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# Essential role of Chk1 in S phase progression through regulation of RNR2 expression

Hiromichi Naruyama [a,b], Midori Shimada [a], Hiroyuki Niida [a], Doaa H. Zineldeen [a], Yoshihiro Hashimoto [b], Kenjiro Kohri [b], Makoto Nakanishi [a,*]

[a] Department of Cell Biology, Graduate School of Medical Sciences, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan
[b] Department of Urology, Graduate School of Medical Sciences, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

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ABSTRACT

Chk1 is an essential kinase for maintaining genome integrity and cell cycle checkpoints through phosphorylating several downstream targets. Recently, we demonstrated that Chk1 is also required for cell proliferation in somatic cells under unperturbed condition through regulating transcription of several genes. Here, we show that Chk1 is required for S phase progression and RNR2 is a critical downstream target of genes transcriptionally regulated by Chk1. Hence, although RNR2 expression reached maximum at S phase in the presence of Chk1, Chk1 depletion arrested the cell cycle at S phase and reduced RNR2 expression at both mRNA and protein levels. Ectopic expression of RNR2 failed to rescue the S phase arrest observed in Chk1 depleted cells, suggesting the presence of an additional Chk1-target(s) for completion of S phase other than RNR2. Therefore, our results suggest that Chk1 is required for DNA replication at least through regulating RNR2 gene transcription.



Progression through the cell cycle is regulated carefully to avoid proliferation or mitosis when adverse conditions exist, such as DNA damage and DNA replication fork stalling [1–3]. Abnormal DNA structures are rapidly sensed and transduced to mediators by either the ATR or ATM PI3-kinase related protein kinases (PIKK) [4,5]. One such mediator is Chk1 kinase that is essential for cell cycle arrest upon DNA damage or DNA replication fork stalling through phosphorylating Cdc25 phosphatases [6,7]. In addition to its role as a checkpoint mediator, Chk1 is a constitutively active enzyme and associates with chromatin under unperturbed condition [8,9]. Chk1 phosphorylates histone H3 at threonine 11 (T11) around the promoter regions of cell cycle regulatory genes including cyclin B1 and Cdk1 [10], which accelerate recruitment of GCN5 histone acetyltransferase and subsequent acetylation at lysine 9 (K9) [11]. Increased acetylation of K9 leads to transcriptional activation.

Ribonucleotide reductase (RNR) is essential for de novo synthesis of deoxyribonucleotides (dNTPs), which are required for DNA replication and repair [12,13]. Most eukaryotic RNRs are composed of two essential and non-identical homodimeric subunits, a large subunit (R1) and a small subunit (R2) [12,14]. The former subunit contains the catalytic site and allosteric regulatory site for both enzyme activity and specificity by binding nucleotide triphosphates [12]. The latter subunit contains a non-heme iron center essential for catalysis. p53-Inducible R2 (53R2) [15], a homologous R2 protein, is also capable of forming an active RNR complex together with the R1 protein [16].

In mammalian cells, the transcription of the R1 and R2 genes is cell cycle dependent, being undetectable in G0/G1 and maximum in S phase [17,18]. The S phase-specific expression of R1 genes is characterized as four different promoter elements, b, a, Inr, and g [19]. Although transcription factor YY1 binds to b and a elements, the cell cycle specific expression is mainly regulated via Inr and g elements. In contrast to R1, the S phase specific expression of R2 is relatively complicated. In mouse cells, S phase-specific transcription of R2 gene requires a repressive E2F-binding site and a promoter-activating region [20]. Interestingly, mutation of the E2F-binding site leads to premature promoter activation in G1 and increase promoter activity. Given that the R1 protein has a long half-life, and its level is apparently constant [21,22] and that the R2 protein is rapidly degraded in mitosis by APC-C [23,24], RNR activity and thus DNA replication are mainly dependent on the level of R2 protein.

