Role of RNase H1 in DNA repair: removal of single ribonucleotide misincorporated into DNA in collaboration with RNase H2

Elias Tannous, Eiko Kanaya & Shigenori Kanaya

Several RNases H1 cleave the RNA-DNA junction of Okazaki fragment-like RNA-DNA/DNA substrate. This activity, termed 3'-junction ribonuclease (3'-JRNase) activity, is different from the 5'-JRNase activity of RNase H2 that cleaves the 5'-side of the ribonucleotide of the RNA-DNA junction and is required to initiate the ribonucleotide excision repair pathway. To examine whether RNase H1 exhibits 3'-JRNase activity for dsDNA containing a single ribonucleotide and can remove this ribonucleotide in collaboration with RNase H2, cleavage of a DNA8-RNA1-DNA9/DNA18 substrate with E. coli RNase H1 and H2 was analyzed. This substrate was cleaved by E. coli RNase H2 at the (5')DNA-RNA(3') junction, regardless of whether it was cleaved by E. coli RNase H2 at the (5')DNA-RNA(3') junction in advance or not. Likewise, this substrate was cleaved by E. coli RNase H2 at the (5')DNA-RNA(3') junction, regardless of whether it was cleaved by E. coli RNase H2 at the (5')DNA-RNA(3') junction in advance or not. When this substrate was cleaved by a mixture of E. coli RNases H1 and H2, the ribonucleotide was removed from the substrate. We propose that RNase H1 is involved in the excision of single ribonucleotides misincorporated into DNA in collaboration with RNase H2.

Ribonuclease H (RNase H) is an endoribonuclease that specifically cleaves the RNA strand of RNA/DNA hybrids. It cleaves the PO-3' bond of the substrate with a two-metal-ion catalysis mechanism, in which two divalent cations, such as Mg2+ and Mn2+, directly participate in the catalytic function. RNase H is widely present in various organisms, including bacteria, archaea and eukaryotes. It is also present in retroviruses as a C-terminal domain of reverse transcriptase (RT). These RNases H are classified into type 1 and type 2 RNases H based on the difference in their amino acid sequences. Type 1 RNases H include prokaryotic and eukaryotic RNases H1 and retroviral RNase H. Type 2 RNases H include prokaryotic and eukaryotic RNases H2 and bacterial RNase H3. These RNases H exist in a monomeric form, except for eukaryotic RNases H2, which exist in a heterotrimeric form.

Most organisms have multiple RNases H within a single cell. They are mostly RNases H1 and H2 and occasionally RNases H2 and H3. Loss of these enzymes has shown different effect on various organisms. While loss of all functional RNases H renders B. subtilis unable to grow, it leads to temperature sensitivity and the accumulation of untreated R loops, leading to DNA replication initiation at sites other than the oriC, in E. coli, and higher sensitivity to alkylating agents in Saccharomyces cerevisiae. In mouse, loss of either RNase H1 or RNase H2 causes embryonic lethality. In human, mutations in RNase H2 subunits cause Aicardi-Goutieres syndrome and mimic congenital viral brain infection. In retroviruses, inactivation of the RNase H domain of RT abolishes virus infectivity and therefore retroviral RNase H is regarded as a target for AIDS therapy.
RNase H2 is required to initiate the ribonucleotide excision repair (RER) pathway both in eukaryotes and prokaryotes because of its junction ribonuclease (JRNase) activity. This activity cleaves dsDNA containing a single rNMP (dsDNA_R1) at the 5’-side of the ribonucleotide. Subsequent removal of the ribonucleotide is catalyzed by several enzymes that all together form the RER pathway. RNase H1 does not exhibit JRNase activity. However, RNases H1 from Halobacterium sp. NRC-1 (Halo-RNase H1), Sulfolobus tokodaii (Sto-RNase H1), Thermotoga maritima, and E. coli exhibit a weak activity that catalyzes the cleavage of an Okazaki fragment-like substrate at the RNA-DNA junction. To distinguish this activity from JRNase activity of RNase H2, that catalyzes the cleavage of an RNA-DNA/DNA substrate at the 5’-side of the ribonucleotide of the RNA-DNA junction, this activity and JRNase activity of RNase H2 will be designated as 3’- and 5’-JRNase activities respectively hereafter.

In this report, we showed that the RNA-DNA junction of the RNA1-DNA9/DNA18 substrate is not cleaved by E. coli RNase H1 but is cleaved by the enzyme when a short RNA fragment is supplied to facilitate the formation of an upstream duplex structure. We also showed that E. coli RNase H1 exhibits 3’-JRNase activity for dsDNA_R1 in the presence of manganese ions much more effectively than in the presence of magnesium ions, regardless of whether this substrate is cleaved by 5’-JRNase activity of E. coli RNase H2 in advance or not. From these results, we propose that single rNMPs misincorporated into the genomes can be excised by a cooperative work of RNases H1 and H2 in prokaryotic cells.

**Results and Discussion**

**Oligomeric substrates.** The oligomeric substrates used in this study are summarized in Fig. 2. The asterisk indicates the fluorescein-labeled site. The RNA9-DNA9*/DNA18 (R9-D9*/D18) substrate represents an Okazaki fragment-like substrate labeled at the 3’-end. The RNA1-DNA9*/DNA18 (R1-D9*/D18) and RNA2-DNA9*/DNA18 (R2-D9*/D18) substrates represent Okazaki fragment-like substrates containing one and two ribonucleotides labeled at the 3’-ends. The R8:R9-D9*/D18 and R7:R2-D9*/D18 substrates represent the R9-D9*/D18 substrates with a nick at the P-O3’ bond between the first and second ribonucleotides from the RNA-DNA junction respectively. The *DNA8-RNA1-DNA9/DNA18 (*D8-R1-D9/D18) and DNA8-RNA1-DNA9*/DNA18 (D8-R1-D9*/D18) substrates represent dsDNA containing a single ribonucleotide (dsDNA_R1) labeled at the 5’- and 3’-ends respectively. For dsDNA_R1, the 5’- and 3’-sides of the ribonucleotide are designated as (5’)DNA-RNA(3’) and (5’)RNA-DNA(3’) junctions respectively.

