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CLP1 acts as the main RNA kinase in mice

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ABSTRACT

CLP1 plays an essential role in the protein complex involved in mRNA 3'-end formation and polyadenylation as well as in the tRNA splicing endonuclease (TSEN) complex involved in the splicing of precursor tRNAs. NOL9 localizes in the nucleolus of cells and plays an essential role in ribosomal RNA maturation. Both CLP1 and NOL9 are RNA kinases that phosphorylate the 5' end of RNAs. From the evidence that phosphorylation of the 5' end of a siRNA is essential for its efficient RNA cleavage, it was expected that CLP1 and NOL9 would be corresponding molecules. However, there had been no direct evidence that this is the case. In this study, murine NOL9 showed no apparent RNA kinase activity in cells or even in an RNA kinase assay using recombinant murine NOL9 protein. Although siRNA efficiency was decreased in CLP1 kinase-dead (*Clp1^{K/K}*) cells, it was not influenced by NOL9 overexpression. These findings indicate that in mouse cells it is CLP1 that mainly acts to phosphorylate the 5' end of RNAs in the siRNA pathway, with no apparent involvement of NOL9.

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1. Introduction

CLP1 and NOL9 are RNA kinases that phosphorylate the 5' ends of RNAs [1–3]. CLP1 is a component of the protein complex responsible for mRNA 3'-end formation and polyadenylation [4,5] and it is also a component of the TSEN complex involved in the splicing of precursor tRNAs [6]. Furthermore, CLP1 is a siRNA kinase that functions to phosphorylate the 5' end of siRNAs, which is essential for their incorporation into the RNA-induced silencing complex (RISC), and mediates target RNA cleavage [2,7–9]. NOL9 on the other hand localizes in the nucleolus and is required for processing of the 32S precursor into 5.8S and 28S rRNAs [3]. Both CLP1 and NOL9 share Walker A and Walker B motifs, which are ATP/GTP binding motifs and which are necessary for RNA phosphorylation [1,10].

In an earlier study, we investigated the *in vivo* function of CLP1 by using CLP1 kinase-dead knock-in mice (*Clp1^{K/K}*), which harbor a lysine-to-alanine substitution at amino acid position 127 in CLP1 [11]. These *Clp1^{K/K}* mice show neuronal defects including microcephaly and progressive motor neuron loss resulting in fatal deterioration of motor function [11]. These CLP1 mutant mice accumulate RNA fragments derived from tyrosine pre-tRNAs in their neurons, which sensitizes the cells to oxidative stress leading to p53 activation. These results indicate that the kinase activity of CLP1 plays an important role in pre-tRNA biogenesis [11,12].

In contrast to CLP1, the molecular function of NOL9 remains elusive. The kinase activity of human NOL9 is required for cleavage of the ITS2 region derived from an rRNA precursor and for the generation of 5.8S and 28S rRNAs, which results in the synthesis of the 60S ribosomal subunit [3]. Grc3, a NOL9 homolog in *Schizosaccharomyces pombe*, forms a super dimer complex with Las1 [13]. Grc3-Las1 complex is required for ITS2 cleavage in the processing of the rRNA precursor [14]. Disruption of the conserved K[T/S] residues within the kinase motif domain results in severe growth defects in *Saccharomyces cerevisiae*, meaning that the RNA kinase

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activity of Grc3 is essential in *S. cerevisiae* [13]. Zebrafish with a null mutation of the *nol9* gene show hypoplasia of the pancreas, liver, and intestine, and they also show defective hematopoiesis [15]. These mutant zebrafish also show defective pre-rRNA processing within ITS2, resulting in the accumulation of ITS2 containing rRNA precursors. These results indicate that the importance of NOL9 in rRNA biogenesis is conserved across yeast, zebrafish, and human cell lines. However, although the kinase activity of NOL9 has been demonstrated by *in vitro* RNA kinase assay using the recombinant protein [3], the functional role of its activity in cells remains elusive.

We investigated the kinase activity of CLP1 and NOL9 in mouse cells and tissues by an *in vitro* RNA kinase assay. Unexpectedly, the kinase activity was not observed in fibroblasts, thymus, or brain of the *Clp1^{K/K}* mice even with an extended reaction time. Recombinant NOL9 protein produced by rabbit reticulocytes or insect cells also did not show RNA kinase activity in this *in vitro* kinase assay. We also showed that siRNA efficiency is decreased in the *Clp1^{K/K}* cells, as was expected [2]. However, NOL9 overexpression did not augment the efficiency of siRNA, suggesting that the siRNA kinase activity of NOL9 contributes little to the siRNA machinery. These data indicate that CLP1 functions as the primary RNA kinase, at least in mice.

