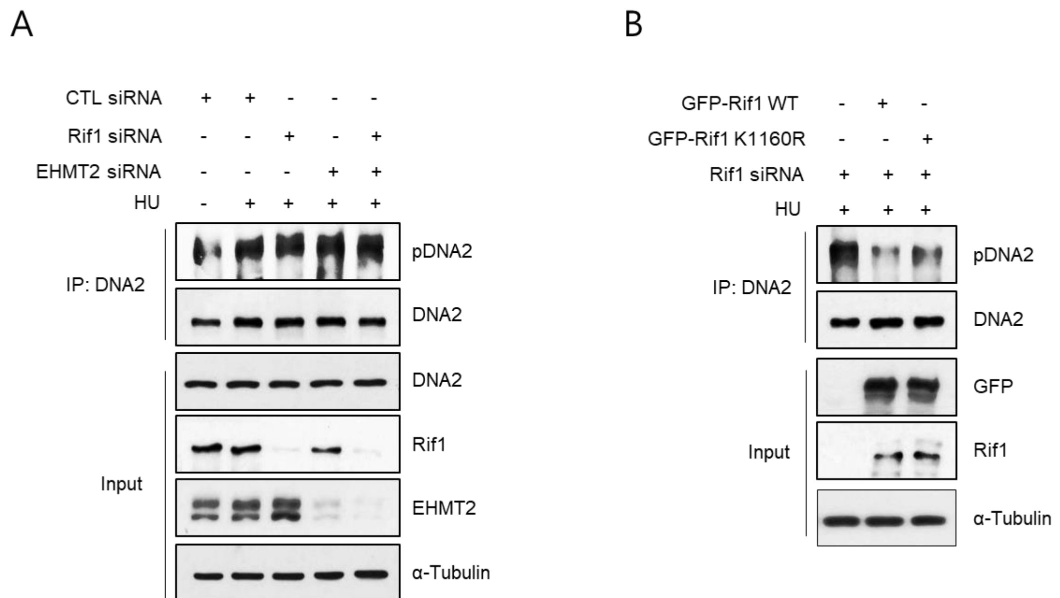

Figure 12. Methylated Rif1 is prevents DNA2−mediated fork degradation.

(A) Hela cells transfected with the indicated siRNA were treated with or without 5mM HU for 3 hours. Cell lysates were subjected to immunoprecipitation with anti−DNA2 antibody followed by immunoblotting with the indicated antibodies. **(B)** Rif1−depleted HEK293T cells co−transfected with GFP−Mock, GFP−Rif1 WT, or GFP−Rif1K1160R were treated with 5mM

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HU for 3 hours. Cell lysates were subjected to immunoprecipitation with anti-DNA2 antibody followed by immunoblotting with the indicated antibodies.

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9. Methylated Rif1 protects nascent DNA at stalled replication forks independently of PP1 interaction.

RIF1 is involved in the regulation of replication timing in mammalian cells[31] by interacting with protein phosphatase 1 (PP1) using two PP1 interaction motifs within its structure[32, 33]. In addition to its contribution to the control of replication timing, RIF1 plays a critical role in protecting nascent DNA at stalled replication forks[11, 13]. The importance of its interaction with PP1 is particularly pronounced in preventing degradation during replication fork stalling[12].

PP1, a serine/threonine protein phosphatase, is known for its involvement in various cellular processes, including DNA repair and cell cycle progression[34]. The interaction between RIF1 and PP1 ensures the timely dephosphorylation of the replication stress response protein DNA2. This process contributes significantly to maintaining replication fork stability and preserving genomic integrity.[12, 13, 28, 35]. In the absence of RIF1, DNA2 nuclease is involved in DNA resection, and this DNA2−mediated degradation leads to DNA damage in cells lacking RIF1. To investigate whether methylation

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of Rif1 affects its interaction with PP1, we performed an co−IP experiment. We found that the presence or absence of methylation on Rif1 did not affect the interaction between Rif1 and PP1 (Figure 13). Taken together, our results indicate that PP1 and Rif1 interact independently of the methylation status of Rif1. Methylated Rif1 plays a critical role in protecting nascent DNA at stalled replication forks, with the interaction with PP1 being essential to prevent degradation upon fork stalling. In the absence of methylated Rif1, DNA2 nuclease mediates resection, and this DNA2−mediated degradation ultimately leads to DNA damage in cells lacking methylated Rif1.