In this study, we found that Chk1 depletion results in incomplete S phase, suggesting that Chk1 may be required for transcription of some essential genes for DNA replication. To address this question, we examined changes in the transcription of known genes involved in S phase progression after Chk1 depletion in MEFs. Only

* Corresponding author. Address: Department of Cell Biology, Graduate School of Medical Sciences, Nagoya City University, Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. Fax: +81 52 842 3955.
*E-mail address:* mkt-naka@med.nagoya-cu.ac.jp (M. Nakanishi).

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RNR2 among genes tested was drastically reduced following loss of Chk1. Therefore, our results suggest an essential function of Chk1 in DNA replication at least through activating RNR2 gene transcription.

## Materials and methods

*Cells and culture condition*. Chk1$^{flox/-}$ MEFs were generated as described previously [10] and were cultured in DMEM supplemented with 10% FBS.

*Immunoblotting*. Cells were lysed as described previously [10], and extracts were subjected to immunoblotting using anti-Chk1 (sc8408; Santa Cruz), anti-RNR2 (sc10844; Santa Cruz), anti-RNR1 (sc11733; Santa Cruz) antibodies.

*Northern blotting*. Total RNA was extracted using ISOGEN (Wako) and northern blotting was performed as described previously [25]. $^{32}$P-labeled fragment of RNR2 and RNR1 was used as a probe.

*Cell cycle analysis*. Cells were incubated with 10 μM BrdU for 30 min, harvested, washed once in PBS and fixed with in 70% ethanol. Cells were prepared for FACS analysis as described previously [26].

*Real time PCR*. Real-time PCR was carried out with single-stranded cDNAs prepared with the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Briefly, total RNA from each sample after transfection was reverse-transcribed with Oligo-DT primer. PCR reactions were performed with Power SYBR Green PCR Master Mix and 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Seuence-Specific primers were designed by Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) as follows; GAPDH, AAC TTTGGCATTGTGGAAGG and GGATGCAGGGATGATGTTCT, Asf1, CAG GCCATTTTACCTTCAGC and GGCTGAGCTTGTTTCTGGAC, MCM4, GACCCTCAGGATGAGGCATA and GGGGCATGATGGTACTATGG, Cdc25C, AAAGACAGGGCTCTGAACCA and TGGTGAAGCATGGGAC AGTA, RNR1, GGTCGTGTCCGAAAAGTTGT and GTTCTGCTGGTTG CTCTTCC RNR2, CGTTGTCTTTCCCATCGAGT and CTCTCATCGGG TTTCAGAGC, Cdc7, GCCCTGCAGAGAAACTCATC and GTTTCCCTC ATCACGCTGTT, Cdc6, TTTCGGAAGTTGATGGGAAC and GGGTCAA AAGCAGCAAAGAG Orc1, ACTGCCATACCCAACCATGT and CAGCA CGTCATTCTGGCTAA, Orc2, TTTGTGCCTTCCTTTTCTGC and CCCA AG CCATAAAGCACAAT, Wee1, GAGAGCTGGAGGACGACTTG and CAGAA AGTAGGCGGTCGAAG, Cdc45, GTTCCTGCCTACGACGACAT and CTC TTCCTGTTTCGCTCCAC. GAPDH primer was used as an internal control. Real-Time PCR was carried out, in duplicate, by 40 cycles of 95 °C for 10 sec and 60 °C for 1 min. Productions of the expected amplification fragments without unanticipated products and primers were confirmed by melting-curve analysis. To determine the relative amounts of the products, we used the comparative Ct (threshold cycle) method according to the instructions supplied by Applied Biosystems. Conventional PCR was performed with the Ex-Taq system (Takara Bio Inc., Shiga, Japan).

*Immunohistochemical analysis*. Chk1$^{flox/-}$ MEFs were infected with adenoviruses expressing either Cre or LacZ (negative control). Immunohistochemical analyses using anti-RNR1 and anti-RNR2 antibodies were performed as described previously [9].

