An RNA dependent RNA polymerase formed by hTERT and the RNase MRP RNA

Yoshiko Maida[1], **Mami Yasukawa**[1], **Miho Furuuchi**[1], **Timo Lassmann**[2], **Richard Possemato**[3], **Naoko Okamoto**[1], **Vivi Kasim**[1], **Yoshihide Hayashizaki**[2], **William C. Hahn**[3,4,†], and **Kenkichi Masutomi**[1,5,†]

[1]Cancer Stem Cell Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045 JAPAN

[2]RIKEN Omics Science Center, RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045 JAPAN

[3]Department of Medical Oncology, Dana-Farber Cancer Institute and Departments of Medicine, Brigham and Women's Hospital and Harvard Medical School, 44 Binney Street, Boston, MA 02115 USA

[4]Broad Institute of Harvard and MIT, 7 Cambridge Center, Cambridge, MA 02142 USA

[5]PREST, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012 JAPAN

Abstract

Constitutive expression of telomerase in human cells prevents the onset of senescence and crisis by maintaining telomere homeostasis. However, accumulating evidence suggests that the human telomerase catalytic subunit (hTERT) contributes to cell physiology independent of its ability to elongate telomeres. Here we show that hTERT interacts with the RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*), a gene that is mutated in the inherited pleiotropic syndrome Cartilage-Hair Hypoplasia. hTERT and *RMRP* form a distinct ribonucleoprotein complex that exhibits RNA dependent RNA polymerase (RdRP) activity and produces double-stranded RNAs that can be processed into small interfering RNA in a Dicer-dependent manner. These observations identify a mammalian RdRP composed of hTERT in complex with *RMRP*.

Keywords

Telomerase reverse transcriptase; RNA dependent RNA polymerase; RMRP; siRNA

Telomerase is a ribonucleoprotein complex that elongates telomeres. Although several proteins interact with telomerase[1–4], the minimal components of active telomerase include the catalytic telomerase reverse transcriptase (TERT) and a non-coding RNA (*TERC*) that encodes the template to synthesize telomeric DNA[5]. Telomere homeostasis mediated by telomerase maintains genomic stability and regulates cell lifespan[6]. Mutations in hTERT, *hTERC* or dyskerin, a telomerase-associated nucleolar protein involved in rRNA maturation[7], are found in dyskeratosis congenita, a syndrome characterized by ectodermal dysplasia and bone marrow failure, and mutations of hTERT have been reported in aplastic anemia and idiopathic pulmonary fibrosis[8]. Moreover, alterations in the regulation of telomeres and telomerase contribute to malignant transformation by affecting genomic integrity and cell immortalization[6].

However, accumulating evidence suggests that hTERT exhibits activities beyond telomere maintenance[9–13] and forms several intracellular complexes[2–4]. In particular, overexpression of TERT induces increased tumor susceptibility[9,10] and disrupts stem cell function independent of telomere maintenance[12] while suppression of hTERT expression alters global chromatin structure[11]. Indeed, some of these telomere-independent functions of TERT do not require the expression of *hTERC*[12].

Identification of a second RNA that interacts with hTERT

To identify hTERT partners, we stably overexpressed a tandem affinity peptide (TAP)-tagged hTERT protein in HeLa-S cells, isolated hTERT immune complexes, and identified a heterogeneous mixture of 38 RNA sequences associated with hTERT (Supplementary Fig. 2; Supplementary Table 1). We found that 5% of the sequences corresponded to *hTERC* and the RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*). *RMRP* is a 267 nt non-coding RNA that is a small nucleolar (sno) RNA, like *hTERC*, and is also found in mitochondria[8,14]. Mutations of *RMRP* are found in the pleiotropic inherited syndrome, Cartilage-Hair Hypoplasia (CHH)[15].

From a single immune complex, we confirmed that either overexpressed or endogenous hTERT interacts with *RMRP* and *hTERC* by isolating TAP-hTERT (Fig. 1a) or endogenous hTERT (Fig. 1b) complexes in both HeLa and 293T cells under conditions where we failed to recover the ribozyme *RNase P*. We also found that the abundance of hTERT-*RMRP* and hTERT-*hTERC* complexes was similar even though *hTERC* was expressed at five-fold higher levels than *RMRP* in these cells (Fig. 1c; Supplementary Fig. 3).

To characterize the interaction of hTERT and *RMRP*, we used TERT truncation mutants and found that the aminoterminal end of hTERT (1–531) was necessary for interactions with *RMRP* (Supplementary Fig. 4). This region overlaps with two regions required for the binding of *hTERC*[8,16]. These observations demonstrate that hTERT and *RMRP* form a novel ribonucleoprotein complex distinct from the hTERT-*hTERC* enzyme.

