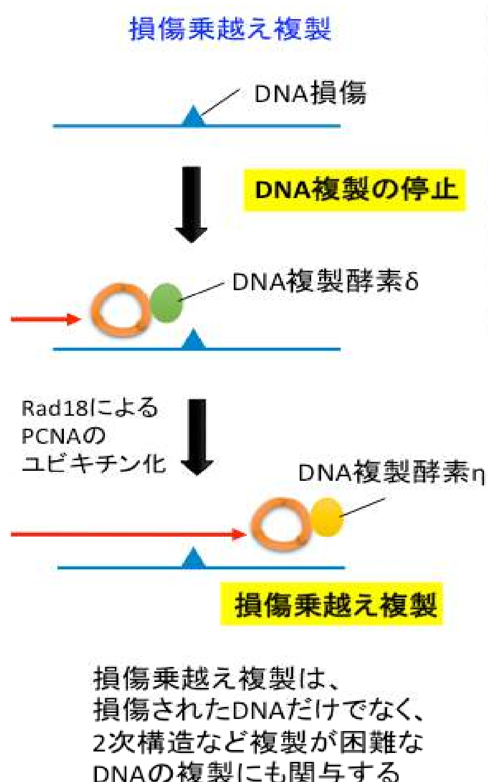
損傷修復分野
Department of Cell Maintenance

細胞への紫外線の照射などにより DNA が損傷されると、DNA 複製は停止してしまう。ユビキチンライゲースである Rad18 は、損傷乗越え複製酵素 η を制御することにより DNA 複製を再開させ(損傷乗越え複製)、ゲノム DNA の安定性を保つ。Rad18 による損傷乗越え複製の制御は、発癌を防ぐ役割の他に生殖機能の維持や造血の場における薬剤耐性などに関与する。その一方で発癌した細胞では、Rad18 タンパクが癌精巣抗原である MAGE-A4 により安定化されることにより、発癌の亢進に「悪用」されている可能性がある。次の 3 つの項目について解明をめざす。(1) 正常な細胞で損傷乗越え複製が発癌を抑制する機構。 (2) 損傷乗越え複製が、体性幹細胞の維持に貢献する機構。(3) 前癌状態の細胞で、Rad18 が発癌の亢進に寄与する機構。

Replicative polymerases stall at damaged template DNA in UV-irradiated cells, which hamper cell proliferation. To circumvent the crisis, ubiquitin ligase Rad18 mono-ubiquitinates PCNA to promote translesion synthesis (TLS) via recruiting polymerase η. Thus, Rad18 maintains genomic DNA stability to prevent tumorigenesis. Rad18 also plays role to maintain spermatogenesis and hematopoiesis. On the other hand, we propose that cancer cells “hijack” Rad18 to reprogram TLS providing a new way for tumors to achieve tumor progression via stabilization of Rad18 by cancer/testis melanoma antigen A-4. We will elucidate following subjects. (1) How Rad18 suppress tumorigenesis in normal cells (2) mechanism of how Rad18 contribute to maintain somatic stem cells (3) how Rad18 contribute to reprogram TLS to achieve tumor progression in pro-cancer cells.



正常な細胞での「損傷乗越え複製」の役割 (モデル)

精子形成能力の維持能力
(精巣生殖細胞の維持)

造血細胞での薬剤耐性

皮膚での毛胞の維持

体性幹細胞または前駆細胞の維持に関与?

発癌の過程での「損傷乗越え複製」の役割 (モデル)

前癌状態にある細胞

癌細胞の増殖の妨げとなる
「複製ストレス」を緩和する

発癌の亢進に貢献?

誤りがち複製を促進する

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研究概略　Projects

細胞への紫外線の照射などにより DNA が損傷されると、DNA 複製は停止してしまう。ユビキチンライゲースである Rad18 は、損傷乗越え複製酵素η(Polη)を制御することにより DNA 複製を再開させる。このように DNA 損傷を乗越えて複製することを損傷乗越え複製(translesion synthesis: TLS)と呼ぶ。TLS は、ゲノム DNA の安定性を保つ役割をもつ一方で、エラー頻度の高い TLS により変異率の上昇をもたらす可能性もある。Rad18 ノックアウトマウスを用いた研究などにより、以下の 1-4 の研究を進めてきた。また本研究室では熊本大学医学部皮膚科などと連携して、光線過敏症・早老症などのゲノム不安定性疾患の診断に携わっている。5 の共同研究により、早老症である UV^{S}S 候群の原因遺伝子を同定に寄与した。

1.DNA 複製停止部位で Polηは、Rad18 を PCNA へ導くことにより、PCNA のモノユビキチン化修飾を促進する。
Polηをコードする遺伝子の異常により、高発癌性疾患である色素性乾皮症バリアントを発症する。細胞に UV が照射されて DNA が損傷を受けると、p53 経路を介して Polηの転写が誘導されて、ゲノム DNA 上で停止した PCNA に対して集積する。Polηは、相互作用により Rad18 を PCNA 部位へ導き、モノユビキチン化を促進する。これにより Polη以外の TLS、ファンコニ経路、鋳型スイッチ経路なども誘導されるモデルを提案した。
(ノースカロライナ大学 CyrusVaziri 博士との共同研究、論文 8 参照)。

2.　Rad18 は造血前駆細胞の DNA 損傷トレランスに寄与することにより、癌抑制作用をもつ
Rad18 による損傷乗越え複製の制御は、発癌を防ぐ役割をもつ。この他に生殖機能の維持など体性幹細胞の維持に貢献すると考えられている。造血細胞についても研究を行った。造血幹細胞および前駆細胞は、Rad18 と FANCD2 タンパクを発現するため、造血の過程で Rad18 はファンコニ経路で役割を果たしていると考えられていた。しかし、Rad18 欠損マウスで造血機能に異常が見られないこと、Rad18 欠損マウス繊維芽細胞をメチル化剤で処理すると FANCD2 タンパクのモノユビキチン化反応が見られることから、造血細胞では Rad18 による経路とファンコニ経路は、独立に機能すると結論した。また、Rad18 欠損マウスに DNA 損傷作用をもつ DMBA を投与すると、B 細胞由来の発癌率が高いことがわかった。このため、Rad18 は造血前駆細胞の DNA 損傷トレランスに寄与することにより、癌抑制作用をもつと結論した。(ノースカロライナ大学 CyrusVaziri 博士との共同研究、　論文 5 参照)。