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Figure 13

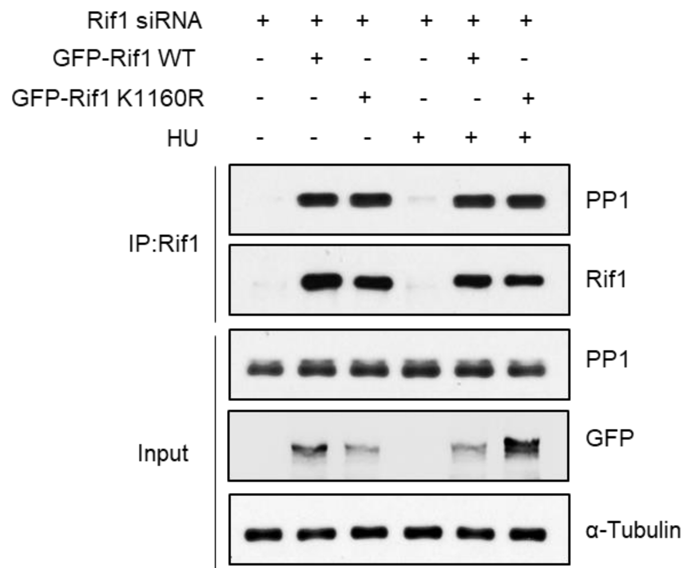

Figure 13. Interaction between Rif1 and PP1 in replication stalled state is independent to methylation status of Rif1.

Rif1−depleted HEK293T cells co−transfected with GFP−Mock, GFP−Rif1 WT or GFP−Rif1 K1160R were exposed to 5mM HU for 3 hours. Total cell lysates were then immunoprecipitated with anti−Rif1 antibody followed by immunoblotting with the indicated antibodies.

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10. Rif1 and EHMT2 promote replication fork restart.

Based on previous findings that EHMT2-mediated methylation prevents hyperphosphorylation of DNA2 and inhibits its degradation, we proposed that this degradation inhibition promotes efficient replication fork restart. To assess the extent of restart, we analysed CldU length in DNA fibre restart experiments in which ongoing replication forks were labelled with IdU (green), fork arrest was induced with HU and subsequent restart was labelled with CldU (red) (Figure 14A). Examining replication fork restart in Rif1 depleted cells, EHMT2 depleted cells and Rif1,EHMT2 double depleted cells (Figure 14B), we observed a significant reduction in CldU length when Rif1 and EHMT2 were depleted individually and no further reduction in the case of double depletion (Figure 14C). This lack of synergy upon co-depletion suggests that the cooperative role of Rif1 and EHMT2 in ensuring replication fork stability during stress is due to the methylation-dependent effect of Rif1 rather than the independent function of EHMT2. This highlights the complex interplay between Rif1 methylation and EHMT2 activity in ensuring replication fork stability under stress conditions.