![Figure 1](https://www.nature.com/scientificreports/)

**Figure 1.** Sites of cleavage by 5’- and 3’-JRNase activities of RNases H. Cleavage sites of an Okazaki fragment-like RNA-DNA/DNA substrate (a) and dsDNA containing a single ribonucleotide (dsDNA_R1) (b) by 5’- and 3’-JRNase activities of RNases H are shown. RNA and DNA strands are shown by red and blue boxes respectively. The ribonucleotide of the (5’)RNA-DNA(3’) junction is indicated by R. The cleavage sites are indicated by arrows.
Purity of the proteins.  

**E. coli** RNases H1 and H2 are encoded by the *rnhA* and *rnhB* genes respectively. The purity of these proteins used in this study were analyzed by SDS-PAGE, followed by silver staining. The results are shown in Fig. 3. *E. coli* RNase H1 migrates in the gel as a single band, indicating that it is homogeneous. *E. coli* RNase H2 does not migrate in the gel as a single band, but migrates as a major band.

### Cleavage of R9-D9/D18 substrate by **E. coli** RNase H1.  
It has been reported that *E. coli* RNase H1 cleaves an Okazaki fragment-like substrate most effectively at R(−2)-R(−1) and less effectively at the
RNA-DNA junction in the presence of 5 mM MnCl₂, indicating that *E. coli* RNase H1 exhibits a weak 3′-JRNase activity for this substrate in the presence of manganese ions. However, two conflicting results that *E. coli* RNase H1 can⁹,²⁶,³⁵ cleave this substrate at the RNA-DNA junction in the presence of magnesium ions have been reported. Therefore, the R9-D9*/D18 substrate was first cleaved by *E. coli* RNase H1 either in the presence of 0.1 mM MnCl₂ or 10 mM MgCl₂, to examine whether *E. coli* RNase H1 exhibits 3′-JRNase activity for this substrate in the presence of magnesium ions. The results are shown in Figs. 3a, b. The D9* fragment is detected as one of the major products when the substrate is extensively cleaved by the enzyme. This band is produced more effectively in the presence of manganese ions than in the presence of magnesium ions. These results indicate that *E. coli* RNase H1 cleaves the R9-D9*/D18 substrate at the RNA-DNA junction either in the presence of manganese or magnesium ions, but more effectively in the presence of manganese ions than in the presence of magnesium ions. This result is consistent with that previously reported. Thus, *E. coli* RNase H1 exhibits 3′-JRNase activity for an Okazaki fragment-like substrate either in the presence of manganese or magnesium ions, but more strongly in the presence of manganese ions. This metal ion preference is opposite to that for the RNase H activity of this enzyme determined using an RNA/DNA substrate.

*E. coli* RNase H1 cleaves the R9-D9*/D18 substrate almost exclusively at R(−2)-R(−1) and RNA-DNA junction in the presence of 0.1 mM MnCl₂, whereas it cleaves this substrate preferentially at R(−3)-R(−2) and R(−2)-R(−1) in the presence of 10 mM MgCl₂. It has been reported that hydrolysis of this substrate by *E. coli* RNase H1 is initiated by the cleavage at R(−5)-R(−4), R(−4)-R(−3), and R(−3)-R(−2) in the presence of 10 mM MgCl₂. These results suggest that the R9-D9*/D18 substrate is cleaved by *E. coli* RNase H1 preferentially at the upstream region of the RNA-DNA junction in the presence of magnesium ions due to its RNase H activity. This substrate is not cleaved by *E. coli* RNase H1 at the upstream region of the RNA-DNA junction except for R(−2)-R(−1), probably due to a very weak RNase H activity. It has been reported that the RNase H activity of *E. coli* RNase H1 determined using an RNA/DNA substrate in the presence of the optimum concentration of manganese ions (5–10 mM) by only 5 fold, whereas the RNase H activity of *E. coli* RNase H1 determined using a 12 base pair RNA/DNA substrate in the presence of 5 mM MnCl₂ is lower than that determined in the presence of 5 mM MgCl₂ by 1000 fold. As a result, *E. coli* RNase H1 cleaves the R9-D9*/D18 substrate more effectively in the presence of 10 mM MgCl₂ than in the presence of 0.1 mM MnCl₂ (Fig. 4a).

Cleavage of R1-D9/D18 and R8:R1-D9/D18 substrates by *E. coli* RNase H1. The R1-D9* fragment is detected as one of the major products when the R9-D9*/D18 substrate is extensively cleaved by *E. coli* RNase H1 (Fig. 4a), suggesting that the R1-D9*/D18 substrate is not cleaved by *E. coli* RNase H1. To examine whether *E. coli* RNase H1 does not cleave this substrate, but cleaves it at the RNA-DNA junction when 8 b RNA (R8) complementary to the single stranded region of the R1-D9*/D18 substrate is supplied, the R1-D9*/D18 and R8:R1-D9*/D18 substrates were cleaved by *E. coli* RNase H1 either in the presence of 0.1 mM MnCl₂ or 10 mM MgCl₂. The results are shown in Figs. 3c, d. *E. coli* RNase H1 does not cleave the R1-D9*/D18 substrate regardless of the metal cofactors. In contrast, it cleaves the R8:R1-D9*/D18 substrate at the RNA-DNA junction, but only in the presence of manganese ions. This result suggests that *E. coli* RNase H1 cleaves an Okazaki fragment-like RNA-DNA/DNA substrate at the RNA-DNA junction regardless of whether this substrate contains a nick at R(−2)-R(−1). The RNA-DNA junction of the R8:R1-D9*/D18 substrate is not fully cleaved by *E. coli* RNase H1, probably because the upstream region of the RNA-DNA junction is cleaved before the RNA-DNA junction is completely cleaved. The RNA-DNA junction of the R9-D9*/D18 substrate is cleaved by the 3′-JRNase activity of *E. coli* RNase H1 less effectively in the presence of magnesium ions than in the presence of manganese ions, probably because the upstream region of the RNA-DNA junction is cleaved by the RNase H activity of *E. coli* RNase H1 more effectively in the presence of magnesium ions than in the presence of manganese ions. The RNA-DNA junction of the R8:R1-D9*/D18 substrate is not cleaved by *E. coli* RNase H1 in the presence of magnesium ions, probably because the presence of a nick at R(−2)-R(−1) alters the interaction between the substrate and metal ion, in such a way that the scissile phosphate group of the substrate and magnesium ions are not arranged ideally.