3.　癌精巣抗原(MAGE-A4)は、Rad18 タンパクを安定化し、損傷乗越え複製を促進することにより、発がん進行過程でのゲノム DNA に影響を及ぼす。
Rad18 は、体性幹細胞の増殖の維持に貢献する一方で、発癌した細胞では異なる役割を果たしている可能性がある。
質量分析により、ヒト培養細胞内で Rad18 タンパクが癌精巣抗原である MAGE-A4 タンパクと複合体を形成していることがわかった。MEGA-A4 は細胞内で Rad18 が分解されるのを抑制することにより、Rad18 を安定化していた。このため、MEGA-A4 は発がん進行の過程で Rad18 を安定化することを介して、損傷乗越え複製を促進させてゲノム DNA に影響を及ぼすと結論した(ノースカロライナ大学 CyrusVaziri 博士との共同研究、論文 4 参照)。
また発がんの過程では細胞の DNA を複製する時にストレスがかかることにより細胞増殖が制限されていると考えられている。これに対して MEGA-A4 により安定化した Rad18 は、損傷乗越え複製を促進することにより、この「複製ストレス」を緩和して癌細胞における DNA 複製および細胞増殖を助けることにより発がんの進行に寄与していることを提唱した(総説 2 参照)。

4. マウス個体で Rad18 が欠損しても体細胞超変異(somatic hypermutation)に影響しない。

体細胞超変異(somatic hypermutation)では、AID酵素が DNA 塩基を脱メチル化して損傷を導入し、その後に正確性の低い複製酵素により損傷が修復されることにより、変異が導入される。Rad18 は PCNA をモノユビキチン化することにより正確性の低い複製酵素を制御する鍵となる分子であることから、体細胞超変異に関与すると考えられてきた。我々は野生型マウスおよび Rad18 欠損マウスに抗原を注入した後に IgG 遺伝子での変異率を測定した結果、両者に差が見られなかった。このため、マウス個体で Rad18 が欠損しても体細胞超変異(somatic hypermutation)に影響しないと結論した。(高知大学の清水博士との共同研究、論文 2 参照)

5. 早老症である UV^{S}S 候群の原因遺伝子の同定

損傷修復分野では熊本大学皮膚科などと協力し、光線過敏または早老症の診断に携わっている。損傷修復分野の前教授である山泉らにより、軽症型の早老症である UV^{S}S 候群が同定された。名古屋大学の荻らは、本研究室と共同でこの疾患の原因遺伝子を特定し、その分子機能を明らかにした (文献 9 参照)。

1. A non-catalytic role of DNA polymerase g in recruiting Rad18 and promoting PCNA monoubiquitination at stalled replication forks

Trans-lesion DNA synthesis (TLS) is a DNA damage-tolerance mechanism that uses low-fidelity DNA polymerases to replicate damaged DNA. The inherited cancer-propensity syndrome xeroderma pig- mentosum variant (XPV) results from error-prone TLS of UV-damaged DNA. TLS is initiated when the Rad6/ Rad18 complex monoubiquitinates proliferating cell nuclear antigen (PCNA), but the basis for recruitment of Rad18 to PCNA is not completely understood. Here, we show that Rad18 is targeted to PCNA by DNA polymerase eta (Polη), the XPV gene product that is mutated in XPV patients. The C-terminal domain of Polη binds to both Rad18 and PCNA and promotes PCNA monoubiquitination, a function unique to Polη among Y-family TLS polymerases and dissociable from its catalytic activity. Importantly, XPV cells expressing full-length catalytically-inactive Polη exhibit increased recruitment of other error-prone TLS polymerases (Poli and Polk) after UV irradiation. These results define a novel non-catalytic role for Polη in promoting PCNA monoubiquitination and provide a new potential mechanism for mutagenesis and genome instability in XPV individuals (Collaborative study with Dr. Vaziri, see reference 8).

2. Rad18 confers hematopoietic progenitor cell DNA damage tolerance independently of the Fanconi Anemia pathway *in vivo*

In cultured cancer cells the E3 ubiquitin ligase Rad18 activates Trans-Lesion Synthesis (TLS) and the Fanconi Anemia (FA) pathway. However, physiological roles of Rad18 in DNA damage tolerance and carcinogenesis are unknown and were investigated here. Primary hematopoietic stem and progenitor cells (HSPC) co-expressed RAD18 and FANCD2 proteins, potentially consistent with a role for Rad18 in FA pathway function during hematopoiesis. However, hematopoietic defects typically associated with fanc- deficiency (decreased HSPC numbers, reduced engraftment potential of HSPC, and Mitomycin C (MMC) -sensitive hematopoiesis), were absent in Rad18$^{-/-}$ mice. Moreover, primary Rad18$^{-/-}$ mouse embryonic fibroblasts (MEF) retained robust Fancd2 mono- ubiquitination following MMC treatment. Therefore, Rad18 is dispensable for FA pathway activation in untransformed cells and the Rad18 and FA pathways are separable in hematopoietic cells. In contrast with responses to crosslinking agents, Rad18$^{-/-}$ HSPC were sensitive to in vivo treatment with the myelo- suppressive agent 7,12 Dimethylbenz[a]anthracene (DMBA). Rad18-deficient fibroblasts aberrantly accu- mulated DNA damage markers after DMBA treatment. Moreover, *in vivo* DMBA treatment led to increased incidence of B cell malignancy in Rad18$^{-/-}$ mice. These results identify novel hematopoietic functions for Rad18 and provide the first demonstration that Rad18 confers DNA damage tolerance and tumor-suppression in a physiological setting. (Collaborative study with Dr. Vaziri, see reference 5).