## Results and discussion

### *Loss of Chk1 resulted in incomplete DNA replication*

Recent findings that Chk1 is a histone H3-T11 kinase unravel a mechanism underlying DNA damage-induced transcriptional repression. In this concept, Chk1 is an essential for transcription of some genes under unperturbed condition. Actually, Chk1 depletion in

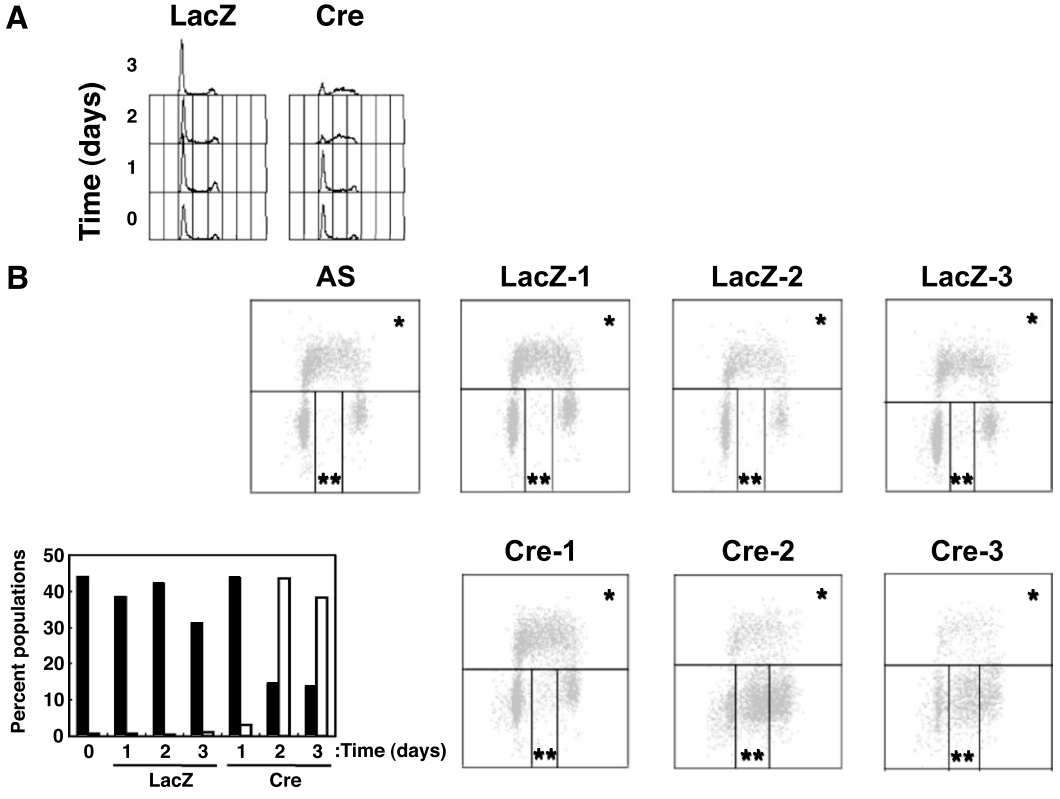


**Fig. 1.** Chk1 depleted MEFs show S phase arrest. (A) Cell cycle profile of Chk1$^{flox/-}$ and Chk1$^{\Delta/-}$ MEFs at the indicated days after adenoviral infection. (B) Chk1$^{flox/-}$ MEFs were treated with 10 μM BrdU for 30 min before cells were harvested at the time indicated after infection and analysed by FACS. The percentages of BrdU positive (*) and negative (**) cells are shown as a graph (black: BrdU positive; white: BrdU negative).