2. Materials and methods

2.1. Mice

Clp1^{K/K} mice and wild-type (*Clp1^{+/+}*) littermates were bred and maintained according to institutional guidelines. All animal experiments were performed according to procedures approved by the Oita University Faculty of Medicine and the Oita University Committees on Animal Research. The approval number is 180502.

2.2. Cell culture

Clp1^{+/+} and *Clp1^{K/K}* embryonic fibroblasts (MEFs) were obtained from E14.5 embryos [11] and cultured in DMEM/Ham's F-12 with L-glutamine and phenol red (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS (Thermo Fisher Scientific, Tokyo, Japan) at 37 °C in a 5% CO₂ incubator. In some experiments, immortalized MEFs established by a standard 3T3 protocol [16] were used.

2.3. In vitro kinase assay

RNA kinase assays were performed with tissue lysates from E18.5 *Clp1^{K/K}* mouse embryos, cell lysates, or recombinant proteins (protein concentration, 2–5 mg/mL) mixed with 15 μM fluorescently labeled RNA (5'-UUCGAAGUAUCCGCGUACGU-TAMRA-3'; Thermo Fisher Scientific) as a substrate in kinase reaction buffer (400 mM KCl, 5 mM DTT, 2 mM MgCl₂, 10 mM ATP, 2 mM GTP, 8 mM ribonucleoside–vanadyl complex [New England Biolabs, Ipswich, MA], and 12 units RNasin Plus RNase inhibitor [Promega, Madison, WI] [1,17]. Ten units of T4 polynucleotide kinase (Thermo Fisher Scientific) was used for positive control. Cell and tissue lysates were prepared using cell lysis buffer (100 mM KCl, 30 mM HEPES [pH 7.4], 5 mM MgCl₂, 1% NP-40, 10% glycerol, 1 mM DTT, and 0.1 mM AEBSF). Phosphorylation reaction mixtures were incubated for 0–240 min at 37 °C and quenched by adding 5 μL 8 M urea, then boiled and loaded onto a 15% polyacrylamide (8 M urea) gel in 0.5 × TBE buffer. Gels were visualized on a Typhoon FLA 7000 (GE Healthcare, Chicago, IL, USA).

2.4. Expression of wild-type and mutant NOL9

cDNA coding for murine CLP1 (mCLP1) and murine NOL9 (mNOL9) were cloned into expression vectors pMXs-IRES-GFP (Cell Biolabs, San Diego, CA) [18], pcDNA3.1 Hygro (+) (Thermo Fisher