3. A neomorphic cancer cell-specific role of MAGE-A4 in trans-lesion synthesis

Trans-lesion synthesis (TLS) is an important DNA-damage tolerance mechanism that permits ongoing DNA synthesis in cells harbouring damaged genomes. The E3 ubiquitin ligase RAD18 activates TLS by promoting recruitment of Y-family

DNA polymerases to sites of DNA-damage-induced replication fork stalling. Here we identify the cancer/testes antigen melanoma antigen-A4 (MAGE-A4) as a tumour cell-specific RAD18-binding partner and an activator of TLS. MAGE-A4 depletion from MAGE-A4-expressing cancer cells destabilizes RAD18. Conversely, ectopic expression of MAGE-A4 (in cell lines lacking endogenous MAGE-A4) promotes RAD18 stability. DNA-damage-induced mono-ubiquitination of the RAD18 substrate PCNA is attenuated by MAGE-A4 silencing. MAGE-A4-depleted cells fail to resume DNA synthesis normally following ultraviolet irradiation and accumulate phosphor-H2AX, thereby recapitulating major hallmarks of TLS deficiency. Taken together, these results demonstrate a mechanism by which reprogramming of ubiquitin signalling in cancer cells can influence DNA damage tolerance and probably contribute to an altered genomic landscape. (Collaborative study with Dr. Vaziri, see reference 4).

4. Somatic hypermutation of immunoglobulin genes in *Rad18* knockout mice

Somatic hypermutation (SHM) of immunoglobulin (Ig) genes is triggered by the activity of activation-induced cytidine deaminase (AID). AID induces DNA lesions in variable regions of Ig genes, and error-prone DNA repair mechanisms initiated in response to these lesions introduce the mutations that characterize SHM. Error-prone DNA repair in SHM is proposed to be mediated by low-fidelity DNA polymerases such as those that mediate trans-lesion synthesis (TLS); however, the mechanism by which these enzymes are recruited to AID-induced lesions remains unclear. Proliferating cell nuclear antigen (PCNA), the sliding clamp for multiple DNA polymerases, undergoes Rad6/Rad18- dependent ubiquitination in response to DNA damage. Ubiquitinated PCNA promotes the replacement of the replicative DNA polymerase stalled at the site of a DNA lesion with a TLS polymerase. To examine the potential role of Rad18-dependent PCNA ubiquitination in SHM, we analyzed Ig gene mutations in *Rad18* knockout (KO) mice immunized with T cell-dependent antigens. We found that SHM in *Rad18* KO mice was similar to wild- type mice, suggesting that *Rad18* is dispensable for SHM. However, residual levels of ubiquitinated PCNA were observed in *Rad18* KO cells, indicating that Rad18- independent PCNA ubiquitination might play a role in SHM. (Collaborative study with Dr. Shimizu, see reference 2).

5. Mutations in *UVSSA* cause UV-sensitive syndrome and impair RNA polymerase IIo processing in transcription- coupled nucleotide-excision repair

UV-sensitive syndrome (UV^{S}S) is a genodermatosis characterized by cutaneous photosensitivity without skin carcinoma. Despite mild clinical features, cells from individuals with UV^{S}S, like Cockayne syndrome cells, are very UV sensitive and are deficient in transcription-coupled nucleotide-excision repair (TC-NER), which removes DNA damage in actively transcribed genes. Three of the seven known UV^{S}S cases carry mutations in the Cockayne syndrome genes *ERCC8* or *ERCC6* (also known as *CSA* and *CSB*, respectively). The remaining four individuals with UV^{S}S , one of whom is described for the first time here, formed a separate UV^{S}S-A complementation group; however, the responsible gene was unknown. Using exome sequencing, we determine that mutations in the *UVSSA* gene (formerly known as *KIAA1530*) cause UV^{S}S-A. The UVSSA protein interacts with TC-NER machinery and stabilizes the ERCC6 complex; it also facilitates ubiquitination of RNA polymerase IIo stalled at DNA damage sites. Our findings provide mechanistic insights into the processing of stalled RNA polymerase and explain the different clinical features across these TC-NER–deficient disorders. (Collaborative study with Dr. Ogi, see reference 9).

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アウトリーチ活動　Outreach activity

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2. 立石 智. 色素性乾皮症、コケイン症候群などの遺伝性難病の細胞診断 (熊本大学医学部皮膚科および名古屋大学との共同作業)、 解析中のものを含めて 11 件.2012-2017, 熊本

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Somatic hypermutation of immunoglobulin genes in *Rad18* knockout mice

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ABSTRACT

Somatic hypermutation (SHM) of immunoglobulin (Ig) genes is triggered by the activity of activation-induced cytidine deaminase (AID). AID induces DNA lesions in variable regions of Ig genes, and error-prone DNA repair mechanisms initiated in response to these lesions introduce the mutations that characterize SHM. Error-prone DNA repair in SHM is proposed to be mediated by low-fidelity DNA polymerases such as those that mediate trans-lesion synthesis (TLS); however, the mechanism by which these enzymes are recruited to AID-induced lesions remains unclear. Proliferating cell nuclear antigen (PCNA), the sliding clamp for multiple DNA polymerases, undergoes Rad6/Rad18-dependent ubiquitination in response to DNA damage. Ubiquitinated PCNA promotes the replacement of the replicative DNA polymerase stalled at the site of a DNA lesion with a TLS polymerase. To examine the potential role of Rad18-dependent PCNA ubiquitination in SHM, we analyzed Ig gene mutations in *Rad18* knockout (KO) mice immunized with T cell-dependent antigens. We found that SHM in *Rad18* KO mice was similar to wild-type mice, suggesting that *Rad18* is dispensable for SHM. However, residual levels of ubiquitinated PCNA were observed in *Rad18* KO cells, indicating that Rad18-independent PCNA ubiquitination might play a role in SHM.