The hTERT-*RMRP* complex exhibits RdRP activity

To test whether *RMRP* substitutes for *hTERC* to reconstitute telomerase activity, we combined recombinant hTERT with *hTERC* or *RMRP* RNAs transcribed *in vitro*. Although we detected telomerase activity with hTERT and *hTERC* (Supplementary Fig. 5), we failed to detect telomerase activity when hTERT and *RMRP* were co-incubated.

TERT has also been shown to act as a terminal transferase[17], and hTERT shares sequence similarity to both viral reverse transcriptases and RNA dependent RNA polymerases (RdRPs)[18]. RdRPs participate in the endogenous RNAi pathway and in the regulation of posttranscriptional gene silencing[19–23]. To examine whether the hTERT-*RMRP* complex exhibits RdRP and/or terminal transferase (TT) activity, we established an RNA synthesis activity assay with recombinant hTERT protein (Supplementary Fig. 6) and RNA molecules transcribed *in vitro*. We predicted three modes that the hTERT-*RMRP* complex might use to elongate RNA: [i] as an RdRP that uses a *de novo* synthesized RNA primer to elongate a complementary strand (Fig. 2a left panel); [ii] as an RdRP that uses a 3' fold-back (back-priming) configuration of template RNA as a primer (middle panel); or [iii] as a TT (right panel). Viral RdRPs[24,25] have been shown to use the first two modes to prime RdRP activity, and cellular RdRP in fission yeast[26] and fungi[23] use similar priming mechanisms to produce double-stranded (ds) RNAs that serve as precursors for RNAi.

We found that recombinant hTERT and *RMRP* produced 2 different products depending on the salt concentration (Fig. 2b; Supplementary Fig. 7). Specifically, we found ~267 nt (corresponding to sense *RMRP*) and ~534 nt sized products (hereafter referred to as sense + antisense *RMRP* products) under high salt conditions and *RMRP*-sized products under low salt conditions. To discriminate among these modes, we treated the products of the RdRP assay with RNase T1 (Fig. 2c), under conditions that favor the digestion of single-stranded RNA. RNase T1 treatment eliminated the ~267 nt *RMRP*-sized RNA products produced under low salt concentrations (data not shown), indicating that 32P-UTP was incorporated by TT activity.

In contrast, under high salt conditions, we found two RNAs (~267 nt and ~534 nt) that collapsed into a single ~267 nt band after treatment with RNase T1 (Fig. 2c). To eliminate the possibility that the sense + antisense product represented partially denatured RNAs, we treated the products of the RdRP assay with bacterial RNase III to digest dsRNA and found that only the input ~267 nt RNA remained (Fig. 2d). Furthermore, when we left out adenine or guanine ribonucleotides, we failed to detect the sense + antisense product (Fig. 2e). These observations confirm that the ~534 nt sense + antisense products are formed by RdRP activity and represent a ds hairpin structure created by an RNA molecule composed of sense and antisense strands of *RMRP*.

To confirm that the interaction of hTERT and *RMRP* was required for RdRP activity, we performed an RdRP activity assay using combinations of recombinant mutant hTERT proteins and *RMRP*. We failed to detect RdRP reaction products when hTERT and *hTERC* were co-incubated (Supplementary Fig. 8). Moreover, when we used the hTERT-HT1 mutant that does not bind *RMRP* (Supplementary Fig. 4), we failed to observe labeled RNA

products (Supplementary Fig. 8) under conditions where we detected two different RNA products in reactions containing wild-type hTERT and *RMRP*. We previously described a catalytically inactive hTERT mutant (DN hTERT) that fails to elongate telomeres[11,27]. We confirmed that the recombinant DN hTERT mutant retained the ability to bind *RMRP* (Fig. 2f) but that the DN hTERT-*RMRP* complex lacked detectable RdRP activity (Fig. 2f). Thus hTERT serves as the catalytic subunit for both the telomerase reverse transcriptase and RdRP activities.

The hTERT-*RMRP* RdRP produces ds RNA

These observations suggest that the hTERT-*RMRP* RdRP synthesizes ds RNA in a template-dependent manner. To confirm the synthesis of the *RMRP* complementary strand, we used the sense strand of *RMRP* as a probe in Northern blotting. We detected the antisense strand of *RMRP* in reactions containing recombinant WT hTERT protein and *RMRP* but not in reactions containing DN hTERT and *RMRP* (Fig. 2g). Furthermore, we detected the sense + antisense product in the RdRP assay using the antisense strand of *RMRP* as a probe (Supplementary Fig. 9). These observations indicate that the hTERT-*RMRP* RdRP produces ds RNAs in template-dependent manner *in vitro*.