Cleavage of R2-D9/D18 and R7:R2-D9/D18 substrates by *E. coli* RNase H1. Inability of *E. coli* RNase H1 to cleave the R1-D9*/D18 substrate at the RNA-DNA junction indicates that multiple upstream ribonucleotides are necessary for the cleavage of an Okazaki fragment-like substrate by the enzyme at the RNA-DNA junction. To examine whether two upstream ribonucleotides are sufficient for this cleavage, the R2-D9*/D18 substrate was cleaved by *E. coli* RNase H1 either in the presence of 0.1 mM MnCl₂ or 10 mM MgCl₂. The results are shown in Figs. 3e, f. *E. coli* RNase H1 cleaves the R2-D9*/D18 substrate at the RNA-DNA junction either in the presence of manganese or magnesium ions, but more effectively in the presence of manganese ions. This result indicates that the presence of two upstream ribonucleotides is sufficient for the cleavage of an Okazaki fragment-like substrate by *E. coli* RNase H1 at the RNA-DNA junction. To examine whether this cleavage site is shifted by supplying an RNA strand that facilitates the formation of an upstream duplex structure, the R7:R2-D9*/D18 substrate was also cleaved by *E. coli* RNase H1. The results are shown in Fig. 3e, f. *E. coli* RNase H1 most effectively cleaves this substrate at R(−2)-R(−1) in the presence of manganese ions. This site is also cleaved in the presence...
of magnesium ions, but much less effectively, probably because the RNA/DNA region of the R7:R2-D9*/D18 substrate is effectively cleaved by the RNase H activity of the enzyme in the presence of magnesium ions. Thus, the primary products of the R7:R2-D9*/D18 substrate upon cleavage with *E. coli* RNase H1 in the presence of manganese and magnesium ions are probably the R7:R1-D9*/D18 and R2-D9*/D18 duplexes respectively. The RNA-DNA junctions of these primary products are cleaved only when the concentration of the enzyme is greatly elevated.

**Cleavage of D8-R1-D9/D18 substrate by *E. coli* RNase H1.** To examine whether *E. coli* RNase H1 exhibits 3'-JRNase activity for dsDNA R1, the D8-R1-D9*/D18 and *D8-R1-D9*/D18 substrates were cleaved by *E. coli* RNase H1 either in the presence of 0.1 mM MnCl₂ or 10 mM MgCl₂. The results are shown in Fig. 5. The D9* and *D8-R1 fragments are detected as the major products more clearly in the presence of manganese ions, indicating that *E. coli* RNase H1 cleaves these substrates mainly at the (5')RNA-DNA(3') junction and much more effectively in the presence of manganese ions than in the presence of magnesium ions to produce the D8-R1-D9/D18 duplex containing a nick at the (5')RNA-DNA(3') junction. The D9* fragment produced from D8-R1-D9* upon cleavage with *E. coli* RNase H1 did not migrate in the urea gel equally with the synthetic D9* fragment having the 5'-OH terminus but migrated equally with the synthetic D9* fragment phosphorylated at the 5'-terminus. The synthetic D9* fragment phosphorylated at the 5'-terminus migrated in the urea gel faster and slower than the synthetic D9* and D8* fragments with the 5'-OH terminus respectively (data not shown). This indicates that the 3'-JRNase activity of *E. coli* RNase H1 hydrolyzes the phosphodiester bond (PO-3') of the (5')RNA-DNA(3') junction producing a 3'-OH and a 5'-phosphate ended products. *E. coli* RNase H1 does not cleave the *D18/D18 substrate at all either in the presence of 0.1 mM MnCl₂ or 10 mM MgCl₂, indicating that the presence of the ribonucleotide is required for the cleavage of the D8-R1-D9/D18 substrate by the 3'-JRNase activity of *E. coli* RNase H1. Determination of the 3'-JRNase activity of *E. coli* RNase

---

**Figure 4.** Cleavage of R9-D9/D18, R1-D9/D18, and R2-D9/D18 substrates. (a, c, and e) The R9-D9*/D18 substrate (a), R1-D9*/D18 and R8:R1-D9*/D18 substrates (c), and R2-D9*/D18 and R7:R2-D9*/D18 substrates (e) were hydrolyzed by the enzyme at 30°C for 15 min and the products were separated on a 20% polyacrylamide gel containing 7 M urea as described in Experimental Procedures. The concentration of the substrate was 1.0 μM. The enzymes used to cleave these substrates are shown above the gel. The concentrations of these enzymes in the reaction mixture (10 μL) are indicated above each lane. The metal cofactors used to cleave these substrates are also shown above the gel together with their concentrations. C, substrate before enzymatic reaction; M, marker. (b, d, and f) The cleavage sites are schematically shown. “R” represents the ribonucleotide, which is inversely numbered from −1 from the RNA-DNA junction to the 5’ end. The open box represents the DNA strand (either D9 or D18). The differences in the lengths of the arrows reflect relative cleavage intensities at the position indicated.
MnCl$_2$ using an oligomeric RNA/DNA substrate is lower than that of RNase H activity of E. coli. It has been reported that the RNase H activity of E. coli cells does not exhibit this activity, suggesting that E. coli RNase H1 is less specific in the presence of manganese ions than in the presence of magnesium ions. Decreased substrate specificity and cleavage-site selectivity in the presence of manganese ions have also been reported for other RNases H.