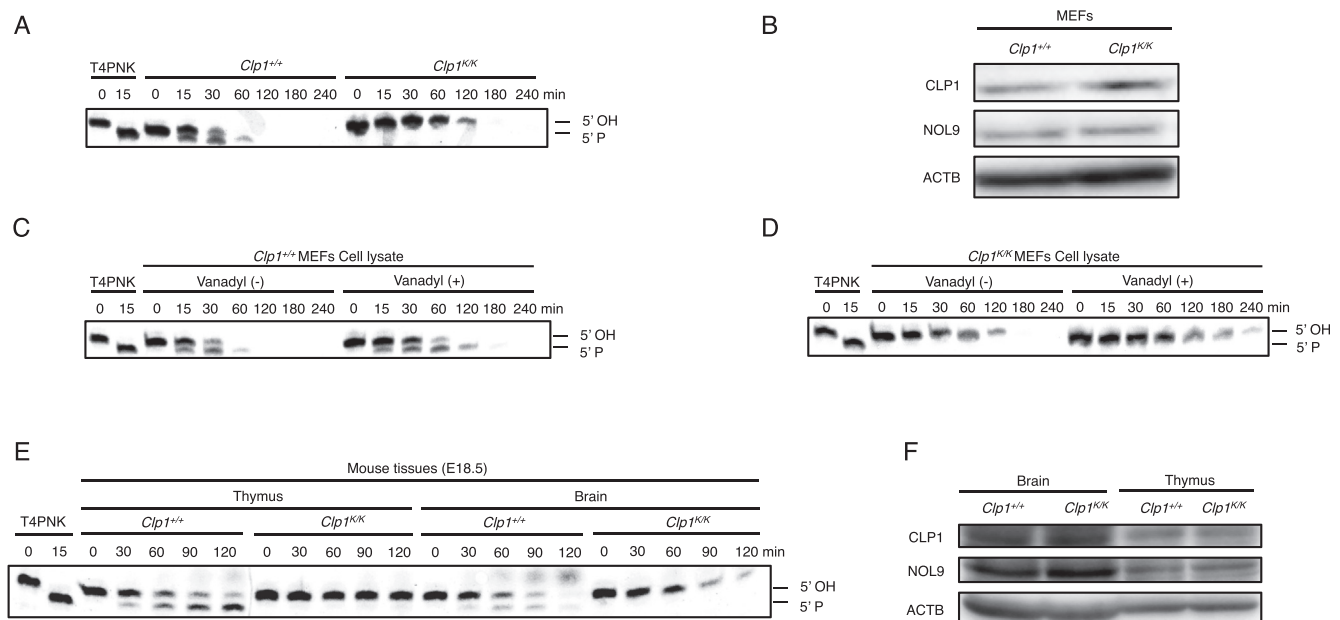


Fig. 1. RNA kinase activity was not detected in CLP1 kinase-dead (*Clp1^{K/K}*) mice. A. RNA kinase activity assay of protein extracts from *Clp1^{+/+}* and *Clp1^{K/K}* MEFs. Whole-cell lysates were incubated with single-stranded 21-nt RNA substrate 3'-end-labeled with TAMRA for the indicated times. Phosphorylation was detected in a denaturing 15% acrylamide gel (8 M urea). T4 polynucleotide kinase (T4PNK) was used as a positive control. B. Western blot analysis of protein extracts from *Clp1^{+/+}* and *Clp1^{K/K}* MEFs. ACTB is shown as a loading control. C. RNA kinase activity assay of protein extracts from *Clp1^{+/+}* MEFs with or without ribonucleoside–vanadyl complex (vanadyl), which is a potent inhibitor of various ribonucleases. D. RNA kinase activity assay of protein extracts from *Clp1^{K/K}* MEFs with or without vanadyl. E. RNA kinase activity assay of protein extracts from *Clp1^{+/+}* and *Clp1^{K/K}* tissues (thymus and brain) of murine embryos (E18.5) with vanadyl. F. Western blot analysis of protein extracts from *Clp1^{+/+}* and *Clp1^{K/K}* tissues (thymus and brain) of murine embryos (E18.5). ACTB is shown as a loading control.