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Figure 14

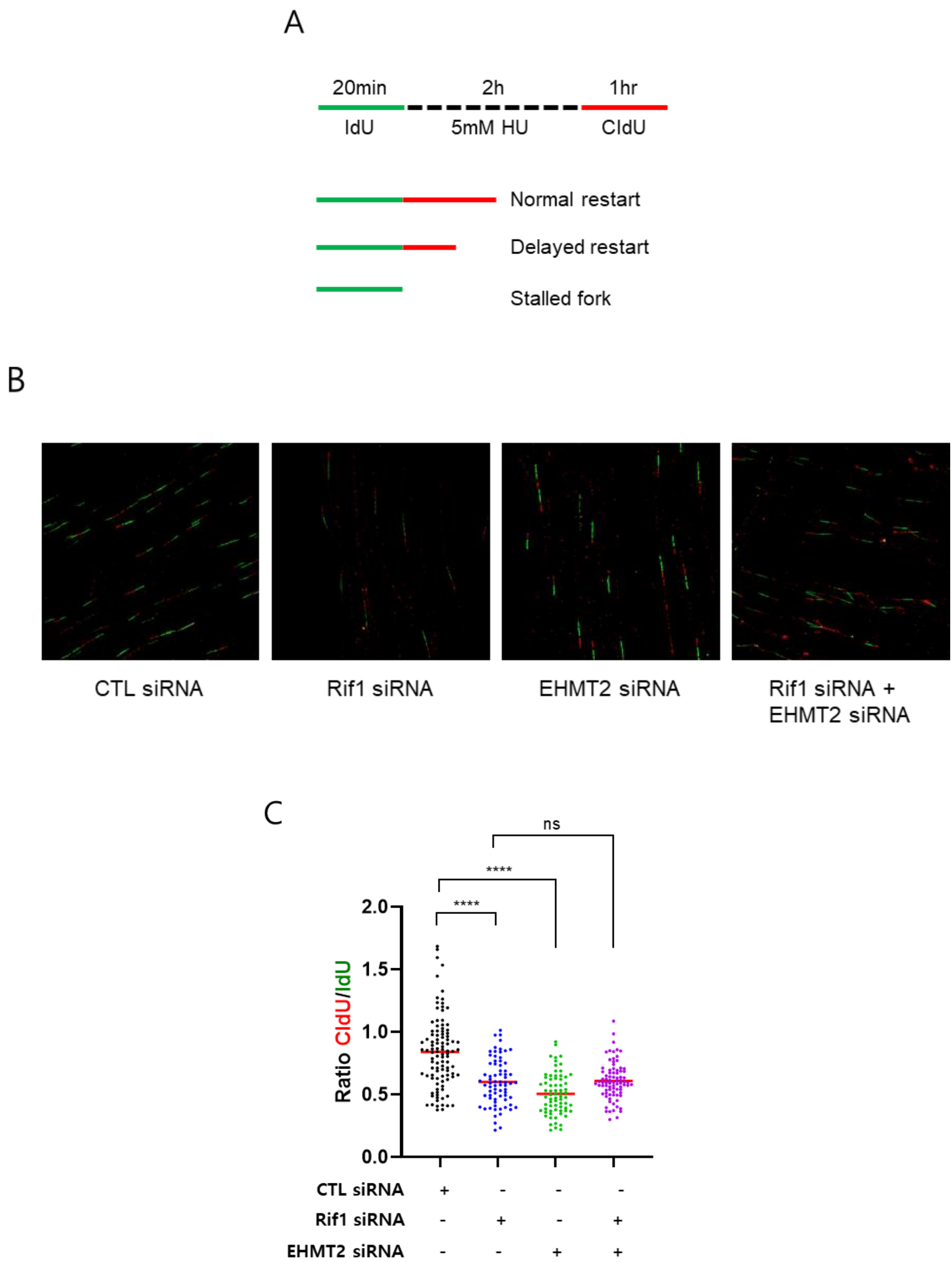

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Figure 14. Rif1 and EHMT2 contributes to replication fork restart.

(A) Schematic illustrating the labeling of cells in the replication fork restart assay. Representative images of normal and delayed restart forks are presented below the schematic. **(B)** Replication fork restart of Rif1−depleted, EHMT2−depleted, Rif1 and EHMT2−depleted HeLa Cells were measured. Cells were labeled with IdU, exposed to 5 mM HU for 3 hours, and then labeled with CIdU. **(C)** The ratio of CIdU to IdU tract length was graphed as an indicator of fork restart. Results are presented as mean ± SD (n = 3), and statistical significance between indicated samples was determined using the Mann−Whitney test.

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11. Methylated Rif1 promotes replication fork restart.

To investigate the effect of EHMT2-mediated Rif1 methylation on replication fork restart, we initiated Rif1 knockdown using siRNA targeting the 3′ UTR region. Subsequently, we reconstituted the depleted Rif1 cells with ectopically expressed GFP-Rif1 WT or GFP-Rif1 K1160R mutant to investigate the role of methylation at the K1160 site. Following knockdown and reconstitution, we performed DNA fiber restart experiments, a technique designed to assess the efficiency of replication fork restart (Figure 15A). The results showed that GFP-Rif1 WT cells exhibited a significant increase in replication fork restart, as indicated by an increased CldU track length compared to Rif1-depleted cells. In contrast, cells reconstituted with the GFP-Rif1 K1160R mutant showed a decreased CldU track length (Figure 15B), suggesting an impaired replication fork restart efficiency. This observation suggests that methylation of Rif1 by EHMT2, particularly at the K1160 site, promotes effective replication fork restart. The data further support the complex regulatory role of EHMT2-mediated Rif1 methylation in maintaining genome stability during replication stress.

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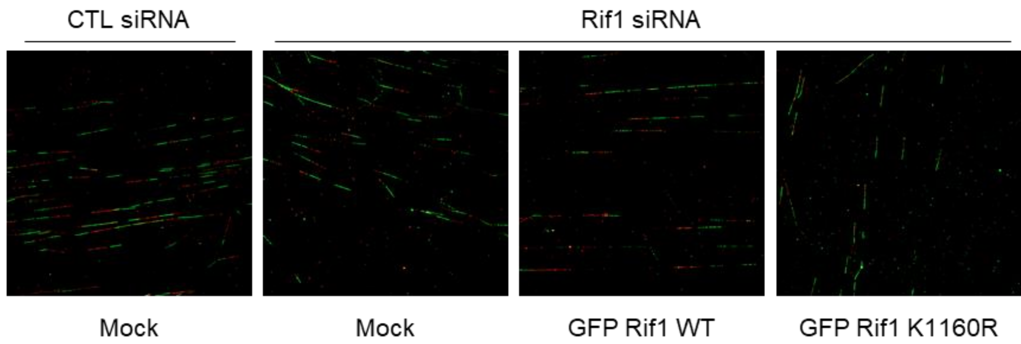
Figure 15



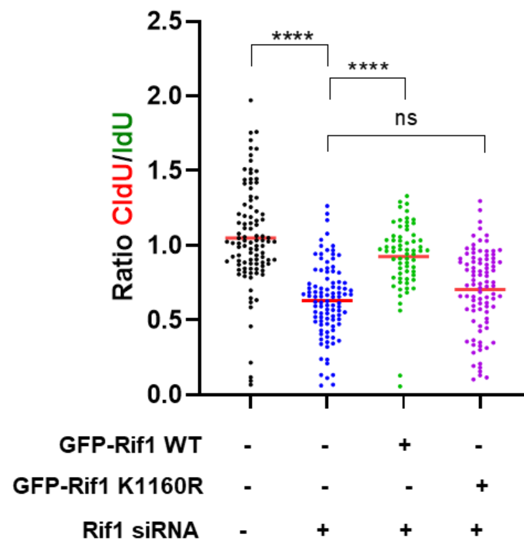


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Figure 15. Methylated Rif1 promotes to Replication fork restart.