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1. Introduction

Affinity maturation of antibodies is mediated by somatic hypermutation (SHM) in immunoglobulin (Ig) genes in B cells followed by affinity-based selection in germinal centers (GCs) [1]. SHM is characterized by the introduction of point mutations in the variable regions of Ig genes at a frequency 10^6 times higher than spontaneous mutations [2–4]. Residual mutations are observed in the intron downstream of the joining region of Ig genes, but not in the constant region, and Ig gene mutations are also introduced in the switch region during class switch recombination (CSR) [5]. Mutations in non-Ig genes can promote tumorigenesis [6].

Activation-induced cytidine deaminase (AID) is required for the initiation of SHM and CSR [7]. AID generates U:G mismatches by converting cytosine to uracil in the variable regions of Ig genes [8,9]. As U:G mismatches can be generated throughout the entire genome via spontaneous cytosine deamination, multiple pathways for excising U from DNA have evolved. The base excision repair (BER) pathway [10] is mediated by the recognition and excision of uracil base by uracil-DNA *N*-glycosylase (UNG) and the creation of a DNA nick by apurinic/apyrimidinic (AP) endonuclease at the resulting abasic site. Subsequent steps in the BER pathway are mediated by either the short-patch or long-patch pathway. In the short-patch pathway, DNA polymerase (Pol) β replaces the abasic site, and DNA ligase 3 seals the nick. In the long-patch pathway, FEN1 generates a gap 2–10 nucleotides in length that is subsequently filled by Polβ or Polδ. The mismatch repair (MMR) pathway [11] is another pathway that mediates the excision of aberrant uracil bases. The MMR pathway is initiated by the recognition of the U:G mismatch by the MSH2/MSH6 heterodimer. MLH1, PMS2, and Exo1 are subsequently recruited to the repair site where they introduce a nick and remove a region of DNA encompassing the aberrant uracil. Polδ fills the gap, and DNA ligase 1 seals the nick. In these pathways, the U:G

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DNA repair factor RAD18 and DNA polymerase Polκ confer tolerance of oncogenic DNA replication stress

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The mechanisms by which neoplastic cells tolerate oncogene-induced DNA replication stress are poorly understood. Cyclin-dependent kinase 2 (CDK2) is a major mediator of oncogenic DNA replication stress. In this study, we show that CDK2-inducing stimuli (including Cyclin E overexpression, oncogenic RAS, and WEE1 inhibition) activate the DNA repair protein RAD18. CDK2-induced RAD18 activation required initiation of DNA synthesis and was repressed by p53. RAD18 and its effector, DNA polymerase κ (Polκ), sustained ongoing DNA synthesis in cells harboring elevated CDK2 activity. RAD18-deficient cells aberrantly accumulated single-stranded DNA (ssDNA) after CDK2 activation. In RAD18-depleted cells, the G2/M checkpoint was necessary to prevent mitotic entry with persistent ssDNA. *Rad18*$^{-/-}$ and *Polκ*$^{-/-}$ cells were highly sensitive to the WEE1 inhibitor MK-1775 (which simultaneously activates CDK2 and abrogates the G2/M checkpoint). Collectively, our results show that the RAD18–Polκ signaling axis allows tolerance of CDK2-mediated oncogenic stress and may allow neoplastic cells to breach tumorigenic barriers.

Introduction

During tumorigenesis, neoplastic cells must endure DNA damage from environmental, metabolic, and other intrinsic sources (Bartkova et al., 2006; Halazonetis et al., 2008). Oncogene-induced DNA replication stress can be a major cause of intrinsic DNA damage and represents a potential source of genome instability in cancer cells. Many oncogenes, including v-RAS, cyclin E, and others, induce DNA replication defects that trigger DNA damage signaling (including ATM–CHK2, ATR–CHK1, and p53) and lead to irreversible cell cycle exit often termed oncogene-induced senescence (OIS; Bartkova et al., 2006; Di Micco et al., 2006).

The precise mechanisms by which oncogenes induce DNA damage are incompletely understood. Oncogene-induced DNA damage has been attributed to induction of genotoxic reactive oxygen species (ROS; DeNicola et al., 2011), depletion of nucleotide pools (Bester et al., 2011), collisions between the DNA replication and transcriptional machinery (Jones et al., 2013), or aberrant reinitiation of DNA synthesis multiple times each per cell cycle—a process usually termed "rereplication" or "hyperreplication" (Di Micco et al., 2006). Rereplication likely generates "onion skin" DNA structures in which head-to-tail collisions between replication forks produce double-strand breaks (DSBs; Davidson et al., 2006). It is unknown whether oncogene-induced rereplication is caused by inappropriate activation of DNA replication licensing factors, initiation factors, or deregulation of both licensing and initiation phases of DNA synthesis. It is also unclear whether common mechanisms mediate rereplication and DNA damage in response to all oncogenes. It is possible that the constitutive mitogenic signals induced by oncogenes culminate in aberrant cyclin-dependent kinase 2 (CDK2) activation, in turn leading to DNA rereplication and other replication defects. Indeed, oncogene-induced DNA replication stress is often modeled experimentally by overexpression of CDK2 activators (Cyclin E and CDC25A) or inhibition of the WEE1 kinase to remove negative constraints over CDK2 (Sogo et al., 2002; Bartkova et al., 2006; Beck et al., 2010, 2012; Jones et al., 2013).

Despite our limited mechanistic understanding of how oncogenes dysregulate DNA synthesis and cause DNA damage, there is general consensus that OIS poses a barrier to tumorigenesis. Clearly, however, the OIS barrier is imperfect and can be breached. The precise mechanisms by which oncogene-expressing cells withstand replication stress and DNA damage are poorly understood. DNA repair and/or DNA dam-

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Abbreviations used: DSB, double-strand break; HR, homologous recombination; IP, immunoprecipitation; MEF, mouse embryonic fibroblast; NHF, normal human fibroblast; OIS, oncogene-induced senescence; PCC, premature chromatin condensation; PCNA, proliferating cell nuclear antigen; PDS, pyridostatin; ROS, reactive oxygen species; sgRNA, single guide RNA; ssDNA, single-stranded DNA; TLS, trans-lesion synthesis; TMEJ, θ-mediated end joining; XPV, XP-variant.