Figure 5. Cleavage of D8-R1-D9/D18 substrate. (a) The *D8-R1-D9*/D18 substrates (1.0μM) were hydrolyzed by E. coli RNase H1 (10ngμL$^{-1}$) or E. coli RNase H2 (1ngμL$^{-1}$ or 5ngμL$^{-1}$) at 30°C for 15 min in the presence of 10 mM MgCl$_2$ or 0.1 mM MnCl$_2$ for E. coli RNase H1 or in the presence of 10 mM MgCl$_2$ or 10 mM MnCl$_2$ for E. coli RNase H2 and the products were separated on a 20% polyacrylamide gel containing 7 M urea. The enzymes, metal cofactors, and substrates are shown above the gel. *R1 and R1* represent *D8-R1-D9/D18 and D8-R1-D9*/D18 respectively. C, substrate before enzymatic reaction; M, marker. (b) The cleavage sites and the products detected by gel electrophoresis are schematically shown. "R" represents the ribonucleotide. The open box represents the DNA strand. The differences in the lengths of the arrows reflect relative cleavage intensities at the position indicated.

Cleavage of R9-D9/D18 and D8-R1-D9/D18 substrates by E. coli RNase H2. The finding that E. coli RNase H1 can cleave the D8-R1-D9/D18 substrate at the (5')RNA-DNA(3') junction promotes us to examine whether a single ribonucleotide of dsDNA can be removed by the combination of the 5'-JRNase activity of E. coli RNase H2 and the 3'-JRNase activity of E. coli RNase H1. E. coli RNase H2 has been shown to exhibit 5'-JRNase activity for an Okazaki fragment-like substrate. A crude extract from E. coli cells exhibits 5'-JRNase activity for dsDNA, whereas that from RNase H2-deficient E. coli cells does not exhibit this activity, suggesting that E. coli RNase H2 also exhibits 5'-JRNase activity for dsDNA. However, it remains to be determined whether the purified protein of E. coli RNase H2 exhibits this activity for dsDNA. Therefore, the *D8-R1-D9/D18 and D8-R1-D9*/D18 substrates were cleaved by E. coli RNase H2 either in the presence of 10 mM MnCl$_2$ or 10 mM MgCl$_2$ to examine whether this enzyme exhibits 5'-JRNase activity for dsDNA. The R9-D9*/D18 substrate was also cleaved by this enzyme for comparative purpose either in the presence of 10 mM MnCl$_2$ or 10 mM MgCl$_2$.

As shown in Fig. 3a, b, E. coli RNase H2 cleaved the R9-D9*/D18 substrate almost exclusively at R(−2)-R(−1) either in the presence of manganese or magnesium ions, but more effectively in the presence of magnesium ions. Likewise, as shown in Fig. 5, E. coli RNase H2 cleaved the *D8-R1-D9/D18 and D8-R1-D9*/D18 substrates almost exclusively at the (5')DNA-RNA(3') junction to produce the D8-R1-D9/D18 duplex containing a nick at the (5')DNA-RNA(3') junction either in the presence of magnesium or magnesium ions, but more effectively in the presence of magnesium ions. Thus, E. coli RNase H2 exhibits 5'-JRNase activity for dsDNA either in the presence of manganese or magnesium ions, but more strongly in the presence of magnesium ions. This metal ion preference is opposite to that for the RNase H activity of this enzyme determined using an RNA/DNA substrate. The RNase H activity of E. coli RNase H2 determined in the presence of 5 mM MgCl$_2$ is lower than that determined in the presence of 5 mM MnCl$_2$, by 10 fold. The RNA/DNA hybrid region of the R9-D9*/D18 substrate is not cleaved by E. coli RNase H2, probably because the RNase H activity of this enzyme is very low. It has been reported that the RNase H activity of E. coli RNase H2 determined in the presence of 5 mM MnCl$_2$ using an oligomeric RNA/DNA substrate is lower than that of E. coli RNase H1 determined in the presence of 5 mM MgCl$_2$ and 5 mM MnCl$_2$ by 2×10$^4$ and 20 fold respectively. Determination of the 5'-JRNase activity of E. coli RNase H2 in the presence of various concentrations (0.1–100 mM) of manganese or magnesium ions using the D8-R1-D9*/D18 substrate indicates that the optimum concentrations of manganese and magnesium ions for this activity are both 10 mM and the activity determined in the presence of 10 mM MgCl$_2$ is higher than that determined in the presence of 10 mM MnCl$_2$ by 15 fold (data not shown).

Stepwise cleavage of D8-R1-D9/D18 substrate by E. coli RNases H1 and H2. To examine whether a single ribonucleotide embedded in dsDNA can be removed by the combination of the 5'-JRNase activity of E. coli RNase H2 and 3'-JRNase activity of E. coli RNase H1, the D8-R1-D9/D18 D8-R1-D9*/D18 substrate indicated that E. coli RNase H1 exhibits 3'-JRNase activity most efficiently in the presence of manganese ions and the optimum concentration of magnesium ions for this activity is 0.1 mM (data not shown). This result suggests that the substrate specificity of E. coli RNase H1 is less specific in the presence of manganese ions than in the presence of magnesium ions. Decreased substrate specificity and cleavage-site selectivity in the presence of manganese ions have also been reported for other RNases.
MgCl₂ at first. The resultant product, *D₈-R₁-D₉*/D₁₈ duplex, was further hydrolyzed by the enzyme in a stepwise manner. The results are shown in Fig. 6. When the *D₈-R₁-D₉/D₁₈ substrate was cleaved by *E. coli* RNase H₁ in the presence of both 0.1 mM MnCl₂ and 1 mM MgCl₂, the products were separated on a 20% polyacrylamide gel containing 7 M urea. *R₁* represents *D₈-R₁-D₉*/D₁₈. C, substrate before enzymatic reaction. (a) The *D₈-R₁-D₉*/D₁₈ substrate was cleaved by *E. coli* RNases H₁ and H₂ in the presence of both 0.1 mM MnCl₂ and 1 mM MgCl₂. The products were separated on a 20% polyacrylamide gel containing 7 M urea. *R₁* represents *D₈-R₁-D₉*/D₁₈. C, substrate before enzymatic reaction. (b) The *D₈-R₁-D₉*/D₁₈ substrate was cleaved by the mixture of *E. coli* RNases H₁ and H₂ in the presence of both 0.1 mM MnCl₂ and 1 mM MgCl₂. The products were separated on a 20% polyacrylamide gel containing 7 M urea. *R₁* represents *D₈-R₁-D₉*/D₁₈. C, substrate before enzymatic reaction. (c) The cleavage sites are schematically shown. *R* represents the ribonucleotide. The open box represents the DNA strand. The open and solid arrows represent the first and second cleavage sites respectively.