(A) Replication fork restart in HEK293T cells, including control and Rif1-depleted cells reconstituted with GFP-Mock, GFP-Rif1 WT, or GFP-Rif1 K1160R constructs, was assessed, and representative images are provided. **(B)** The ratio of CIdU to IdU tract length was utilized as an indicator for fork restart. Results are expressed as means ± SD (n = 3), and statistical significance between the indicated samples was determined using the Mann-Whitney test.

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12. Rif1 methylation by EHMT2 promotes genomic stability during replication stress.

Replication fork restart defects due to lack of Rif1 methylation can lead to genomic instability and increased sensitivity to replication stress. To investigate the contribution of fork restart defects to genomic instability, specifically in the context of Rif1 methylation, we performed clonal survival experiments following treatment with HU, a replication stress inducer. HEK293T cells were infected with control or Rif1-deficient cells reconstituted with GFP-Mock, GFP-Rif1 WT, or GFP-Rif1 K1160R DNA. Cells were then exposed to various concentrations of HU for clonal survival assays (Figure 16A). The increased sensitivity to HU observed in Rif1 knockdown cells was rescued upon re-expression of GFP-Rif1 WT, but not GFP-Rif1 K1160R (Figure 16B).

To further analyze the chromosomal aberrations, we also performed a metaphase spread experiment. The results of this experiment were consistent with those obtained from the clonal survival assay. While re-expression of GFP-Rif1 WT decreased the aberrant chromosome phenotype induced by Rif1

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deficiency, GFP−Rif1 K1160R increased the aberrant chromosome phenotype (Figure 17), further highlighting the critical role of Rif1 methylation in maintaining genome stability under replication stress conditions. This experimental setup allowed us to determine the effect of Rif1 methylation, specifically at the K1160 site, on the cellular response to replication stress, highlighting its crucial role in maintaining genomic stability and regulating sensitivity to stress. Taken together, our results indicate that Rif1 is methylated by EHMT2 under conditions of replication stress. Methylated Rif1 interacts with PP1, preventing hyperphosphorylation by the DNA nuclease DNA2 and protecting the reverse fork from degradation, thereby maintaining genomic stability (Figure 18). In conclusion, our study highlights the critical role of Rif1 methylation in orchestrating the cellular response to replication stress and represents a potential therapeutic target for enhancing genomic stability.

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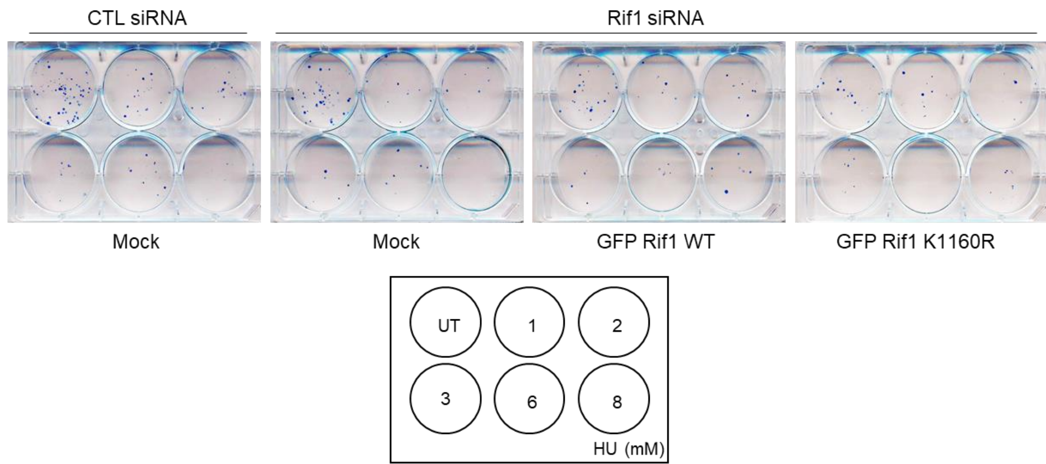
Figure 16



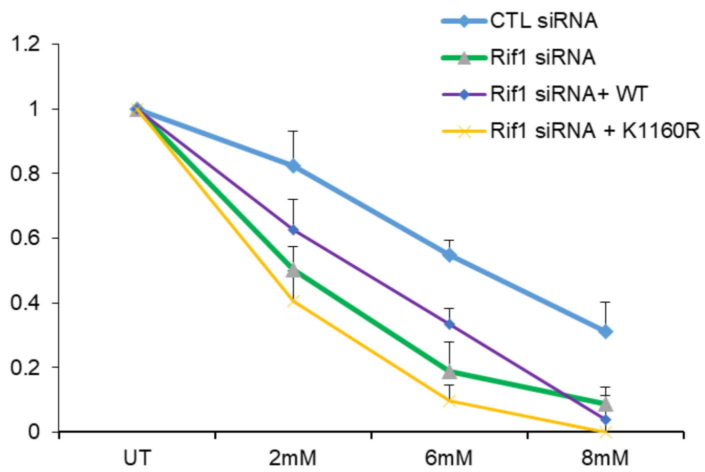


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Figure 16. Methylated Rif1 rescues replication damage sensitivity.