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A neomorphic cancer cell-specific role of MAGE-A4 in trans-lesion synthesis

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Trans-lesion synthesis (TLS) is an important DNA-damage tolerance mechanism that permits ongoing DNA synthesis in cells harbouring damaged genomes. The E3 ubiquitin ligase RAD18 activates TLS by promoting recruitment of Y-family DNA polymerases to sites of DNA-damage-induced replication fork stalling. Here we identify the cancer/testes antigen melanoma antigen-A4 (MAGE-A4) as a tumour cell-specific RAD18-binding partner and an activator of TLS. MAGE-A4 depletion from MAGE-A4-expressing cancer cells destabilizes RAD18. Conversely, ectopic expression of MAGE-A4 (in cell lines lacking endogenous MAGE-A4) promotes RAD18 stability. DNA-damage-induced mono-ubiquitination of the RAD18 substrate PCNA is attenuated by MAGE-A4 silencing. MAGE-A4-depleted cells fail to resume DNA synthesis normally following ultraviolet irradiation and accumulate γH2AX, thereby recapitulating major hallmarks of TLS deficiency. Taken together, these results demonstrate a mechanism by which reprogramming of ubiquitin signalling in cancer cells can influence DNA damage tolerance and probably contribute to an altered genomic landscape.

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Rad18 confers hematopoietic progenitor cell DNA damage tolerance independently of the Fanconi Anemia pathway *in vivo*

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ABSTRACT

In cultured cancer cells the E3 ubiquitin ligase Rad18 activates Trans-Lesion Synthesis (TLS) and the Fanconi Anemia (FA) pathway. However, physiological roles of Rad18 in DNA damage tolerance and carcinogenesis are unknown and were investigated here. Primary hematopoietic stem and progenitor cells (HSPC) co-expressed RAD18 and FANCD2 proteins, potentially consistent with a role for Rad18 in FA pathway function during hematopoiesis. However, hematopoietic defects typically associated with *fanc*-deficiency (decreased HSPC numbers, reduced engraftment potential of HSPC, and Mitomycin C (MMC) -sensitive hematopoiesis), were absent in *Rad18*$^{-/-}$ mice. Moreover, primary *Rad18*$^{-/-}$ mouse embryonic fibroblasts (MEF) retained robust Fancd2 mono-ubiquitination following MMC treatment. Therefore, Rad18 is dispensable for FA pathway activation in untransformed cells and the Rad18 and FA pathways are separable in hematopoietic cells. In contrast with responses to crosslinking agents, *Rad18*$^{-/-}$ HSPC were sensitive to *in vivo* treatment with the myelosuppressive agent 7,12 Dimethylbenz[a]anthracene (DMBA). Rad18-deficient fibroblasts aberrantly accumulated DNA damage markers after DMBA treatment. Moreover, *in vivo* DMBA treatment led to increased incidence of B cell malignancy in *Rad18*$^{-/-}$ mice. These results identify novel hematopoietic functions for Rad18 and provide the first demonstration that Rad18 confers DNA damage tolerance and tumor-suppression in a physiological setting.

INTRODUCTION

Cells are frequently subject to DNA damage from environmental, intrinsic and therapeutic sources. Failure to tolerate and accurately repair DNA damage can lead to loss of cell viability or genome instability, an enabling characteristic of cancer cells (1). The E3 ubiquitin ligase RAD18 plays key roles in Trans-Lesion Synthesis (TLS), a DNA damage tolerance mechanism that allows cells to replicate genomes harboring bulky DNA lesions including polycyclic aryl hydrocarbon (PAH) adducts (2). In response to DNA damage, RAD18 redistributes to stalled DNA replication forks (3,4) and mono-ubiquitinates the DNA polymerase processivity factor PCNA (5). DNA damage-tolerant 'Y-family' TLS DNA polymerases possess ubiquitin-binding domains and associate preferentially with mono-ubiquitinated PCNA (6) to promote replicative bypass of DNA lesions and DNA damage tolerance (7). However, TLS polymerases are inherently error-prone when compared to replicative DNA polymerases and can generate mutations. Thus, RAD18 and its effector TLS polymerases can confer viability, but also have the potential to compromise genome stability (7). Indeed *Rad18*-deficient cells are genotoxin-sensitive and hypomutagenic for bypass of various DNA lesions, including PAH (8,9). The RAD18-mediated TLS pathway has been stud-

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RESEARCH ARTICLE

RAD18 Activates the G2/M Checkpoint through DNA Damage Signaling to Maintain Genome Integrity after Ionizing Radiation Exposure

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Abstract

The ubiquitin ligase RAD18 is involved in post replication repair pathways via its recruitment to stalled replication forks, and its role in the ubiquitylation of proliferating cell nuclear antigen (PCNA). Recently, it has been reported that RAD18 is also recruited to DNA double strand break (DSB) sites, where it plays novel functions in the DNA damage response induced by ionizing radiation (IR). This new role is independent of PCNA ubiquitylation, but little is known about how RAD18 functions after IR exposure. Here, we describe a role for RAD18 in the IR-induced DNA damage signaling pathway at G2/M phase in the cell cycle. Depleting cells of RAD18 reduced the recruitment of the DNA damage signaling factors ATM, γH2AX, and 53BP1 to foci in cells at the G2/M phase after IR exposure, and attenuated activation of the G2/M checkpoint. Furthermore, depletion of RAD18 increased micronuclei formation and cell death following IR exposure, both *in vitro* and *in vivo*. Our data suggest that RAD18 can function as a mediator for DNA damage response signals to activate the G2/M checkpoint in order to maintain genome integrity and cell survival after IR exposure.