Figure 6. Stepwise cleavage of D₈-R₁-D₉/D₁₈ substrate with *E. coli* RNases H₁ and H₂. (a) The *D₈-R₁-D₉*/D₁₈ substrate was cleaved by *E. coli* RNase H₁ (20 ng μL⁻¹) at 30 °C for 30 min in the presence of 0.1 mM MnCl₂ at first. The resultant product, *D₈-R₁-D₉*/D₁₈ duplex, was further hydrolyzed by *E. coli* RNase H₂ (10 ng μL⁻¹) at 30 °C for the period indicated above each lane in the presence of 0.1 mM MnCl₂ and 1 mM MgCl₂, and the products were separated on a 20% polyacrylamide gel containing 7 M urea. *R₁* represents *D₈-R₁-D₉*/D₁₈. C, substrate before enzymatic reaction. (b) The *D₈-R₁-D₉*/D₁₈ substrate was cleaved by *E. coli* RNase H₁ (20 ng μL⁻¹) at 30 °C for 30 min in the presence of 1 mM MgCl₂ at first. The resultant product, D₈-R₁-D₉*/D₁₈ duplex, was further hydrolyzed by *E. coli* RNase H₂ (10 ng μL⁻¹) at 30 °C for the period indicated above each lane in the presence of 0.1 mM MnCl₂ and 1 mM MgCl₂, and the products were separated on a 20% polyacrylamide gel containing 7 M urea. *R₁* represents D₈-R₁-D₉*/D₁₈. C, substrate before enzymatic reaction. (c) The cleavage sites are schematically shown. *R* represents the ribonucleotide. The open box represents the DNA strand. The open and solid arrows represent the first and second cleavage sites respectively.

DNA (deoxyribonucleic acid) is a universal hereditary material that encodes genetic information essential for the existence of all cellular life and some viruses. It is characterized by its ability to store and transfer information and to self-replicate, which is catalyzed by enzymatic machinery. To keep the integrity of the transferred information, DNA should be kept unmodified. However, DNA is frequently subject to various modifications that can render it unstable if left unmodified. Of these modifications, the presence of single ribonucleotide monophosphates (rNMPs) misincorporated into the DNA backbone shows both negative and positive consequences.
H1 did not exhibit 3’-JRNase activity for dsDNA R1 either in the presence of magnesium or manganese RNase H2 and completed by the 3’-JRNase activity of RNase H1, or vice versa. However, human RNase removal of the single ribonucleotides misincorporated into DNA is initiated by the 5’-JRNase activity of in which both enzymes are included, are schematically shown in Fig. 8. According to these pathways, that not only RNase H2 but also RNase H1 is involved in the RER pathway. Two possible RER pathways, which removes those intruders and restores the DNA back to its original form with the assistance of several other enzymes. Recent studies indicate that RNase H2 saves the genome by initiating the pathway which removes those intruders and restores the DNA back to its original form with the assistance of several other enzymes.

In this study, we showed for the first time that E. coli RNase H1 exhibits 3’-JRNase activity for dsDNA much more effectively in the presence of manganese ions than in the presence of magnesium ions, regardless of whether this substrate is cleaved by 5’-JRNase activity of E. coli RNase H2 in advance or not, and can excise the single ribonucleotide in collaboration with E. coli RNase H2. These results suggest that not only RNase H2 but also RNase H1 is involved in the RER pathway. Two possible RER pathways, in which both enzymes are included, are schematically shown in Fig. 8. According to these pathways, removal of the single ribonucleotides misincorporated into DNA is initiated by the 5’-JRNase activity of RNase H2 and completed by the 3’-JRNase activity of RNase H1, or vice versa. However, human RNase H1 did not exhibit 3’-JRNase activity for dsDNA either in the presence of magnesium or manganese ions (E. Tannous, unpublished result), suggesting that RNase H1 is involved in the RER pathway only in the prokaryotic cells. This result and the result that Hal0-RNase H1 exhibits 3’-JRNase activity for dsDNA in the presence of Mn ions (E. Tannous, unpublished result) may exclude the possibility that the 3’-JRNase activity of E. coli RNase H1 is derived from another E. coli enzyme contaminated, which is not detected as a band on SDS-PAGE (Fig. 4), because human RNase H1 is a basic protein similar to E. coli RNase H1 and is purified using cation-exchange column chromatography (E. Tannous, unpublished result), whereas Halo-RNase H1 is an acidic protein and is purified by anion-exchange chromatography. Because E. coli RNase H1 is purified using cation-exchange column chromatography, it is unlikely that another E. coli enzyme with 3’-JRNase activity is co-purified with E. coli RNase H1 and Halo-RNase H1 but not co-purified with human RNase H1. Disruption of the rnhA gene has been reported to increase a basal level of SOS expression in E. coli, probably due to persistence of R-loops on the chromosome. However, the 3’-JRNase activity of E. coli RNase H1 may not be involved in SOS response, because this activity may not be required for R-loop resolution.