(A) Control or Rif1-depleted HEK293T cells were reconstituted with GFP-Mock, GFP-Rif1 WT, or GFP-Rif1 K1160R constructs. Subsequently, cells were treated with varying concentrations of HU for a clonal survival assay. **(B)** The experiment was repeated three times, and error bars indicate the standard deviation.

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Figure 17

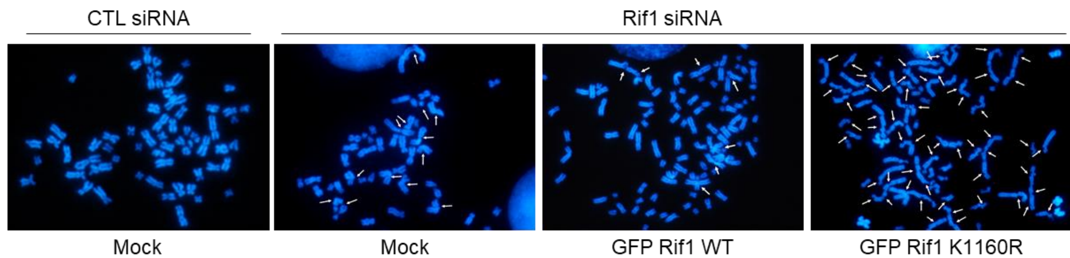

Figure 17. Methylated Rif1 attenuates to chromosome aberrations under replication stress.

Control or Rif1−depleted cells were reconstituted with GFP−Mock, GFP−Rif1 WT, or GFP−Rif1 K1160R constructs, a meta−spread experiment was performed to assess chromosome aberrations. Representative images are shown. Abnormal chromosomes were indicated with white arrows.

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Figure 18

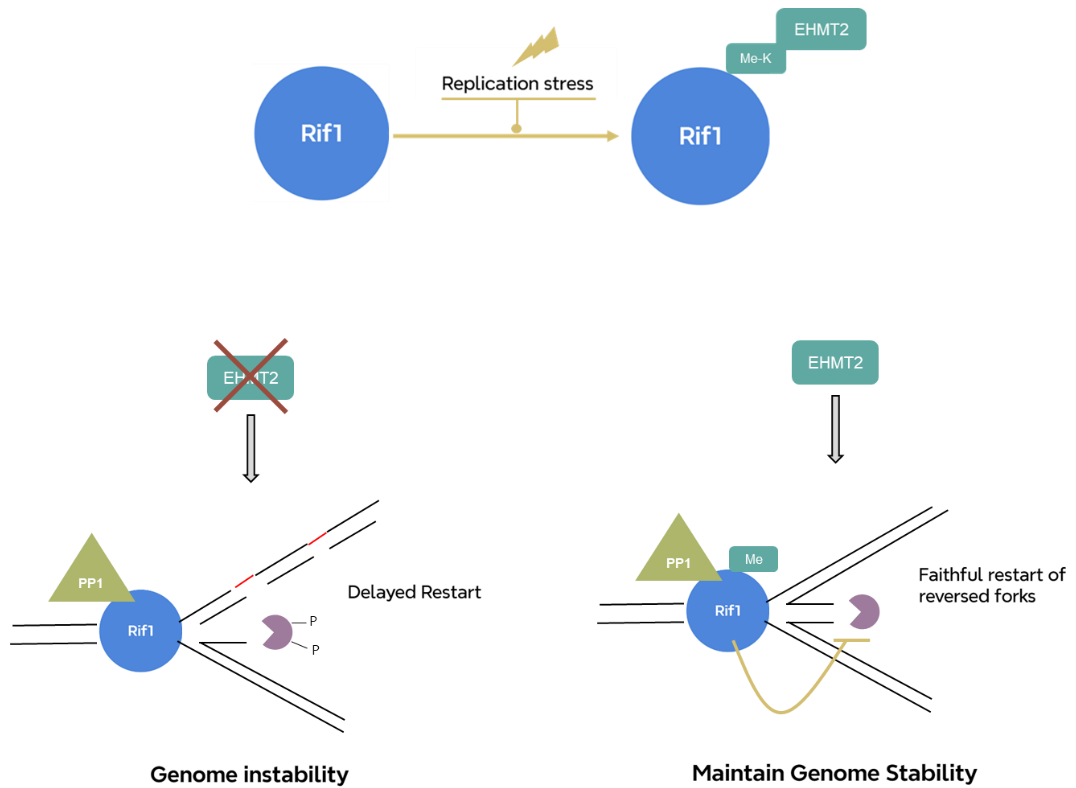

Figure 18. Schematic model of Rif1 methylation in the regulation of genomic stability under replication stress.