Introduction

The RING-type (E3) ubiquitin ligase RAD18 is a key player involved in post-replication repair (PRR) that regulates ubiquitylation of proliferating cell nuclear antigen (PCNA) in response to

LETTER

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Two replication fork maintenance pathways fuse inverted repeats to rearrange chromosomes

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Replication fork maintenance pathways preserve chromosomes, but their faulty application at nonallelic repeats could generate rearrangements causing cancer, genomic disorders and speciation[1–3]. Potential causal mechanisms are homologous recombination and error-free postreplication repair (EF-PRR). Homologous recombination repairs damage-induced DNA double-strand breaks (DSBs) and single-ended DSBs within replication. To facilitate homologous recombination, the recombinase RAD51 and mediator BRCA2 form a filament on the 3′ DNA strand at a break to enable annealing to the complementary sister chromatid[4] while the RecQ helicase, BLM (Bloom syndrome mutated) suppresses crossing over to prevent recombination[5]. Homologous recombination also stabilizes[6,7] and restarts[8,9] replication forks without a DSB[10,11]. EF-PRR bypasses DNA incongruities that impede replication by ubiquitinating PCNA (proliferating cell nuclear antigen) using the RAD6–RAD18 and UBC13–MMS2–RAD5 ubiquitin ligase complexes[12]. Some components are common to both homologous recombination and EF-PRR such as RAD51 and RAD18[13,14]. Here we delineate two pathways that spontaneously fuse inverted repeats to generate unstable chromosomal rearrangements in wild-type mouse embryonic stem (ES) cells. Gamma-radiation induced a BLM-regulated pathway that selectively fused identical, but not mismatched, repeats. By contrast, ultraviolet light induced a RAD18-dependent pathway that efficiently fused mismatched repeats. Furthermore, TREX2 (a 3′→5′ exonuclease) suppressed identical repeat fusion but enhanced mismatched repeat fusion, clearly separating these pathways. TREX2 associated with UBC13 and enhanced PCNA ubiquitination in response to ultraviolet light, consistent with it being a novel member of EF-PRR. RAD18 and TREX2 also suppressed replication fork stalling in response to nucleotide depletion. Interestingly, replication fork stalling induced fusion for identical and mismatched repeats, implicating faulty replication as a causal mechanism for both pathways.

The identical and mismatched repeat reporters (IRR and MRR, Fig. 1a, b) were designed to investigate pathways that rearrange chromosomes through repeat fusion. Both reporters contain a 313-base-pair major satellite repeat (MSR) at each junction of an inversion in *miniHPRT*. These repeats are indirect so repeat fusion restores *miniHPRT* to enable survival in hypoxanthine, aminopterin, thymidine (HAT)-selection media by a potential mechanism shown in Fig. 1c. The only difference between these reporters is that the MRR 3′ repeat contains seven mismatches with the longest contiguous homology being 67 bases. The IRR and MRR were stably transfected into wild-type AB2.2 and IB10 ES cells. About the same number of HAT-resistant colonies spontaneously grew for both reporters (Fig. 1d, $P > 0.85$, Student's *t*-test), indicating that spontaneous repeat fusion occurred in wild-type cells.

The fused 5′ repeat for the MRR was sequenced to determine the switch location (Fig. 1e, Extended Data Fig. 1). Strand exchange in fission yeast predominantly occurred at the palindrome centre after replication forks were induced to stall, an event called a U turn[9]. We found 6 of 14 switches had this U-turn at the base of a putative hairpin (all green), whereas two occurred at the apex (all orange) and six occurred in the stem (green-orange). Thus, strand exchange occurred at multiple locations.

It is possible that the switched strand replicated to the telomere, forming a dipericentric (Fig. 1c). Two-colour fluorescence *in situ* hybridization (FISH) was performed on clones with the IRR and MRR using a pericentromeric and telomeric probe. Dipericentrics and chromosomes with extra pericentromeres and telomeres (EPTs)[15] were observed for cells with both reporters (Extended Data Fig. 2a and Extended Data Tables 1 and 2). EPTs seemed unstable because the pericentromere number and location varied between metaphase spreads from the same clone, implicating secondary events consistent with breakage–fusion–bridge cycles[16]. Spectral karyotyping on three MRR clones showed multiple fusion points confirming rearrangement complexity (Extended Data Table 3). Duplications of chromosome 1 (Fig. 1f, left) and translocations between chromosomes 14 and 11 (Fig. 1f, right) or 14 and 13 were frequently observed from the same clone and even in the same metaphase spread, indicating a role in genome topology[17]. Two-colour FISH was performed on a single clone (clone 18 from Extended Data Tables 2 and 3) with the MRR probe and either chromosome 1 or 14. This analysis revealed unstable structures because the MRR could be found at either chromosomes 1 or 14 (Extended Data Fig. 2b), indicating faulty DNA synthesis[18]. Furthermore, the MRR pattern changed from a discrete dot to multiple dots interspersed with chromosomal sequences similar to segmental duplications described during evolution[19]. Thus, both reporters caused unstable and complex rearrangements, yet the causal pathways are not known.

Complex genomic rearrangements could arise from faulty chromosome maintenance. Therefore, we tested whether γ-radiation or ultraviolet light enhanced repeat fusion for wild-type AB2.2 cells with the IRR or MRR. Exposure to 4 Gy γ-radiation induced repeat fusion for the IRR (Fig. 2a, left, $P = 0.017$, Student's *t*-test) but not the MRR (Fig. 2a, right, $P = 0.16$), whereas exposure to 20 J m^{-2} ultraviolet light had the opposite effect on the IRR (Fig. 2b, left, $P = 0.35$) and MRR (Fig. 2b right, $P = 0.006$). This contrast suggests different pathways fused identical and mismatched repeats.