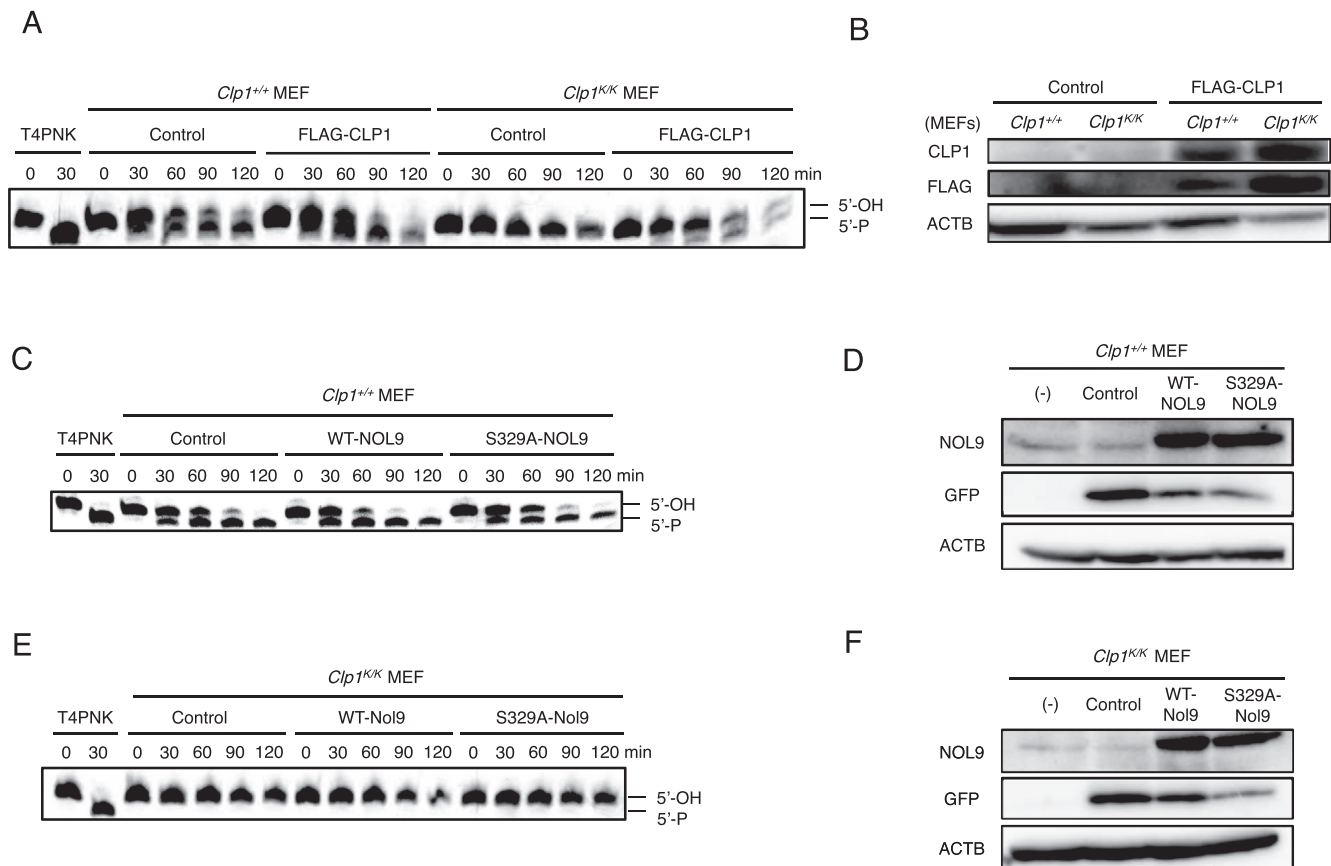


Fig. 2. Overexpression of NOL9 did not affect endogenous kinase activity in MEFs lysates A and B. RNA kinase activity assay of lysates from *Clp1*^{+/+} and *Clp1*^{K/K} MEFs infected with a retroviral vector containing FLAG-tagged mCLP1 (FLAG-CLP1) (A). Infected cells were lysed and incubated with an RNA substrate for the indicated times. T4PNK was used as a positive control. FLAG-CLP1 protein levels in each of the MEFs were evaluated with Western blotting (B). ACTB is shown as a loading control. C and D. RNA kinase activity assay of lysates from *Clp1*^{+/+} MEFs infected with a retroviral vector containing wild-type mNOL9 (WT-NOL9) or mutated mNOL9 (S329A-NOL9) with IRES-GFP (C). WT-NOL9 and S329A-NOL9 protein levels in *Clp1*^{+/+} MEFs were evaluated with Western blotting (D). ACTB is shown as a loading control. E and F. RNA kinase activity assay of lysates from *Clp1*^{K/K} MEFs infected with a retroviral vector containing WT-NOL9 or S329A-NOL9 with IRES-GFP (E). WT-NOL9 and S329A-NOL9 protein levels in *Clp1*^{K/K} MEFs were evaluated with Western blotting (F). ACTB is shown as a loading control.

Scientific), or pF25AICE (Promega). NOL9 kinase-dead (S329A) mutations were introduced using the QuikChange Site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). All sequences were confirmed by ABI 3130 DNA Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.5. Establishment of stable transfected cell lines, and retroviral transduction

The retroviral vector pMXs-IRES-GFP containing mCLP1 or mNOL9 was transfected into Plat-E packaging cells with FuGENE HD (Promega) for retrovirus production [18]. Virus-containing medium was collected and filtered 48 h after transfection. MEFs were incubated for 4 h with the virus-containing medium to which 8 mg/mL polybrene was added, and then 48 h later, infection efficiency was assessed using flow cytometry. Newly established stable cell lines (within 10 passages) were used for experiments. Western blot analysis was conducted to validate expression of the target gene.

2.6. Cell-free protein expression system

To achieve the synthesis of mNOL9 (wild-type mNOL9 and S329A mutant mNOL9) and mCLP1, we used a TNT (rabbit reticulocyte lysate) T7 Quick Coupled Transcription/Translation system

(Promega) or a TNT (insect cell extract) T7 Quick Coupled Transcription/Translation system (Promega) with plasmid DNA containing a T7 promoter (pcDNA3.1 Hygro(+)) or pF25A ICE T7 Flexi Vector, respectively, according to the manufacturer's manual. Briefly, a reaction mixture containing 40 μ L TNT Master Mix, each plasmid DNA template, and nuclease-free water (final volume 50 μ L) were incubated at 30 $^{\circ}$ C. The *in vitro* translated products were analyzed by sodium dodecyl sulfate (SDS-PAGE, 10%) and used for RNA kinase assay, immunoprecipitation, or Western blotting.