The concentration of magnesium ions (10 mM) optimum for in vitro 5’-JRNase activity of E. coli RNase H2 is comparable to that in E. coli cells, whereas the concentration of manganese ions (0.1 mM) optimum for in vitro 3’-JRNase activity of E. coli RNase H1 is higher than that in E. coli cells. However, the fact that the presence of magnesium ions is not inhibitory for the 3’-JRNase activity of E. coli RNase H1 may suggest that E. coli RNase H1 exhibits this activity inside the cells. The in vivo studies showing that E. coli RNase H1 helps in sanitizing the genome from errant rNMPs misincorporated in dsDNA when the rnhB gene encoding E. coli RNase H2 is inactivated and thus limiting the consequences of the excessive accumulation of ribonucleotides in the E. coli genome supports this hypothesis. Thus, the significance of this work is that it highlights a new possible role for bacterial type I RNases H in DNA repair, which should be further investigated in vivo, as well as for RNases H from different organisms.

**Methods**

**Protein preparation.** Overproduction and purification of E. coli RNase H1 using E. coli MIC3009 transformants with pJAL600 and E. coli RNase H2 using E. coli MIC2067(DE3) transformants with pTYB600E were performed as described previously. The purity of each protein was analyzed by SDS-PAGE using 15% (w/v) polyacrylamide gel, followed by staining with silver staining, using Silver.
Stain II Kit Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The protein concentration was determined from UV absorption using a cell with an optical path length of 1 cm and an $A_{280}$ value for $E. coli$ RNase H1 and 0.56 for $E. coli$ RNase H2. The value of $E. coli$ RNase H1 was experimentally determined. The value of $E. coli$ RNase H2 was calculated by using absorption coefficients of 1576 M$^{-1}$ cm$^{-1}$ for Tyr and 5225 M$^{-1}$ cm$^{-1}$ for Trp at 280 nm.

Oligonucleotides. 18 b RNA$_5$-DNA$_9$ (R9-D9*), 11 b RNA$_7$-DNA$_9$ (R2-D9*), and 10 b RNA$_8$-DNA$_9$ (R1-D9*), 5'- and 3'-FAM-labeled 18 b DNA$_8$-RNA$_1$-DNA$_9$ (*D8-R1-D9 and D8-R1-D9* respectively), 5'-FAM-labeled 18 b DNA (*D18), 7 b RNA (R7), 8 b RNA (R8), and 18 b DNA (D18) were synthesized by Hokkaido System Science (Sapporo, Japan). Of these oligonucleotides, R2-D9* and R1-D9* are 5’-phosphorylated, while the others are not. The sequences of these oligonucleotides are 5’-uugcaugccTGCAGGTCG-3’ for R9-D9*, 5’-ccTGCAGGTCG-3’ for R2-D9*, 5’-TGCAAGTGCAGGTCG-3’ for R1-D9*, 5’-uugaugc-3’ for R7, 5’-uugcaugc-3’ for R8, and 5’-TGCAATGGcTGCAGGTCG-3’ for *D8-R1-D9 and D8-R1-D9*, where deoxyribonucleotides and ribonucleotides are shown by uppercase and lowercase letters respectively. The sequence of *D18 is identical to that of *D8-R1-D9. The sequence of D18 is complementary to those of R9-D9* and *D8-R1-D9. FAM represents 6-carboxyfluorescein.

Enzymatic activity. The R9-D9*/D18, R2-D9*/D18, R1-D9*/D18, *D8-R1-D9/D18, *D8-R1-D9/D18, *D8-R1-D9*/D18, and *D18 duplexes were prepared by hybridizing R9-D9*, R2-D9*, R1-D9*, *D8-R1-D9, D8-R1-D9*, and *D18 with a 2 molar equivalent of D18 respectively. The R7:R2-D9*/D18 and R8:R1-D9*/D18 duplexes were prepared by hybridizing R2-D9* and R1-D9* with a 2 molar equivalent of D18 in the presence of a 2 molar equivalent of R7 and R8 respectively. These duplexes were used as substrates. Hydrolysis of the substrate at 30°C for 15 min and separation of the products on a 20% polyacrylamide gel containing 7 M urea were carried out as described previously. The reaction buffer contained 10 mM Tris-HCl (pH 8.5), 1 mM 2-mercaptoethanol, 0.01% BSA, 10 mM NaCl, and MnCl$_2$ or MgCl$_2$ at the

Figure 8. A new RER pathway directed by RNases H1 and H2. dsDNA containing a single ribonucleotide (dsDNA$^{R1}$) is schematically shown. DNA strand and a single ribonucleotide are shown by blue and red boxes respectively. Single ribonucleotide and flanking deoxyribonucleotides are labeled R and D respectively. Two possible pathways, in which a single ribonucleotide embedded in dsDNA is removed by RNases H1 and H2 in a stepwise manner, are shown. In pathway A, dsDNA$^{R1}$ is first cleaved by RNase H2 at the 5’ side of the ribonucleotide to produce dsDNA$^{R1}$ with a nick at the 5’ side of the ribonucleotide. Then dsDNA$^{R1}$ with this nick is subsequently cleaved by RNase H1 at the 3’ side of ribonucleotide of the (5’)RNA-DNA(3’) junction to release a single ribonucleotide. In pathway B, dsDNA$^{R1}$ is first cleaved by RNase H1 at the 3’ side of the ribonucleotide to produce dsDNA$^{R1}$ with a nick at the 3’ side of the ribonucleotide. Then dsDNA$^{R1}$ with this nick is subsequently cleaved by RNase H2 at the 5’ side of the ribonucleotide to release a single ribonucleotide. The gaps in dsDNAs produced by removal of a single ribonucleotide from dsDNA$^{R1}$ in pathway A or B are probably filled by DNA polymerase and ligase.
concentration indicated. The substrate concentration was 1 μM. The products were detected by Typhoon 9240 Imager (GE Healthcare, Tokyo, Japan).