The figure illustrates the proposed model depicting the role of Rif1 methylation by EHMT2 in the cellular response to replication stress. Under conditions of replication stress, EHMT2 methylates Rif1 at K1160. Methylated Rif1 interacts

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with PP1, preventing hyperphosphorylation by the DNA nuclease DNA2 and subsequently protecting the reverse fork from degradation. This molecular mechanism contributes to the maintenance of genomic stability during replication stress.

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DISCUSSION

The main objective of this study was to clarify the complex interactions involving Rif1, EHMT2, and their combined impact on DNA replication fork protection. While previous studies have offered valuable insights into the role of Rif1, there is an urgent need for a more comprehensive understanding of the molecular mechanisms underlying its function in safeguarding DNA replication forks. To address this, we performed a detailed analysis of the underlying mechanisms responsible for Rif1's contribution to DNA replication fork protection. Our results demonstrate that a thorough investigation of the interaction between Rif1 and EHMT2 has revealed intricate mechanisms governing the DNA replication process and genome stability. We identified EHMT2 as an interactor of Rif1, a lysine histone methyltransferase[19, 36, 37]. Rif1 methylated by EHMT2 plays a critical role in replication fork protection, preventing DNA2-mediated fork degradation, increasing replication restart, and promoting genomic stability. The K1160R mutation, targeting the methylation site in Rif1, consistently impaired replication fork protection,

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DNA2-mediated fork degradation, restart efficiency, and genome stability. Previous studies have highlighted the critical role of phosphorylation at specific sites in Rif1 in maintaining stable replication forks and genome integrity[11]. In our recent investigation, we focused on EHMT2-mediated Rif1 methylation and demonstrated that it is a robust defense mechanism that enhances replication fork protection and overall genome stability.

Understanding the complex interactions and regulatory networks of phosphorylation and methylation in the context of replication fork stability is crucial. Exploring how these modifications fit into broader cellular signaling pathways will provide a comprehensive understanding of the complex mechanisms orchestrating genome stability and potentially open avenues for therapeutic intervention targeting these regulatory processes. The significance of Rif1 methylation in modern cancer therapy will be highlighted by its close association with gene stability in BRCA-deficient tumors. BRCA gene deficiency limits the effective response to DNA damage, causing cancer cells to explore alternative survival mechanisms[38-42]. It has been suggested that methylation of Rif1, which serves as a key regulator, may be particularly

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pronounced in BRCA−deficient tumors. To verify this hypothesis, further investigation specifically in the context of BRCA−deficient tumors is imperative.

Our study suggests that Rif1 methylation functions primarily through a replication fork protection mechanism, effectively responding to DNA damage and enhancing replication fork stability—a crucial aspect in the context of anticancer drug resistance. The correlation between Rif1 methylation and BRCA−deficient tumors is expected to provide valuable insights into the development of cancer therapeutic strategies and shed light on the emergence and progression of resistance to anticancer drugs. As a result, Rif1 methylation is clinically relevant and may contribute to the development of personalized treatment strategies.

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MATERIALS AND METHODS

Cell culture and Transient transfection

HeLa and 293T cells were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C with 5% CO2 in a humidified incubator. For transient transfections, TurboFect (Thermo Fisher Scientific), a highly efficient transfection reagent, was employed following the manufacturer's recommended protocol. Briefly, cells were seeded in culture dishes for the experimental design. The transfection mixture, composed of plasmid DNA or siRNA of interest along with TurboFect reagent, was carefully prepared according to the specified ratios. The transfection mixture was then added to the cultured cells, allowing for the efficient uptake and expression of the introduced genetic material.

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Plasmid constructs

Full-length human wild-type Rif1 was obtained from Addgene (plasmid #52506, Addgene, USA). The pCMV6 Entry Myc DDK-EHMT2 construct was obtained from OriGene (RC224625, OriGene Technologies, USA), and the EHMT2 WT, EHMT2 ⊿SET, and EHMT2 SET constructs were amplified by PCR and cloned into pcDNA3 HA-tagged vector. All constructs were confirmed by automated DNA sequencing.

Mutagenesis

The full-length human wild-type Rif1 was obtained from Addgene (Plasmid #52506, Addgene, USA). To generate the Rif1 1160KR mutant, mutagenesis was performed using the GENEART® site-directed mutagenesis system (Thermo Fisher Scientific, USA) following the manufacturer's instructions.

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RNA interference

For knockdown of Rif1 or EHMT2, siRNA was designed.

The sequences of siRNAs targeting Rif1 and EHMT2 are as follows:

Rif1 siRNA, 5′ −GACUCACAUUUCCAGUCAAdTdT−3′ ,

Rif1 UTR siRNA 5′ −UCUUAUGAGACGUAUAGUAUUdTdT−3′

EHMT2 siRNA, 5′ −GCUCCAGGAAUUUAACAAGAUdTdT−3′

EHMT2 UTR siRNA, 5′ −GAGAGAGUUCAUGGCUCUUdTdT−3′

Control siRNA , 5′ −CCUACGCCACCAAUUUCGUdTdT−3′ .