2.7. Western blot analysis

Cells were washed in cold 1 \times PBS two times, lysed in appropriate amounts of lysis buffer (100 mM KCl, 30 mM HEPES [pH 7.4], 5 mM MgCl₂, 1% NP-40, 10% glycerol, 1 mM DTT, and 0.1 mM AEBBSF). Proteins were separated by 10% SDS-PAGE and transferred onto Immobilon membranes (Millipore, Burlington, MA, USA). Blots were blocked with 5% non-fat milk at room temperature for 30 min, and subsequently incubated with primary antibodies targeting NOL9 (1:200), CLP1 (1:200), or β -actin (1:10,000), overnight at 4 $^{\circ}$ C. After washing with TBST, the blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA) at room temperature for 1 h. The detection was done by Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan) with Fusion SOLO S (Vilber, Collégien, France).

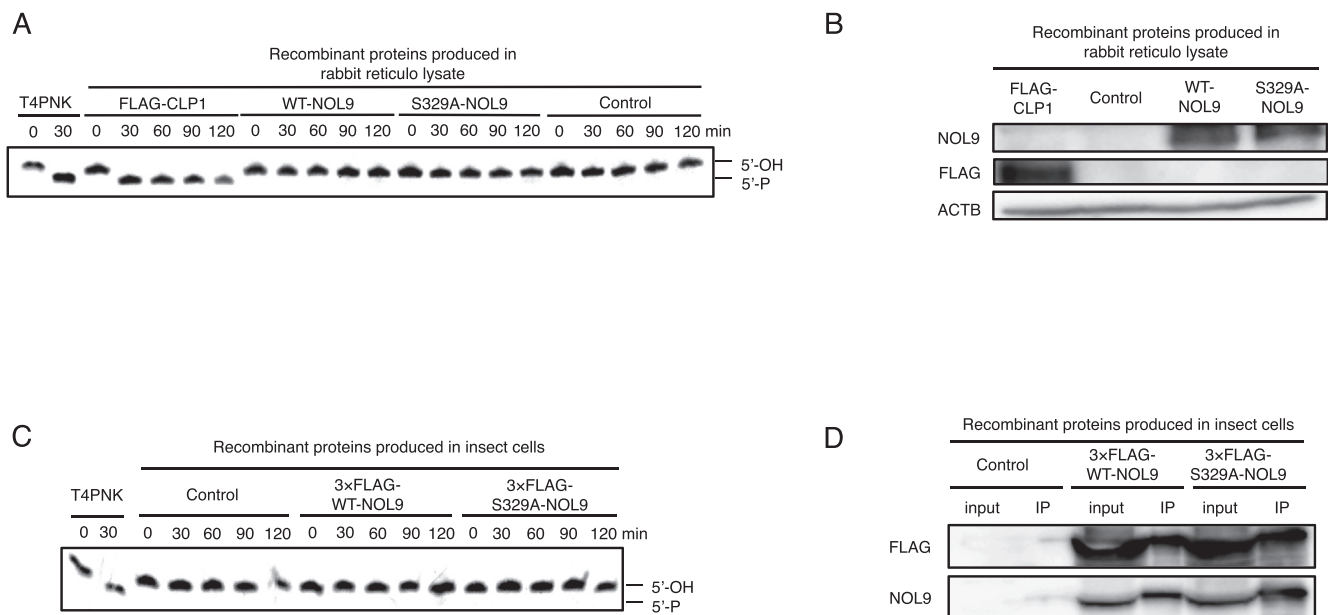


Fig. 3. RNA kinase activity of recombinant NOL9 protein was not detected A and B. RNA kinase activity assay of recombinant WT-NOL9, S329A-NOL9, and FLAG-CLP1 protein produced using a rabbit reticulocyte lysate system (A). Reaction mixture was incubated with an RNA substrate for the indicated times. T4PNK and FLAG-CLP1 were used as positive controls. pcDNA3.1 Hygro (+) empty vector was used as a control. Recombinant FLAG-CLP1, WT-NOL9 and S329A-NOL9 were evaluated with Western blotting (B). ACTB is shown as a loading control. C and D. RNA kinase activity assay of 3xFLAG-tagged WT-NOL9 or S329A-NOL9 protein produced using an insect cell lysate system (C). Equal amounts of FLAG-tagged WT-NOL9 and S329A-NOL9 purified by immunoprecipitation (IP) with anti-FLAG antibody beads were incubated with an RNA substrate for the indicated times. pF25A ICE T7 luciferase control vector was used as control. Immunoprecipitated recombinant 3xFLAG-tagged WT-NOL9 and S329A-NOL9 were evaluated with Western blotting (D).