To determine whether the hTERT-*RMRP* RdRP uses a back priming mechanism, we examined the priming process using hTERT and *RMRP* as a model system and found that elongation products appeared in time-dependent manner (Fig. 2h; Supplementary Fig. 10). To assess whether the *RMRP* RNA forms a 3' fold-back configuration, we generated 3' *RMRP* truncation mutants and failed to find any reaction products (Fig. 2i). Thus, unlike what has been described for other cellular RdRPs, the hTERT-*RMRP* RdRP exhibits a restricted preference for RNA molecules that can be used as a template. Indeed, when we incubated purified recombinant hTERT together with total cellular RNA and 32P-UTP, we identified a limited number of labeled RNAs (Fig. 2j). Although the secondary structure adopted by *RMRP* to create the 3' fold-back is not known, these observations suggest that *RMRP* can itself serve as a primer for the polymerization process using a 3' fold-back structure.

To ascertain whether this RdRP activity also occurs *in vivo*, we used the sense strand of *RMRP* as a probe and found ~534 nt RNAs that contain antisense *RMRP* in RNA derived from 293T, HeLa and MCF7 cells (Fig. 3a; Supplementary Fig. 11, Supplementary Fig 12). Moreover, we detected both sense + antisense and sense products using *RMRP* antisense strand probe (Fig. 3b). These observations confirmed that the ~534 nt products contain both sense and antisense *RMRP* sequences. To determine whether hTERT was necessary for the appearance of antisense *RMRP* in cells, we examined the levels of the complementary *RMRP* strand in cells: (i) that do not express hTERT and *hTERC* (VA-13)[28]; (ii) that transiently express low levels of hTERT (BJ)[27,29,30]; and (iii) that constitutively express hTERT (293T and HeLa). We also introduced a control vector or a vector that encodes hTERT in VA-13 and BJ cells. We detected the complementary *RMRP* strand using both a quantitative RNase protection assay with a sense strand probe that detects antisense *RMRP* (Fig. 3c; Supplementary Fig. 13) and Northern blotting with both sense and antisense strand-specific *RMRP* probes (Fig. 3d and Supplementary Fig. 11a). The levels of antisense *RMRP*

correlated with the expression of hTERT (Fig. 3c,d). These observations confirmed that the hTERT-*RMRP* RdRP produces ds *RMRP in vivo*.

Effects of the hTERT-*RMRP* complex on *RMRP* expression

To assess the consequences of overexpressing the hTERT-*RMRP* complex on *RMRP* levels, we introduced *RMRP* into cells that lack hTERT expression (VA-13), that transiently express hTERT in a cell-cycle dependent manner (BJ fibroblasts) and that constitutively express hTERT (VA-13 and BJ fibroblasts expressing ectopic hTERT, HeLa and MCF7 cells). Upon expressing *RMRP* in cells lacking hTERT (VA-13), we found that *RMRP* levels were increased (Fig. 4a; Supplementary Fig. 14). In contrast, in cells that express hTERT, we found that the steady state levels of *RMRP* were decreased when *RMRP* was overexpressed regardless of the promoter that was used to express *RMRP* (Fig. 4a; Supplementary Fig. 14). We also found that forced expression of hTERT in VA-13 or BJ cells suppressed *RMRP* expression (Fig. 4b; Supplementary Fig. 15). Consistent with these findings, suppression of hTERT in HeLa cells led to increased *RMRP* expression (Fig. 4c).

Since the 3' end of *RMRP* was essential for hTERT-*RMRP* activity (Fig. 2i), we examined the effects of expressing *RMRP* truncation mutants lacking 3' ends and found that only truncation mutants lacking intact 3' ends were readily overexpressed (Fig. 4d). These observations demonstrate that *RMRP* expression levels are dependent on the hTERT-*RMRP* RdRP and suggest that *RMRP* levels are controlled by an RdRP-dependent, negative feedback control mechanism.

Identification of siRNAs derived from *RMRP*

In other organisms, RdRPs synthesize ds RNAs that are processed into active siRNAs[31]. Since manipulating hTERT and *RMRP* levels affected *RMRP* expression, we hypothesized that the hTERT-*RMRP* complex produces *RMRP*-specific siRNA to regulate *RMRP* levels. To test this possibility, we used sense and antisense probes corresponding to *RMRP* (nucleotides 21–40) in Northern blotting and found ds 22 nt RNAs (Fig. 4e; Supplementary Fig. 11b). Since siRNAs contain 5' monophosphates and 3' hydroxyl groups[32–34], we characterized the chemical nature of the small RNA ends. We found that calf intestinal phosphatase (CIP) slowed the migration of these short RNAs and subsequent incubation with polynucleotide kinase (PNK) and ATP restored the mobility of the short RNAs, indicating that either the 5' or 3' end of this small RNA is monophosphorylated (Fig. 4f and data not shown). Moreover, incubation with PNK in the absence of ATP did not alter the migration (Fig. 4f), and oxidation and β-elimination treatment increased the migration of these small RNAs (Fig. 4g), indicating that the 3' ends bear vicinal 2', 3' dihydroxyls. Together, these observations confirm that these small RNAs contain 5' monophosphate and 3' hydroxyl groups and therefore share the size and chemical composition of known siRNAs.