We tested whether homologous recombination proteins fused identical repeats because homologous recombination corrects damage caused by γ-radiation but not ultraviolet light[4]. We tested BLM-defective ES cells ($blm^{tm3Brd/tm4Brd}$, simply called $blm^{-/-}$)[20] because BLM regulates homologous recombination through Holliday junction dissolution[5]. Repeat fusion was significantly higher in $blm^{-/-}$ cells as compared to AB2.2 cells for the IRR (Fig. 2c, compare lanes 1 and 2, $P < 0.0001$), but not the MRR (Fig. 2c, compare lanes 6 and 7, $P = 0.47$). Next we tested $blm^{-/-}$ cells haploinsufficient for RAD51 or BRCA2 because BRCA2 enables RAD51 filament formation on DNA single stands to mediate strand annealing and Holliday junction formation. We found $blm^{-/-}$ $Rad51^{+/\Delta ex2\text{-}4}$ cells

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A non-catalytic role of DNA polymerase η in recruiting Rad18 and promoting PCNA monoubiquitination at stalled replication forks

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ABSTRACT

Trans-lesion DNA synthesis (TLS) is a DNA damage-tolerance mechanism that uses low-fidelity DNA polymerases to replicate damaged DNA. The inherited cancer-propensity syndrome xeroderma pigmentosum variant (XPV) results from error-prone TLS of UV-damaged DNA. TLS is initiated when the Rad6/Rad18 complex monoubiquitinates proliferating cell nuclear antigen (PCNA), but the basis for recruitment of Rad18 to PCNA is not completely understood. Here, we show that Rad18 is targeted to PCNA by DNA polymerase eta (Polη), the *XPV* gene product that is mutated in *XPV* patients. The C-terminal domain of Polη binds to both Rad18 and PCNA and promotes PCNA monoubiquitination, a function unique to Polη among Y-family TLS polymerases and dissociable from its catalytic activity. Importantly, XPV cells expressing full-length catalytically-inactive Polη exhibit increased recruitment of other error-prone TLS polymerases (Polκ and Polι) after UV irradiation. These results define a novel non-catalytic role for Polη in promoting PCNA monoubiquitination and provide a new potential mechanism for mutagenesis and genome instability in XPV individuals.

INTRODUCTION

Living organisms are constantly exposed to ubiquitous genotoxins from endogenous and external sources (1). However, cells have evolved numerous DNA damage response (DDR) pathways that protect genomic DNA and prevent genetic instability (2). Trans-lesion synthesis (TLS) is a DDR mechanism involving specialized DNA polymerases that can replicate damaged DNA templates (3).

TLS relies on inherently error-prone DNA polymerases of the Y family to replicate damaged DNA (4). TLS by Y-family polymerases (Polη, Polι, Polκ and Rev1) (5) maintains replication in cells harbouring damaged DNA, albeit at the cost of reduced fidelity. Each TLS polymerase performs relatively error-free replication past a preferred cognate lesion; in the absence of the appropriate TLS polymerase for its preferred lesion, mutagenic replication by error-prone polymerases predisposes to genetic instability (2).

Polη is unique among Y-family polymerases in its ability to perform accurate replication past UV-damaged DNA (6,7). Lack of Polη in the inherited cancer-propensity syndrome xeroderma pigmentosum variant (XPV) (8) results in error-prone replication by other Y-family polymerases in sunlight-exposed cells (9,10). Thus, UV-induced mutagenesis due to Polη deficiency compromises genetic integrity to manifest as exquisite sunlight sensitivity and early skin cancer propensity.

A prerequisite for error-prone replication in TLS is the Rad6/Rad18-mediated monoubiquitination of proliferating cell nuclear antigen (PCNA) at the highly conserved lysine K164 (11,12). Y-family polymerases contain ubiquitin-binding (UBZ) domains that confer affinity to monoubiquitinated PCNA (13,14). Failure to monoubiquitinate PCNA at K164 phenocopies XPV by compromising TLS and sensitizing cells to UV light and other ubiquitous genotoxins (15–18). Several other DDR pathways also depend on PCNA monoubiquitination, including SHPRH/HTLF-mediated template switching (19), ZRANB3-dependent replication fork restart (20), SNM1A-dependent intrastrand cross-link repair (21) and the Fanconi Anaemia pathway activation (22).

Despite its pivotal role in the DDR, the molecular mechanisms regulating Rad18-mediated PCNA monoubiquitination are incompletely understood. The Rad18–Rad6 complex is thought to be recruited to the vicinity of damaged DNA via direct interactions with RPA-coated

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Mutations in *UVSSA* cause UV-sensitive syndrome and impair RNA polymerase IIo processing in transcription-coupled nucleotide-excision repair

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UV-sensitive syndrome (UV^{S}S) is a genodermatosis characterized by cutaneous photosensitivity without skin carcinoma[1–4]. Despite mild clinical features, cells from individuals with UV^{S}S, like Cockayne syndrome cells, are very UV sensitive and are deficient in transcription-coupled nucleotide-excision repair (TC-NER)[2,4,5], which removes DNA damage in actively transcribed genes[6]. Three of the seven known UV^{S}S cases carry mutations in the Cockayne syndrome genes *ERCC8* or *ERCC6* (also known as *CSA* and *CSB*, respectively)[7,8]. The remaining four individuals with UV^{S}S, one of whom is described for the first time here, formed a separate UV^{S}S-A complementation group[1,9,10]; however, the responsible gene was unknown. Using exome sequencing[11], we determine that mutations in the *UVSSA* gene (formerly known as *KIAA1530*) cause UV^{S}S-A. The UVSSA protein interacts with TC-NER machinery and stabilizes the ERCC6 complex; it also facilitates ubiquitination of RNA polymerase IIo stalled at DNA damage sites. Our findings provide mechanistic insights into the processing of stalled RNA polymerase and explain the different clinical features across these TC-NER–deficient disorders.