Antibodies

Pan methyl Lysine (abcam, ab7315), Rif1 (Bethyl, #A300−568A), EHMT2 (Cell signaling, #3306), HA (Cell signaling, #3724), GFP (Novus, NB600−308) β−actin (Santa cruz, sc−47778), DNA2 (Abcam, ab96488), Phospho−(Ser/Thr) Phe (Cell signaling, #9631) α−tubulin (Bethyl, A305−798A).

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Western blot analysis

Cells were lysed in NP-40 buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 5 mM EDTA) with protease inhibitors (aprotinin, PMSF, leupeptin, pepstatin A; Sigma) or RIPA buffer (50 mM Tris-HCl [pH 7. 5], 150 mM NaCl, 1% NP-40, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with protease inhibitors and sonicated once for 3 seconds. Lysates were incubated on ice for 20 minutes and then centrifuged at 15,000 rpm for 30 minutes. Equal amounts of protein were separated by 6-15% SDS-PAGE followed by electrotransfer to a polyvinylidene difluoride membrane (Pall). Membranes were blocked in 5% skim milk in 1× TBST (1× TBS supplemented with 0.1% Tween 20) for at least 30 minutes and incubated with primary antibodies in 5% skim milk at 4°C overnight. The blots were washed six times for 10 minutes with TBS-T and then incubated with peroxidase-conjugated secondary antibodies for 2 hours at room temperature. The membranes were then washed six more times and developed using an enhanced chemiluminescence detection system (ECL; Dogen Bio, Korea).

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Immunoprecipitation (IP)

For the immunoprecipitation assays, cell extracts were precleared with Protein A−Sepharose beads (17−0780−01, GE Healthcare, USA) in normal serum and incubated for 1 hour at 4° C with rotation. The bead pellet was then discarded, and the extracts were incubated with the appropriate antibodies overnight at 4° C with rotation. The next day, fresh Protein A−Sepharose beads were added to the reaction mixture, and incubation continued for 2 hours at 4° C with rotation. The beads were washed at least three times in NP40 buffer, resuspended in 2X SDS sample buffer, and boiled for 5 minutes. Immune complexes were then analyzed by Western blotting.

Immunofluorescence (IF)

HeLa cells were labeled with EdU (10 μM) for 10 minutes to visualize cells in the S phase. After this labeling step, the cells were washed and then incubated in fresh medium containing HU (5 mM) for 3 hours. After the 3hour incubation, the cells were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized in 0.5% Triton−X solution in PBS for 15 minutes. Coverslips

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were washed three times and EdU incorporation was detected using a Click−IT reaction (Click−IT EdU Imaging Kit, Invitrogen) with a 488 nm fluorescent azide, according to the manufacturer's protocol. The coverslips were then washed and stained with EHMT2 primary antibody (1:200, rabbit) for overnight at 4° C. The next day, the cells were washed with PBS and incubated with a secondary antibody conjugated to Alexafluor−594 for 2 hours at room temperature. Finally, the cells were washed with PBS, and coverslips were mounted with Fluoroshield™ containing DAPI (Sigma) before imaging with a Carl Zeiss LSM 900 confocal microscope.

Yeast two− hybrid screening

The Bait A plasmid, representing the fused Rif1 N−terminal domain (2−980 aa), was introduced into the PBN204 strain and positive clones were subsequently validated. The cDNA library was then introduced into the PBN204 strain containing Rif1 (Bait A). Interactions were evaluated on selective media lacking Leu, Trp, Ade, and His, including the reporter expression markers lacZ, URA3, and ADE2. This experiment was submitted to PanBioNET for analysis.

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DNA fiber analysis

Briefly, HeLa cells were sequentially pulse−labeled with 30 μM IdU (Sigma−Aldrich) and 300 μM CIdU (Sigma−Aldrich) for 30 minutes and treated with HU (5 mM) for 3 hours for the fork degradation assay. For the fork restart assay, cells were first labeled with IdU and treated with 5 mM HU for 3 hours and then with CIdU. After harvesting the labeled cells, they were lysed in a lysis buffer (50 mM EDTA, 0.5% SDS, 200 mM Tris–HCl, pH 7.5), and DNA fibers were stretched onto glass slides and fixed in methanol:acetic acid (3:1). The coverslips were then denatured (2.5 M HCl for 1 hour), washed with PBS, and blocked (5% BSA and 0.5% Tween 20 in PBS) for 1 hour. The labeled IdU and CIdU tracks were revealed with anti−BrdU antibodies recognizing BrdU from mouse (1:25, 347580, BD) and BrdU from rat (1:200, ab6326, Abcam), followed by 1−hour incubation with secondary antibodies at room temperature in the dark: anti–mouse Alexa Fluor 488 (1:200, A−21200, Invitrogen) and anti–rat Alexa Fluor 594 (1:200, A−21209, Invitrogen). Images were obtained using a confocal microscope (Zeiss LSM 900), and fiber lengths were analyzed using Zeiss microscopic imaging software ZEN (Carl Zeiss).