To demonstrate that ds RNAs produced by the hTERT-*RMRP* RdRP are processed into siRNA, we suppressed the expression of Dicer with two distinct Dicer-specific shRNAs. Suppression of Dicer to levels that partially inhibited the processing of miR-16 (Fig. 5a; Supplementary Fig. 16) led to diminished levels of the siRNAs derived from *RMRP* (Fig.

5a). When we suppressed Dicer expression in HeLa, 293T or MCF7 cells, we found that endogenous *RMRP* levels increased up to 3.7 fold (Fig. 5b). Suppressing Dicer expression in VA-13 cells that lack hTERT did not affect the levels of single-stranded *RMRP* (Fig. 5b) but did increase levels of the elongated sense + antisense *RMRP* products in cells that constitutively express hTERT (Supplementary Fig. 17). Moreover, we found that only the sense strands of these endogenous *RMRP*-specific siRNAs were associated with human Ago2 (Fig. 5c). These observations indicate that the endogenous *RMRP*-specific siRNAs are processed by the RNA-induced silencing complex, similar to other small RNAs that are processed into siRNA.

To confirm that these small RNAs act as siRNA, we identified small RNAs from total RNA that hybridized to probes spanning *RMRP*, synthesized siRNA corresponding to the identified sequences and tested the consequences of introducing this siRNA in HeLa, 293T and MCF7 cells. We found that the synthesized siRNA suppressed endogenous *RMRP* levels (Supplementary Fig. 18). These observations provide evidence that similar to other cellular RdRPs, the TERT-*RMRP* RdRP synthesizes ds RNAs that serve as a precursor for siRNA.

Discussion

Here we demonstrate that hTERT and *RMRP* form distinct ribnucleoprotein complex that exhibits the ability to produce ds RNAs (Supplementary Fig. 1). Like RdRPs found in other organisms, the hTERT-*RMRP* complex produces ds RNAs that serve as substrates for the generation of siRNA. However, unlike other cellular RdRPs23,26,31,35,36, the hTERT-*RMRP* RdRP exhibits a strong preference for RNA templates that can form 3' fold-back structures. Since other cellular RdRPs have been identified using assays that require primer independent RdRP activity23,26,36, the substrate specificity of the hTERT-*RMRP* RdRP may, in part, account for the difficulty in identifying mammalian enzymes that exhibit RdRP activity.

Although the cellular RdRPs described to date do not exhibit a primer requirement, several viral RdRPs use both primer-dependent and primer-independent mechanisms, and fungal and yeast RdRPs are also able to employ a back-priming mechanism23,26. Since TERT is a closed right-handed polymerase37 evolutionarily related to both reverse transcriptases and viral RdRPs18, these observations are consistent with prior observations that indicate that right-handed RdRPs exhibit primer-dependent RdRP polymerase activity38.

Using *RMRP* as a template, the hTERT-*RMRP* RdRP produces ds RNAs that are processed by Dicer into 22 nt ds RNAs that contain 5' monophosphate and 3' hydroxyl groups and are loaded into Ago2, confirming that these short RNAs represent endogenous siRNAs. Recent work has shown that in oocytes and embryonic stem cells, endogenous siRNA can also be formed by the transcription of complementary sense and antisense strands39–41. Thus, in mammals at least two mechanisms lead to the production of ds RNAs that are processed into siRNA. Further work will be necessary to determine whether there are tissue-dependent differences in the use of these two mechanisms and if other mammalian RdRPs exist.

We found that the hTERT-*RMRP* RdRP regulates *RMRP* levels through a negative feedback control mechanism. The identities and functions of the RNAs other than *RMRP* that serve as templates for the hTERT-*RMRP* RdRP remain to be identified (Fig. 2j). However, since endogenously encoded siRNAs suppress L1 retrotransposition in human cells42, these observations suggest that the hTERT-*RMRP* complex may regulate the expression of other genes by generating siRNAs.

Since mutations in *RMRP* are found in CHH15, these findings suggest that perturbation of the hTERT-*RMRP* complex is involved in the pathogenesis of this disorder. The involvement of hTERT in two syndromes characterized by stem cell failure (CHH and dyskeratosis congenita)7,8,43 suggests that ribonucleoprotein complexes containing hTERT play a critical role in stem cell biology. Indeed, overexpression of mTERT in mice lacking m*TERC* leads to defects in normal hair follicle stem cell function12 at least in part by altering gene expression programs related to stem cell function44. In mammals, TERT may regulate both telomere biology and gene expression through these two ribonucleoprotein complexes.