2.8. Antibodies

Antibodies against FLAG-tags, β -actin (ACTB), and GAPDH were purchased from Sigma-Aldrich (St. Louis, MO). Antibody against GFP was purchased from Invitrogen (Carlsbad, CA). For immunoprecipitation, anti-FLAG M2 affinity agarose gel from Sigma-aldrich was used. Rabbit polyclonal antibodies were raised against peptides of mNOL9 (N-TTLKKIGIRRRQKRKAIC-C) and mCLP1 (N-VVERSKDFRREC-C), and were subsequently affinity purified from sera for Western blot analysis.

2.9. Immunoprecipitation

Equilibrated anti-FLAG M2 agarose affinity gel (Sigma-Aldrich) was added onto all indicated proteins (FLAG-tagged mNOL9, S329A mNOL9) and incubated under rotation for 3 h at 4 °C. After washing five times with the cell lysis buffer used for kinase assay, the retained FLAG-tagged proteins were used for RNA kinase assay.

2.10. Effects of siRNA-Mediated knockdown of GAPDH

MEFs (wild-type and $Clp1^{K/K}$) were plated in a six-well dish at 1×10^5 cells per well 1 day prior to transfection. Cells were transfected with siRNA-GAPDH (Sigma-Aldrich) or the control RNA using Lipofectamine RNAiMax (Invitrogen) following the procedure recommended by the manufacturer. At various time points after transfection, cells were scraped, collected, and lysed with the lysis buffer. The effect of siRNA-GAPDH on MEFs was evaluated by Western blot analysis.

3. Results

3.1. RNA kinase activity in CLP1 kinase-dead mice

CLP1 and NOL9 both have RNA 5'-end kinase activity in human

cells [2,3]. To assess the kinase activity of NOL9 in mouse cells, first, we performed an *in vitro* RNA kinase assay using $Clp1^{K/K}$ MEFs. We could not observe RNA phosphorylation in $Clp1^{K/K}$ cell lysate (Fig. 1A). The NOL9 protein levels were comparable between the wild-type and $Clp1^{K/K}$ MEFs in Western blots (Fig. 1B).

To evaluate the RNA kinase activity at later time points, ribonucleoside–vanadyl complex, which is a potent inhibitor of various ribonucleases, was added to the assay reaction mixture [19–21]. As expected, it effectively blocked the degradation of RNA substrates, resulting in the assay extending over a longer reaction period (Fig. 1C and D). Even under this condition, RNA kinase activity was not detected in $Clp1^{K/K}$ MEFs until 240 min of reaction time (Fig. 1C and D).

Further, we performed the assay using mouse thymus and brain tissue lysates. RNA kinase activity was observed in the wild-type thymus and brain (Fig. 1E). However, no phosphorylated RNAs were detected in the $Clp1^{K/K}$ thymus or brain (Fig. 1E). Levels of both CLP1 and NOL9 protein were comparable between wild-type and $Clp1^{K/K}$ thymus and brain (Fig. 1F). These data demonstrate that $Clp1^{K/K}$ cells and tissues show undetectable RNA kinase activity, at least in our *in vitro* RNA kinase assay.

3.2. Undetectable RNA kinase activity in CLP1 kinase-dead cells with NOL9 overexpression

We assumed that the undetectable RNA kinase activity in $Clp1^{K/K}$ cells was due to the low level of NOL9 protein in the cells.

Firstly, to confirm the RNA kinase activity of mCLP1, wild-type and $Clp1^{K/K}$ MEFs were retrovirally transfected with wild-type mCLP1. The overexpression of mCLP1 augmented the RNA kinase activity in wild-type MEFs (Fig. 2A and B). It clearly restored the RNA kinase activity in $Clp1^{K/K}$ MEFs (Fig. 2A and B). In humans, the serine-to-alanine substitution at position 313 in the Walker A motif diminishes the kinase activity [3]. To evaluate the kinase activity of mNOL9, we generated MEFs stably overexpressing wild-type