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In situ interactions at replication forks (SIRF) assay

293T cells were transfected with the indicated siRNAs and/or constructs, treated with 20 μM EdU (Invitrogen) for 10 minutes, washed two times with PBS, and incubated in fresh media containing HU (5mM) for 3 hours at 37°C. After 3 hours, cells were fixed, permeabilized with 0.5% Triton-X, and a Click-iT reaction (Invitrogen) was performed using biotin azide (Life Technologies Corp.) according to the manufacturer's instructions. Cells were then washed with PBS and blocked in a blocking solution at 37℃ in a humidified chamber for 1 hour. After washing with PBS, cells were incubated with two primary antibodies. The primary antibodies used were as follows: mouse monoclonal anti-GFP (Santa Cruz, 1:100) or mouse monoclonal anti-HA (Abcam, 1:100) and rabbit polyclonal anti-biotin (Cell Signaling, 1:100). The negative control was used with no EdU treatment. After incubation with primary antibodies, anti-rabbit MINUS, and anti-mouse PLUS PLA probes (1:5 dilution, Duolink, Sigma-Aldrich) were used to detect the two primary antibodies according to the manufacturer's instructions. Briefly, the coverslip was blocked in Duolink blocking buffer overnight at 4℃ and then incubated with the two

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primary antibodies. After washing the coverslip twice in PBS for 5 minutes, anti−mouse PLUS and anti−rabbit MINUS PLA probes (Sigma−Aldrich) were coupled to the primary antibodies for 1 hour at 37 ºC. Next, amplification using the 'Duolink In Situ Detection Reagents Red' (Sigma−Aldrich) was performed at 37°C. After amplification, coverslips were mounted using DAPI−containing mounting media (Sigma) and imaged on a Carl Zeiss LSM 900 confocal microscope. The data are representative of three independent experiments.

Coomassie Blue Staining

GFP−Rif1 WT plasmid was transfected into 293T cells, and 48 hours after transfection, the cells were treated with ionizing radiation (IR) and hydroxyurea (HU) according to the experimental design. After treatment, cells were harvested and lysed in protein extraction buffer, and proteins were loaded onto a polyacrylamide gel for electrophoresis. EZ−Gel Staining Solution was applied for gel staining. The gel was immersed in the staining solution for approximately 1 hour, followed by destaining until protein bands were clearly

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visible against a clear background. Gel images were captured using an imaging system to facilitate identification and analysis of GFP-Rif1 WT bands.

Chromosome aberration assay

The indicated cells were treated with 5 mM hydroxyurea (HU) for 3 hours. After HU treatment, cells were washed in PBS and exposed to 200 ng/mL colcemid (Sigma-Aldrich) for 16 hours at 37° C to arrest the cells in metaphase. Cells were then harvested by trypsinization, incubated in 75 mM KCl for 15 minutes at room temperature, and fixed in a methanol/acetic acid solution (3:1). After removing the supernatant, the pellets were resuspended in the fixative solution, dropped onto slides to obtain chromosome spreads, and air dried overnight. Slides were mounted with DAPI-containing medium (Sigma), and metaphase images were captured using a fluorescence microscope (ECLIPSE 80i, Nikon). Visible chromatid breaks/gaps were then counted as an indicator of chromosome integrity.

Clonal survival assay

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HEK293T cells were exposed to various concentrations of hydroxyurea (HU). Subsequently, 2×10^2 cells were immediately seeded on a 6−well plate and cultured at 37°C for two weeks to facilitate colony formation. After this incubation period, colonies were fixed with 4% paraformaldehyde, stained with 1% methylene blue, and counted. The entire experiment was repeated independently in triplicate. Data are presented with error bars representing the standard deviation (SD), which indicates the variability observed.

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ABSTRACT

Rif1 methylation by EHMT2 maintains replication fork stability

Min Ji Kim

Advisor : Prof. Jung-Hee Lee, Ph.D

Department of Biomedical sciences,

Graduate school of Chosun University

Rif1 maintains genome integrity by protecting the reversal fork at stalled replication forks, ensuring the protection of nascent DNA from over-degradation in the process. Several mechanisms have been proposed for how Rif1 protects the reversal fork, but it is likely that we do not yet fully understand the process. Our results provide evidence that the replication fork-protective function of Rif1 is regulated by the methyltransferase EHMT2. We

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found that EHMT2 interacts with Rif1 and induces methylation of Rif1 at Lysine residue 1160 in response to replication stress. The methylation-defective K1160R mutant consistently disrupts replication fork protection, DNA2-mediated fork degradation and replication fork restart, and induces genome instability. This suggests that methylation of Rif1 by EHMT2 protects the reversal fork and maintains genome stability. Our results indicate that EHMT2 is a novel regulator of Rif1, and highlight that EHMT2-mediated methylation of Rif1 is a crucial step in the Rif1-mediated replication fork protection and maintenance of genome stability.

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