METHODS SUMMARY

RNAs that bind hTERT were identified from HeLa-S cells expressing a TAP-epitope tagged version of hTERT. RNAs that bound to hTERT after two rounds of purification were analyzed using an Experion capillary electrophoresis device (Bio-Rad Laboratories, Inc. CA, USA) to visualize RNA species. For RNA cloning and the sequencing, the same samples were separated using a 7 M Urea/15% polyacrylamide gel, and RNAs recovered from gel were cloned using the small RNA cloning Kit (TaKaRa). Purified GST-hTERT was isolated from *E. coli* and incubated with either *hTERC* or *RMRP* transcribed *in vitro* to assess the ability of such complexes to exhibit telomerase or RdRP activity. RNAi was used to suppress hTERT and to show that the hTERT-*RMRP* complex also produces ds RNA in cells. Northern blotting with sense and anti-sense probes specific for RMRP (nt 21–40) identified 22 nt ds, small RNAs that contained a 5' monophosphate and a 3' hydroxyl group, which were loaded into human Ago2. To determine the function of these *RMRP*-derived small RNAs, a chemically synthesized siRNA corresponding to these small RNAs (siRNA: 5'-ggctacacactgaggactc-3'; Dharmacon) was transfected into HeLa, 293T and MCF7 cells. Full Methods accompany this paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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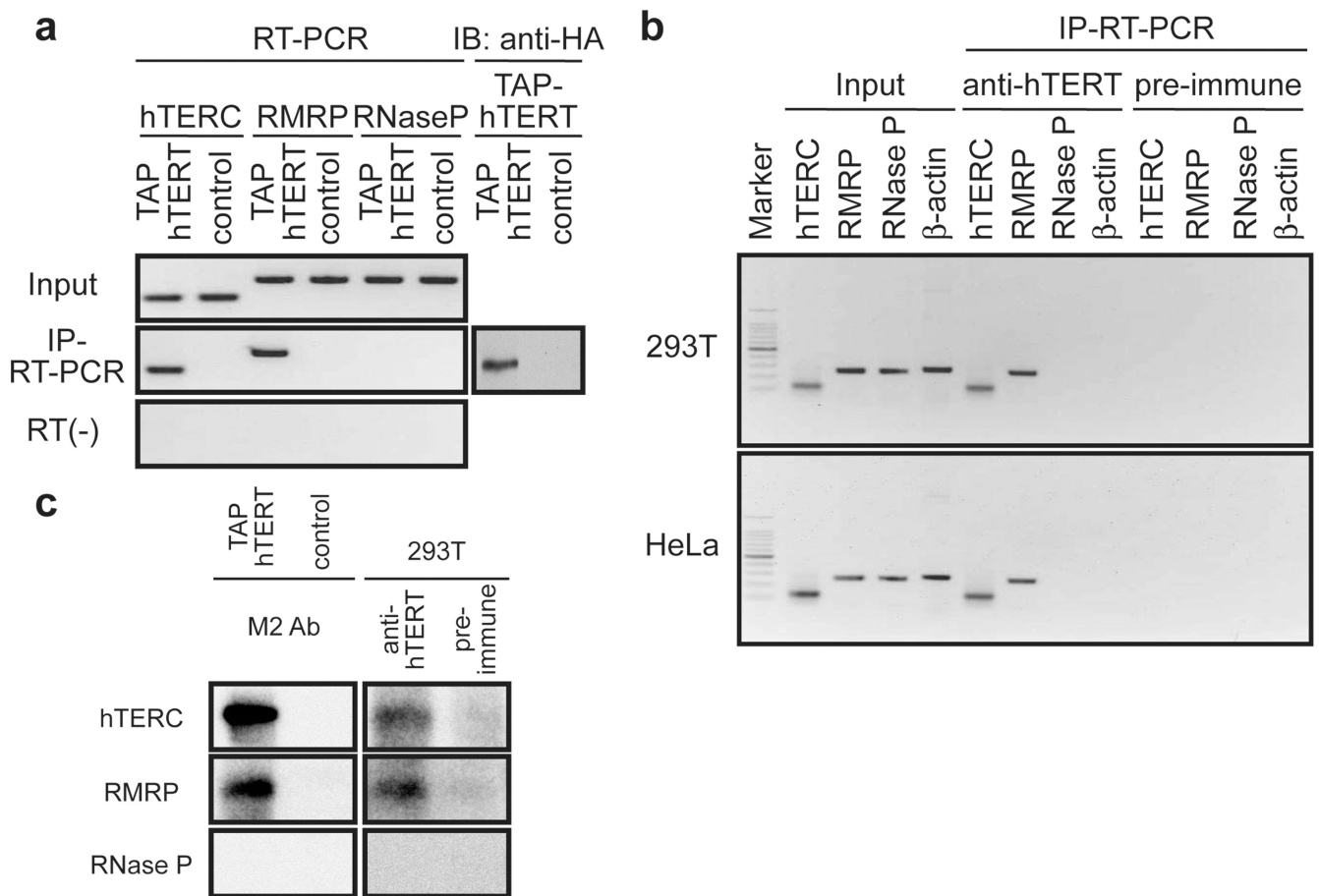
Figure 1. hTERT and *RMRP* interact