References

1. Crouch, R. J. & Dirksen, M. L. Ribonuclease H. In: Nuclease (S. M. Linn & R. J. Roberts, eds), pp. 211–241. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1982)

2. Yang, W., Lee, J. Y. & Nowotny, M. Making and breaking nucleic acids: two-Mg2+ ion catalysis and substrate specificity. Mol. Cell. 22, 5–13 (2006).

3. Ohtani, N., Haruki, M., Morikawa, M. & Kanaya, S. Molecular diversities of RNases H1. J. Biosci. Bioeng. 88, 12–19 (1999).

4. Tadokoro, T. & Kanaya, S. Ribonuclease H2: molecular diversities, substrate binding domains, and catalytic mechanism of the prokaryotic enzymes. FEBS J. 276, 1482–1493 (2009).

5. Figiel, M. et al. The structural and biochemical characterization of human RNase H2 complex reveals the molecular basis for substrate recognition and Aicardi-Goutières syndrome defects. J. Biol. Chem. 286, 10540–10550 (2011).

6. Rubek, D. et al. PCNA directs type 2 RNase H activity on DNA replication and repair substrates. Nucleic Acids Res. 39, 3652–3666 (2011).

7. Iyama, M. et al. Isolation of RNase H genes that are essential for growth of Bacillus subtilis 168. J. Bacteriol. 181, 2118–2123 (1999).

8. Kogoma, T. & Foster, P. L. Physiological functions of E. coli RNase H1. In: Ribonuclease H2 (R. J. Crouch & J. J. Toulme, eds), pp. 39–66. (INSERM, Paris, 1998).

9. Aruduchandran, A. et al. The absence of ribonuclease H1 or H2 alters the sensitivity of Saccharomyces cerevisiae to hydroxyurea, caffeine and ethyl methanesulfonate: implications for roles of RNases H in DNA replication and repair. Genes Cells 5, 789–802 (2000).

10. Cerritelli, S. M. et al. Failure to produce mitochondrial DNA results in embryonic lethality in Rnaseh1 null mice. Mol. Cell 11, 807–815 (2003).

11. Reijns, M. A. et al. Enzymatic removal of ribonucleotides from DNA is essential for mammalian genome integrity and development. Cell 149, 1008–1022 (2012).

12. Hiller, B. et al. Mammalian RNase H2 removes ribonucleotides from DNA to maintain genome integrity. J. Exp. Med. 209, 1419–1426 (2012).

13. Crow, Y. J. et al. Mutations in genes encoding ribonuclease H2 subunits cause Aicardi-Goutières syndrome and mimic congenital viral brain infection. Nat. Genet. 38, 910–916 (2006).

14. Repaske, R., Hartley, J. W., Kavlick, M. F., O'Neill, R. R. & Austin, J. B. Inhibition of RNase H activity and viral replication by single mutations in the 3' region of Moloney murine leukemia virus reverse transcriptase. J. Virol. 63, 1460–1464 (1989).

15. Tsidale, M., Schulzé, T., Larder, B. A. & Modlin, K. Mutations within the RNase H domain of human immunodeficiency virus type 1 reverse transcriptase abolish virus infectivity. J. Gen. Virol. 72 (Pt 1), 59–66 (1991).

16. Tramontano, E. & Di Santo, R. HIV-1 RT-associated RNase H function inhibitors: Recent advances in drug development. Curr. Med. Chem. 17, 2837–2853 (2010).

17. Esposito, F., Corona, A. & Tramontano, E. HIV-1 Reverse transcriptase still remains a new drug target: Structure, function, classical inhibitors with innovative mechanisms of actions. Mol. Biol. Int. 2012, 586401 (2012).

18. Sparks, J. L. et al. RNase H2-initiated ribonucleotide excision repair. Mol. Cell 47, 980–986 (2012).

19. Lazzaro, F. et al. RNase H and postreplication repair protect cells from ribonucleotides incorporated in DNA. Mol. Cell 45, 99–110 (2012).

20. McDonald, J. P., Vaisman, A., Kuban, W., Goodman, M. F. & Woodgate, R. Mechanisms employed by Escherichia coli to prevent ribonuclease incorporation into genomic DNA by Pol V. PLoS Genet. 8, e1003036; DOI:10.1371/journal.pgen.1003036 (2012).

21. Haruki, M., Tsunaka, Y., Morikawa, M. & Kanaya, S. Cleavage of a DNA-RNA-DNA/DNA chimeric substrate containing a single ribonucleotide at the DNA-RNA junction with prokaryotic RNases H1. FEBS Lett. 531, 204–208 (2002).

22. Rydberg, B. & Game, J. Excision of misincorporated ribonucleotides in DNA by RNase H and FEN-1 in cell-free extracts. Proc. Natl. Acad. Sci. U.S.A. 99, 16654–16659 (2002).

23. Rychlik, M. P. et al. Crystal structures of RNase H2 in complex with nucleic acid reveal the mechanism of RNA-DNA junction recognition and cleavage. Mol. Cell. 40, 638–670 (2010).

24. Permanasari, E. D., Angkawidjaja, C., Koga, Y. & Kanaya, S. Role of N-terminal extension of Thermotoga maritima RNase H2 and C-terminal extension of Thermotoga maritima RNase H2. FEBS J. 280, 5065–5079 (2013).

25. Tadokoro, T., Chon, H., Koga, Y., Takano, K. & Kanaya, S. Identification of the gene encoding a type 1 RNase H with an N-terminal double-stranded RNA binding domain from a psychrotrophic bacterium. FEBS J. 274, 3715–3727 (2007).

26. Jongruja, N. et al. The N-terminal hybrid binding domain of RNase H1 from Thermotoga maritima is important for substrate binding and Mg2+–dependent activity. FEBS J. 277, 4474–4489 (2010).

27. Nguyen, T. N. et al. Activity, stability, and structure of metagenome-derived LC1-L-Rnase H1, a homolog of Sulfolobus tokodaii RNase H1. Protein Sci. 21, 553–561 (2012).