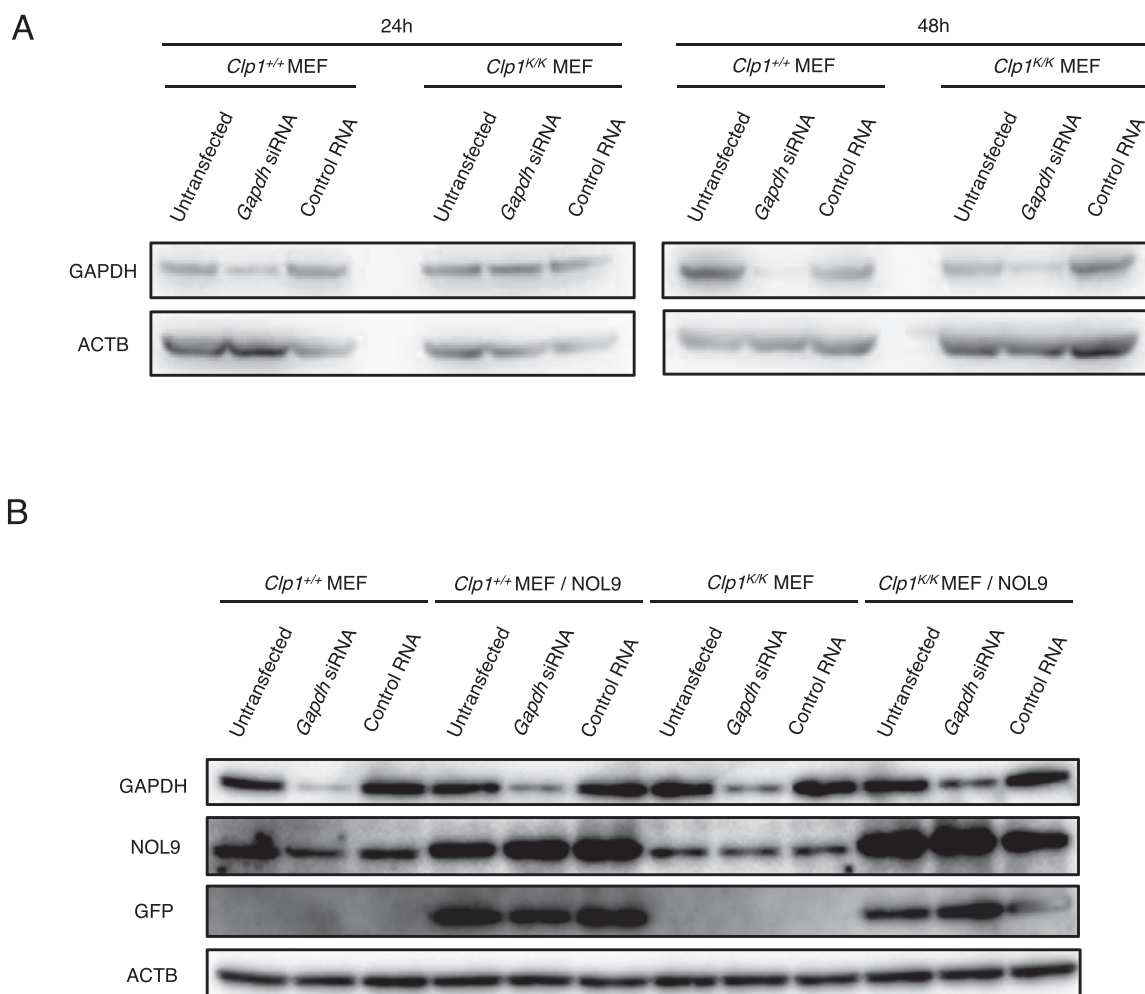


Fig. 4. Efficiency of siRNA was decreased in CLP1 kinase-dead (Clp1K/K) MEFs A. Primary MEFs (*Clp1^{+/+}* and *Clp1^{K/K}*) were transfected with siRNA for *Gapdh* or the control RNA. GAPDH and ACTB protein levels in each of the MEFs were evaluated with Western blotting at the indicated time points. B. Immortalized MEFs (*Clp1^{+/+}* and *Clp1^{K/K}*) overexpressing mNOL9 were transfected with siRNA for *Gapdh* or the control RNA at 24 h. GAPDH and ACTB protein levels in each of the MEFs were evaluated with Western blotting. ACTB is shown as a loading control.

mNOL9 and its expected kinase dead form, S329A. However, wild-type mNOL9 as well as the S329A mutant mNOL9 had no effect on RNA kinase activity in both wild-type or *Clp1^{K/K}* MEFs despite both MEFs having high levels of mNOL9 overexpression (Fig. 2C–F). Thus, RNA kinase activity in mouse cells is mostly attributable to CLP1.

3.3. *In vitro* RNA kinase assay using recombinant NOL9 protein

The RNA kinase activity of human NOL9 and its *S. cerevisiae* orthologue, Grc3, have been demonstrated by using recombinant proteins produced by insect cell-based protein expression [22]. These results may imply that a greater quantity of NOL9 protein was needed for detecting RNA kinase activity in our assay. Therefore, we performed the RNA kinase assay with recombinant mNOL9 protein produced by either insect cell lysate or rabbit reticulocyte lysate in an *in vitro* translation system. However, we could not detect the RNA kinase activity of mNOL9 protein produced by either the insect cell lysate or the rabbit reticulocyte lysate (Fig. 3A–D). As a note, insect cell lysate contains CLP1 protein sufficient for RNA kinase activity in our RNA kinase assay. Thus, the NOL9 proteins were purified by using an affinity-purified antibody to exclude kinase activity derived from CLP1 protein.