a, Detection of *RMRP* and *hTERC*. RNA species associated with TAP-hTERT complexes from a single immunoprecipitation were isolated and subjected to RT-PCR. RT (−) indicates the absence of reverse transcriptase. Right panel shows the levels of TAP-hTERT. **b,** hTERT interacts endogenous *RMRP*. hTERT complexes from 293T and HeLa cells were isolated with an anti-hTERT antibody and associated RNAs were subjected to RT-PCR. **c,** RNAs purified from hTERT complexes isolated from HeLa-S cells expressing TAP-hTERT or a control vector or 293T cells were subjected to Northern blotting.
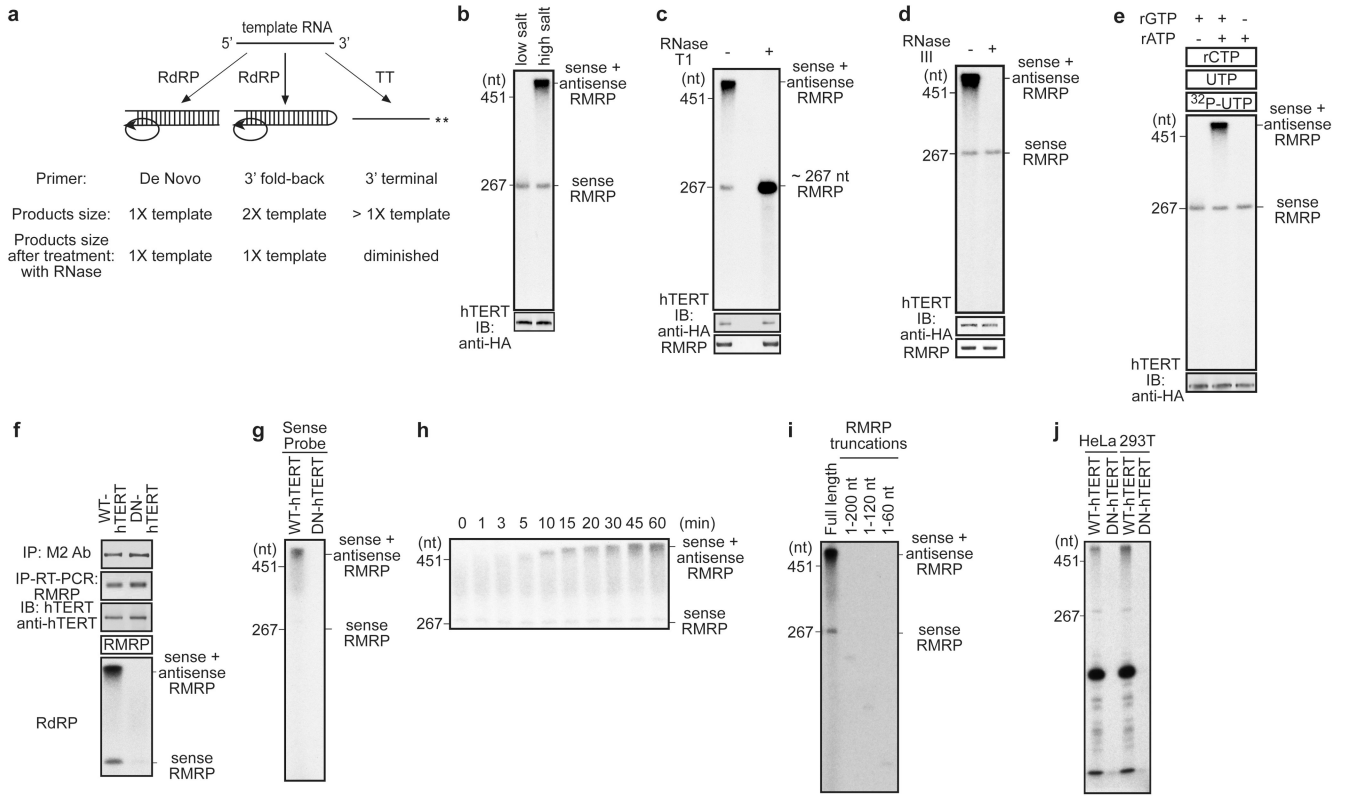
Figure 2. hTERT and *RMRP* exhibit RdRP activity
a, Predicted RNA products produced by RdRP or TT activity. **b,** RNA products produced by
the RdRP activity derived from recombinant hTERT and *RMRP*. **c,d** Treatment of RNA
products with RNase T1 (**c**) or bacterial RNase III (**d**). **e,** RdRP assay performed in the