We performed exome sequencing on two cell lines, Kps3 and XP24KO, derived from two individuals with UV^{S}S-A (cell lines described in **Supplementary Table 1**; exome described in **Table 1**, Online Methods, **Supplementary Table 2a–c** and **Supplementary Note**). Using a recessive model of inheritance, we directly identified overlapping mutations in *KIAA1530* (NCBI Gene 57654), a predicted gene at 4p16.3 encoding a 709 amino acid protein of unknown function (**Table 1** and **Supplementary Table 2c**). *KIAA1530* was subsequently renamed *UVSSA* (encoding UV-stimulated scaffold protein A) because of this finding, with support from the Human Gene Nomenclature Committee (HGNC). Affected individuals were homozygous for a c.367A>T mutation, which led to the introduction of a premature stop codon, p.Lys123*, in the UVSSA protein (**Fig. 1a**,**b**). We identified the same homozygous mutation in subject Kps2 (a sibling of Kps3) and a homozygous c.87delG mutation causing a p.Ile31Phefs*9 frameshift alteration in Israeli subject UV^{S}S24TA (**Fig. 1b**,**c**, **Supplementary Fig. 1** and **Supplementary Note**). The identified mutations are summarized (**Fig. 1d**). We did not detect the 80-kDa UVSSA protein in any of the individuals with UV^{S}S-A (**Fig. 1e**). We also examined several mild xeroderma pigmentosum cases; in one case, XP70TO[12] (**Supplementary Table 1**), we identified a homozygous missense mutation (encoding p.Cys32Arg) in *UVSSA* (**Fig. 1c**,**d**), which implies that XP70TO is also in the UV^{S}S-A complementation group. The mutant protein was stably expressed in XP70TO cells, although band intensity was faint (**Fig. 1f** and **Supplementary Fig. 2a–d**).

The mutated allele encoding p.Lys123* was observed in the heterozygous state in 1 of 576 control individuals (allele frequency of 0.09%) evaluated by direct sequencing or high-resolution melting

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The Vital Role of Polymerase ζ and REV1 in Mutagenic, but Not Correct, DNA Synthesis across Benzo[*a*]pyrene-dG and Recruitment of Polymerase ζ by REV1 to Replication-stalled Site*[S]

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Background: dG lesion derived from potent carcinogen benzo[*a*]pyrene causes mutations through DNA replication.
Results: Pol ζ and REV1 are essential to mutagenic, but not accurate, translesion DNA synthesis.
Conclusion: DNA synthesis across identical DNA damage can be catalyzed by a different set of polymerases.
Significance: The results have revealed an important role for DNA polymerases, pol ζ and REV1, in inducing mutations.

The DNA synthesis across DNA lesions, termed translesion synthesis (TLS), is a complex process influenced by various factors. To investigate this process in mammalian cells, we examined TLS across a benzo[*a*]pyrene dihydrodiol epoxide-derived dG adduct (BPDE-dG) using a plasmid bearing a single BPDE-dG and genetically engineered mouse embryonic fibroblasts (MEFs). In wild-type MEFs, TLS was extremely miscoding (>90%) with G → T transversions being predominant. Knockout of the *Rev1* gene decreased both the TLS efficiency and the miscoding frequency. Knockout of the *Rev3L* gene, coding for the catalytic subunit of pol ζ, caused even greater decreases in these two TLS parameters; almost all residual TLS were error-free. Thus, REV1 and pol ζ are critical to mutagenic, but not accurate, TLS across BPDE-dG. The introduction of human *REV1* cDNA into *Rev1*$^{-/-}$ MEFs restored the mutagenic TLS, but a *REV1* mutant lacking the C terminus did not. Yeast and mammalian three-hybrid assays revealed that the REV7 subunit of pol ζ mediated the interaction between REV3 and the REV1 C terminus. These results support the hypothesis that REV1 recruits pol ζ through the interaction with REV7. Our results also predict the existence of a minor REV1-independent pol ζ recruitment pathway. Finally, although mutagenic TLS across BPDE-dG largely depends on RAD18, experiments using *Polk*$^{-/-}$ *Polh*$^{-/-}$ *Poli*$^{-/-}$ triple-gene knockout MEFs unexpectedly revealed that another polymerase(s) could insert a nucleotide opposite BPDE-dG. This indicates that a non-Y family polymerase(s) can insert a nucleotide opposite BPDE-dG, but the subsequent extension from miscoding termini depends on REV1-polζ in a RAD18-dependent manner.

The human genome constantly suffers from DNA damage induced by endogenous and exogenous sources, and the damage often blocks DNA synthesis catalyzed by replicative DNA polymerases unless it is removed before they reach damage sites. Upon blocking, a group of specialized DNA polymerases takes over DNA synthesis across a lesion, which is termed translesion DNA synthesis (TLS).[5] TLS DNA polymerases can synthesize DNA across a damaged base often at a cost of mutations that mostly are targeted at the lesion site. Among 15 mammalian DNA polymerases (1), Y family DNA polymerases (2) play major roles in TLS. This family includes pol η, pol ι, pol κ, and REV1. These polymerases have in common a wide catalytic space that accommodates unusual base pairs (3, 4). Human pol η is the product of the gene responsible for xeroderma pigmentosum variant, an inherited disorder highly predisposed to skin cancer caused by sunlight exposure (5, 6). This polymerase conducts a very efficient and relatively accurate DNA synthesis across UV-induced cyclobutane pyrimidine dimers. Thus, pol η plays an important role in protecting cells from the deleterious effects of unrepaired cyclobutane pyrimidine dimers. Although pol ι has been reported to play a role in dealing with unrepaired oxidative DNA damage (7), the physiological substrates for pol ι, as well as pol κ, have not yet been established.

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[5] The abbreviations used are: TLS, translesion DNA synthesis; pol, DNA polymerase; BPDE-dG, benzo[*a*]pyrene dihydrodiol epoxide-derived dG adduct; MEF, mouse embryonic fibroblast; TKO, triple gene knockout; DKO, double gene knockout; PCNA, proliferating cell nuclear antigen; AD, activation domain; BD, DNA-binding domain; BRCT, carboxyl-terminus of BRCA1 protein.

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