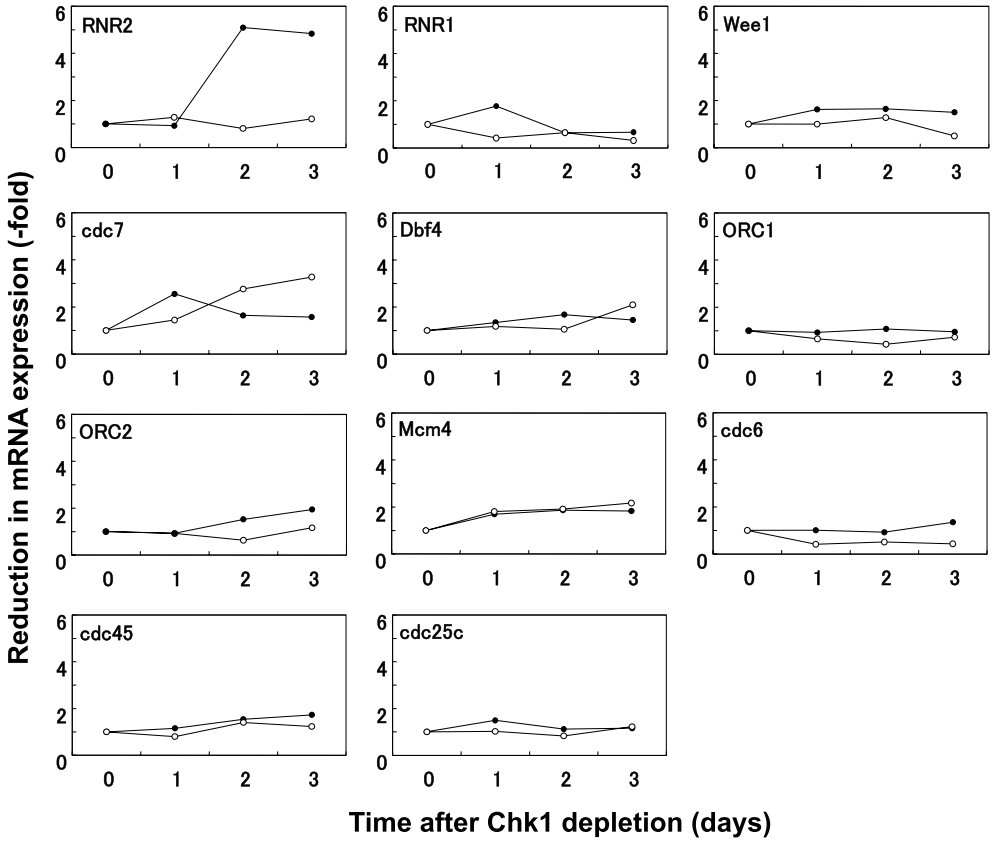


**Fig. 2.** RNR2 mRNA transcripts were decreased in Chk1 depleted MEFs. Chk1$^{flox/-}$ MEFs were harvested at the indicated times after infection of adenoviruses expressing LacZ (open circles) or Cre (closed circles) and their total RNA was extracted. After treatment with reverse transcriptase using random primers, real time PCR was performed using their specific primers to measure the transcripts involved in DNA replication or cell cycle control. The results are presented as a fold-reduction of controls (before infection of adenoviruses).