28. Nguyen, T. N., You, D. J., Kanaya, E., Koga, Y. & Kanaya, S. Crystal structure of metagenome-derived LCR-RNase H1 with atypical DEDD active site motif. FEBS Lett. 587, 1418–1423 (2013).

29. Ohtani, N., Yanagawa, H., Tomita, M. & Iyama, M. Identification of the first archaeal Type 1 RNase H gene from Halobacterium sp. NRC1-1: archaeal RNase H1 can cleave an RNA-DNA junction. Biochem. J. 381, 795–802 (2004).

30. Ohtani, N., Yanagawa, H., Tomita, M. & Iyama, M. Cleavage of double-stranded RNA by RNase H1 from a thermoclophilic archaeon, Sulfolobus tokodaii 7. Nucleic Acids Res. 32, 5809–5819 (2004).

31. Ohtani, N., Tomita, M. & Iyama, M. Junction ribonuclease activity specified in RNases HII/2. FEBS J. 275, 5444–5455 (2008).

32. Kanaya, S. Enzymatic activity and protein stability of E. coli ribonuclease H1. In: Ribonucleases H (R. J. Crouch & J. J. Toulme, eds), pp. 1–38. (INSERM, Paris, 1998).

33. Neck, J. L. & Marcquere, S. The putative substrate recognition loop of Escherichia coli ribonuclease H is not essential for activity. J. Biol. Chem. 271, 19883–19887 (1996).

34. Nowotny, M. et al. Structure of human RNase H1 complexed with an RNA/DNA hybrid: insight into HIV reverse transcription. Mol. Cell. 28, 264–276 (2007).

35. Ohtani, N., Haruki, M., Muroya, A., Morikawa, M. & Kanaya, S. Characterization of ribonuclease H1 from Escherichia coli overproduced in a soluble form. J. Biochem. 127, 895–899 (2000).

36. Potenski, G. J. & Klein, H. L. How the misincorporation of ribonucleotides into genomic DNA can be both harmful and helpful to cells. Nucleic Acids Res. 42, 10226–10234 (2014).

37. Watt, D. L., Johansson, E., Burgers, P. M. & Kunkel, T. A. Replication of ribonucleotide-containing DNA templates by yeast replicative polymerases. DNA Repair (Amst) 10, 897–902 (2011).

38. Ghodgaonkar, M. M. et al. Ribonucleotides misincorporated into DNA act as strand-discrimination signals in eukaryotic mismatch repair. Mol. Cell. 50, 323–332 (2013).
39. Lujan, S. A., Williams, J. S., Clausen, A. R., Clark, A. B. & Kunkel, T. A. Ribonucleotides are signals for mismatch repair of leading-strand replication errors. Mol. Cell. 50, 437–443 (2013).
40. Brown, J. A. et al. A novel mechanism of sugar selection utilized by a human X-family DNA polymerase. J. Mol. Biol. 395, 282–290 (2010).
41. Ruiz, J. E. et al. Lack of sugar discrimination by human Pol mu requires a single glycine residue. Nucleic Acids Res. 31, 4441–4449 (2003).
42. Nick McElhinny, S. A. & Ramsden, D. A. Polymerase mu is a DNA-directed DNA/RNA polymerase. Mol. Cell. Biol. 23, 2309–2315 (2003).
43. Nick McElhinny, S. A. et al. Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. Proc. Natl. Acad. Sci. U.S.A. 107, 4949–4954 (2010).
44. Nick McElhinny, S. A. et al. Genome instability due to ribonucleotide incorporation into DNA. Nat. Chem. Biol. 6, 774–781 (2010).
45. Kim, N. et al. Mutagenic processing of ribonucleotides in DNA by yeast topoisomerase I. Science 332, 1561–1564 (2011).
46. Eder, P. S., Walder, R. Y. & Walder, J. A. Substrate specificity of human RNase H and its role in excision repair of ribose residues misincorporated in DNA. Biochimie 75, 123–126 (1993).
47. Rice, G. I. et al. Mutations involved in Aicardi-Goutières syndrome implicate SAMHD1 as regulator of the innate immune response. Nat. Genet. 41, 829–832 (2009).
48. Tannous, E., Yokoyama, K., You, D. J., Koga, Y. & Kanaya, S. A dual role of divalent metal ions in catalysis and folding of RNase H1 from extreme halophilic archaeon Halobacterium sp. NRC-1. FEBS Open Bio. 2, 345–352 (2012).
49. Kanaya, S., Oobatake, M., Nakamura, H. & Ikehara, M. pH-dependent thermostabilization of Escherichia coli ribonuclease H1 by histidine to alanine substitutions. J. Biotechnol. 28, 117–136 (1993).
50. McCool, J. D. et al. Measurement of SOS expression in individual Escherichia coli K-12 cells using fluorescence microscopy. Mol Microbiol. 53, 1343–1357 (2004).
51. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685 (1970).
52. Kanaya, S., Kimura, S., Katsuda, C. & Ikehara, M. Role of cysteine residues in ribonuclease H from Escherichia coli. Site-directed mutagenesis and chemical modification. Biochem. J. 271, 59–66 (1990).
53. Goodwin, T. W. & Morton, R. A. The spectrophotometric determination of tyrosine and tryptophan in proteins. Biochem. J. 40, 628–632 (1946).
54. Ohtani, N. et al. Identification of the genes encoding Mn2+-dependent RNase HII and Mg2+-dependent RNase HIII from Bacillus subtilis: classification of RNases H into three families. Biochemistry 38, 605–618 (1999).

Acknowledgements
We thank Dr. Y. Koga for helpful discussion. This work was supported by a Grant (24380055) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Author Contributions
Conception and design of the work and final approval of the version to be published: S.K. Acquisition and analysis of data and preparation of the paper: E.T. Interpretation and discussion of data: E.T., E.K. and S.K.

Additional Information
Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Tannous, E. et al. Role of RNase H1 in DNA repair: removal of single ribonucleotide misincorporated into DNA in collaboration with RNase H2. Sci. Rep. 5, 9969; doi: 10.1038/srep09969 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/