In the end, we could not detect RNA kinase activity of mNOL9, even using an abundant amount of recombinant proteins.

3.4. Efficiency of siRNA in CLP1 kinase-dead MEFs

CLP1 phosphorylates 5' hydroxyl ends of siRNAs in human cells [2], which is required for assembling the siRNA duplex into an active RISC [7,23,24]. To assess the potential roles of CLP1 and NOL9 in the siRNA machinery, the siRNA targeting *Gapdh* mRNA was transfected into wild-type and *Clp1^{K/K}* MEFs. The protein levels of GAPDH were knocked down in the wild-type MEFs 24 h and 48 h after siRNA transfection. As expected, the efficiency of siRNAs was decreased in the *Clp1^{K/K}* MEFs after the transfection of siRNAs (Fig. 4A). In order to examine the involvement of NOL9 in siRNA machinery, we established MEFs stably overexpressing NOL9 protein. The overexpression of NOL9 protein could not influence the efficiency of siRNA in either wild-type or *Clp1^{K/K}* MEFs (Fig. 4B). These results indicated that the kinase activity of CLP1, but not NOL9, is essential for the siRNA pathway.

4. Discussion

In this study, we investigated the RNA kinase function of CLP1

and NOL9 in murine cells and tissues by *in vitro* RNA kinase assay. Adding the ribonucleoside–vanadyl complex into this assay system enabled us to examine the reaction over a more extended period. By using this system, we found CLP1 to be the primary RNA kinase in mice. Cells and tissue samples derived from *Clp1*^{K/K} mice had no RNA kinase activity. The efficiency of siRNA activity in *Clp1*^{K/K} cells was decreased compared with that in wild-type cells, and the overexpression of NOL9 protein did not augment the siRNA activity. These results indicate that CLP1 plays an essential role as an RNA kinase *in vivo*, at least in mice.

It has been reported that human NOL9 functions as a polynucleotide 5' kinase and is essential for efficient processing of the 32S precursor into 5.8S and 28S rRNAs [3]. K312A and S313A mutations of the NOL9 Walker A motif abolish this kinase activity, resulting in defective 32S precursor processing [3]. Grc3, which is a NOL9 orthologue in *S. cerevisiae*, has also been demonstrated to be a polynucleotide kinase that phosphorylates the 5' ends of RNA, which is essential for efficient degradation of cleaved pre-rRNAs mediated by exonucleases [25]. Unexpectedly, however, we could not detect the kinase activity of mNOL9. Similarly, there has also been no direct evidence of Grc3 kinase activity demonstrated in *S. pombe* [14].

LAS1L is an endonuclease that interacts with NOL9. The LAS1L–NOL9 complex plays an essential role in the processing of ITS2 (the internal spacer sequence flanked by 5.8S and 28S rRNAs) [26,27]. Intriguingly, each of these molecules controls the mutual enzymatic activity of the other [27]. The similarity of amino acid sequences between mNOL9 and the human orthologue is about 59% according to BLAST. It is possible that this relatively low similarity might influence the association with LAS1L and might result in the difference in RNA kinase activity. Further studies are needed to clarify the difference in kinase activity between human and murine NOL9.

Although the *in vitro* RNA kinase assay in this study showed that *Clp1*^{K/K} cells no longer had RNA kinase activity, NOL9 kinase-dead mice would be useful for confirming the *in vivo* function of NOL9 kinase activity. This model provides a valuable piece of information about the role of NOL9 in rRNA processing and its relevance in pathogenesis, such as hematopoietic disorder and pancreatic defects reported in the zebrafish *nol9* gene knockout model [15].

In summary, our experiments indicate that mNOL9 has undetectable RNA kinase activity in cells, even in cells with overexpression of mNOL9 protein. Further, we demonstrated for the first time that the efficiency of siRNA was decreased in *Clp1*^{K/K} cells. However, the reduced efficiency of siRNA could not be overcome by the overexpression of NOL9. Therefore, these results indicate that CLP1 is the principal kinase to phosphorylate the 5' ends of RNAs, including siRNAs, in mice.

Declaration of competing interest

The authors declare no conflict of interests.

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