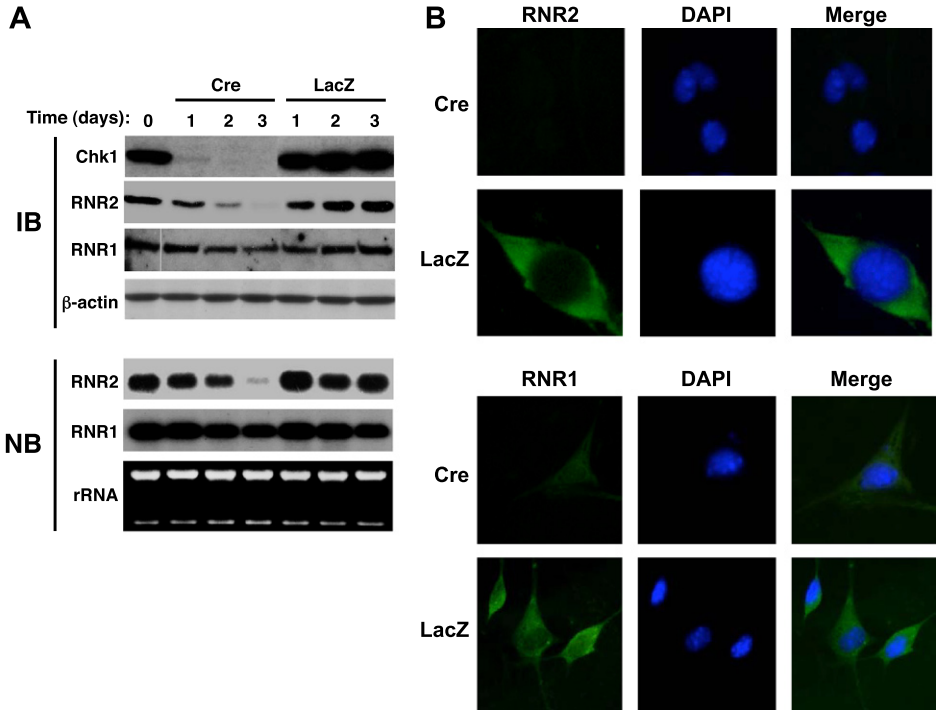


**Fig. 3.** Specific reduction in RNR2 protein in Chk1 depleted MEFs. Chk1$^{flox/-}$ MEFs were harvested at the indicated times after infection of adenoviruses expressing LacZ or Cre. The resultant whole cell extracts and total RNA were prepared and subjected to immunoblotting (IB) using the indicated antibodies or northern blotting (NB) analysis using RNR2 or RNR1 probes. (B) Immunohistochemical analysis of RNR2 and RNR1 in Chk1 depleted cells. Chk1$^{flox/-}$ MEFs were infected with adenoviruses expressing either LacZ or Cre. Three days after infection the resultant cells were fixed and stained with anti-RNR2 or anti-RNR1 antibodies. Cells were also counterstained with DAPI.