presence of ribonucleotides (middle) or in the absence of adenine (left lane) or guanine
(right lane) ribonucleotides. A and G are present within the first 5 nt of the predicted
complementary strand of *RMRP*. **f,** DN-hTERT binds *RMRP* but lacks RdRP activity. TERT
immune complexes were isolated from 293T cells expressing FLAG-tagged hTERT or
FLAG-tagged DN hTERT. RdRP activity is shown in lower panel. **g,** Northern blotting to
detect complementary sequence of *RMRP*. **h**, Time course of RdRP activity. **i,** RNA
products produced by recombinant hTERT and truncation mutants of *RMRP* transcribed *in
vitro*. Faint signals at the 200 nt, 120 nt and 60 nt are hTERT TT products. **j,** RNA products
produced by the RdRP activity derived from recombinant hTERT or DN hTERT and total
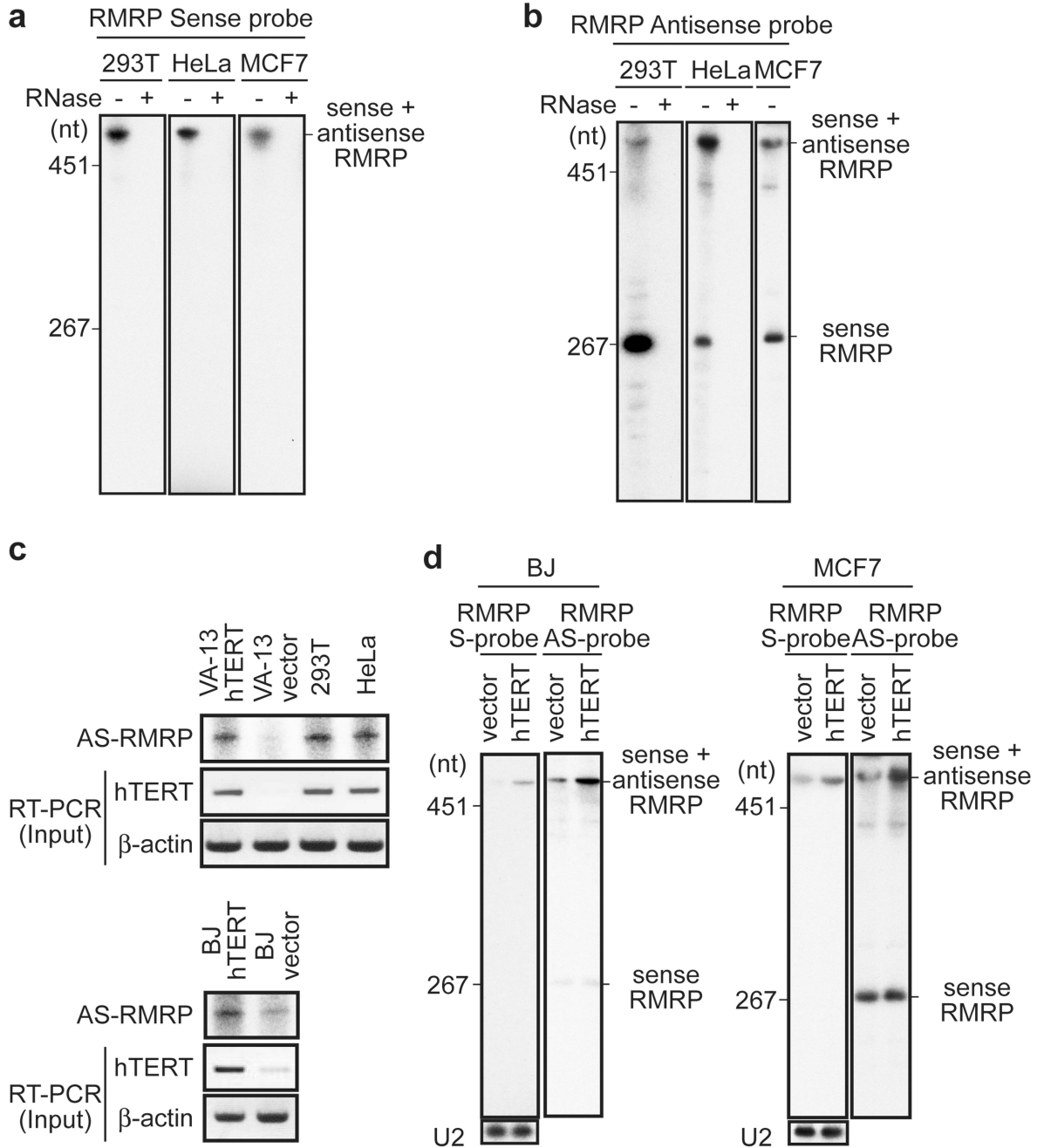RNA. A limited pool of RNAs serve a templates for RdRP activity.