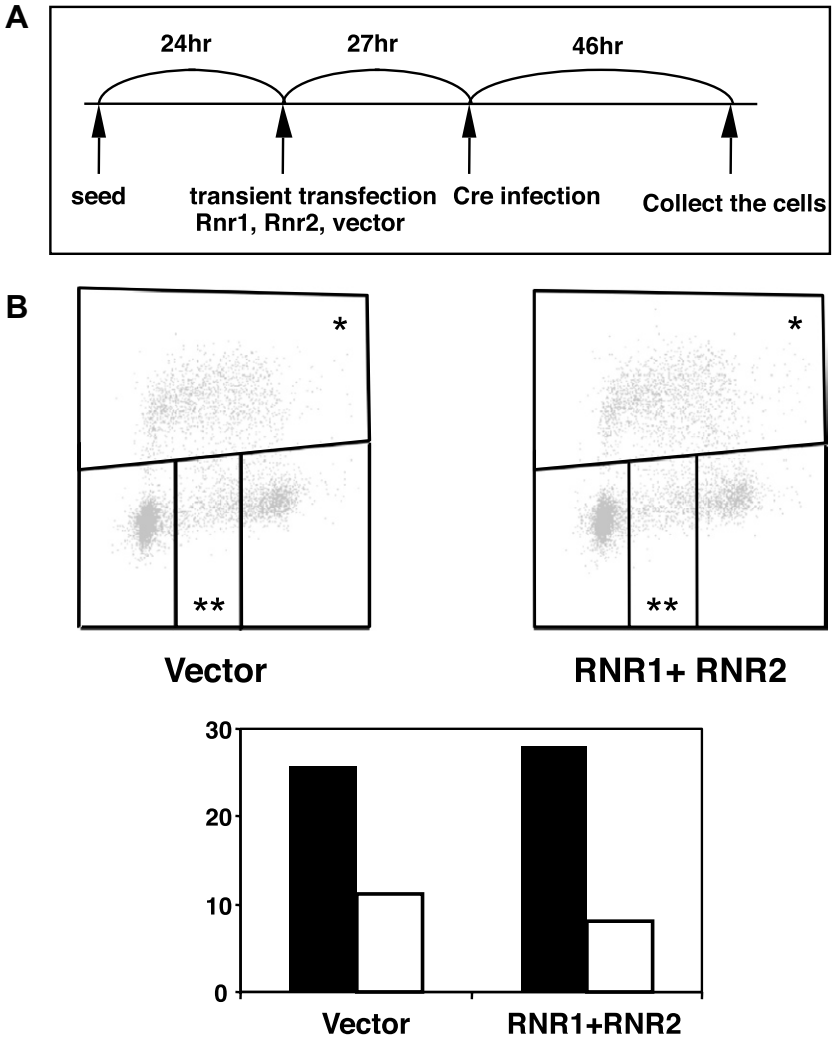


**Fig. 4.** Ectopic expression of RNR2 and RNR1 is insufficient for complement S phase arrest in Chk1 depleted MEFs. (A) Time course strategy for transient transfection of RNR2 and RNR1, and Chk1 depletion by Ade-Cre infection. (B) BrdU staining and FACS analysis were performed as indicated in Fig. 1. The percentages of each cell cycle distribution were also shown (black: BrdU positive; white: BrdU negative).

somatic cells by infection of adenoviruses expressing Cre reduced expression of more than 200 transcripts [10]. Therefore, increased S phase population in Chk1 depleted MEFs (Fig. 1A) are likely due to S phase arrest but not enhanced initiation of DNA replication. To address this question, we first examined BrdU incorporation in Chk1 depleted MEFs. After infection of adenoviruses expressing Cre, Chk1$^{del/-}$ MEFs were incubated with culture medium containing 10 μM BrdU for 30 min. Cells were then fixed, immunostained with anti-BrdU antibodies, and analyzed by FACScan. As shown in Fig. 1B, majority of S phase cells in control MEFs were BrdU positive and infection of adenoviruses expressing LacZ did not affect cell cycle progression. In contrast, although most of S phase cells in Chk1$^{del/-}$ MEFs were BrdU positive at 1 day after adenoviral infection, they became negative at two days or thereafter, indicating that increased S phase population is due to S phase arrest.

*Transcriptional changes in genes involved in DNA replication after Chk1 depletion*

We next examined the changes in the expression of genes involved in DNA replication after Chk1 depletion. Quantitative real time PCR revealed that the expression of RNR2, but not RNR1, was significantly reduced at two days after Chk1 depletion, whereas those of the other genes tested were almost constant during this experimental period (Fig. 2). Given that expression of many genes involved in RNR2, ASK, ORC1, cdc6, and cdc25c were dependent on the functional E2Fs [20,27–30], reduction in RNR2 expression was not likely due to the impairment of E2F functions. In addition, because RNR2 expression is cell cycle dependent, reaching at a maximum at S phase [20], decreased expression of RNR2 did not solely reflect the changes in cell cycle profile following Chk1 depletion (Fig. 1A).

The reduction in RNR2 protein as well as its mRNA was also confirmed by northern blotting, immunoblotting, and immunohistochemistry using specific antibodies to RNR2, respectively (Fig. 3A and B). The reduction in RNR2 protein was obvious at two days after Chk1 depletion, supporting the observation that S phase arrest in Chk1 depleted cells was detected at two days after infection. RNR2 was predominantly localized at cytoplasm and RNR1 was localized at both cytoplasm and nucleus, consistent with the previous reports [12], but signal of RNR2, but not RNR1, was almost disappeared after Chk1 depletion. Thus, S phase arrest following Cre infection appeared to be correlated well with the reduction in RNR2 protein.

*Ectopic expression of RNR2 is not sufficient for complement S phase arrest in Chk1$^{del/-}$ MEFs*

We finally asked whether ectopic expression could reverse the S phase arrest in Chk1$^{del/-}$ MEFs. Experimental protocols were

summarized in Fig. 4A. In brief, MEFs were transfected with both expression vectors for RNR1 and RNR2 and then infected with Cre. Forty six hours after infection, cells were incubated with the medium containing 10 μM BrdU and analyzed by FACScan. As shown in Fig. 4B, coexpression of RNR1 and RNR2 was not capable of reducing BrdU negative S phase population. These results suggests that ectopic expression of RNR1 and RNR2 is not sufficient for rescue the S phase arrest in Chk1 deficient MEFs and thus existence of unidentified transcriptional targets of Chk1 which is essential for completion of DNA replication. Clearly, additional work is necessary to clarify those Chk1 targets and their role in S phase progression.

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