Figure 3. Identification of ds RNA synthesized by the hTERT-*RMRP* RdRP
a, Northern blotting to detect complementary sequence of *RMRP* in cell lines. (+) indicates samples treated with RNase. **b**, Northern blotting to detect the *RMRP* sense strand. **c**, hTERT expression correlates with the levels of antisense *RMRP* detected by RNase protection assay. Vector denotes cells infected with a control vector. **d**, hTERT expression correlates with the levels of the sense (S) + antisense (AS) *RMRP* products detected by Northern blotting. The bottom panel shows U2 RNA levels.
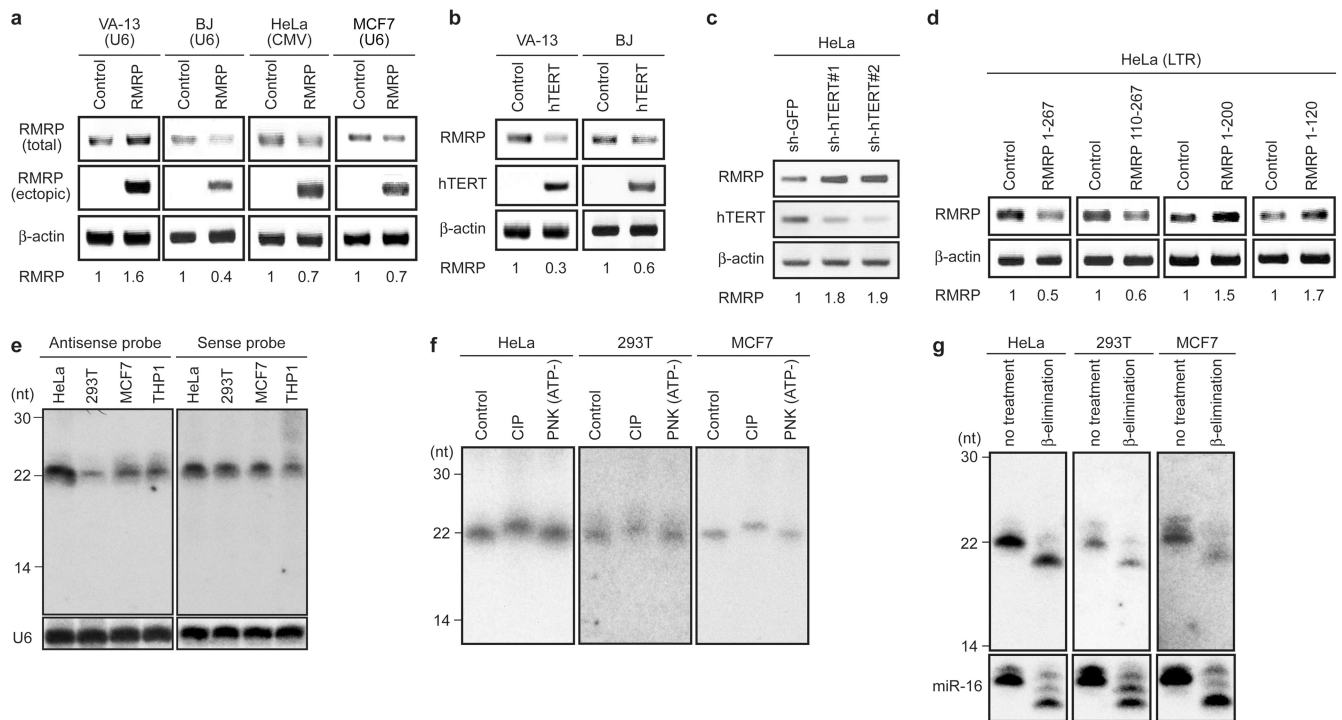
Figure 4. Effects of ds RNA produced by the hTERT-*RMRP* RdRP

a, Semi-quantitative RT-PCR for total *RMRP* and retrovirally delivered *RMRP* (ectopic) in cell lines expressing control or *RMRP* expression vectors. Promoters used to express *RMRP* are indicated. The relative intensity of *RMRP* is noted below each panel. See Supplementary Fig. 14. **b**, RT-PCR for total *RMRP*. See Supplementary Fig. 15. **c**, Effects of suppressing hTERT on *RMRP* levels. A control shRNA (sh-GFP) or 2 different *hTERT*-specific shRNAs were stably introduced into HeLa cells. **d**, Effects of *RMRP* mutants on *RMRP* levels. RT-PCR was used to detect *RMRP* levels in (**c, d**). **e**, Detection of small RNA species in human cells. Northern blotting to detect small RNAs (22 nt in length) using antisense (left panel) and sense (right panel) probes derived from nt 21–40 of *RMRP*. **f, g**, Analysis of the termini of the small RNA species identified in (**e**). Total RNA was incubated with the indicated enzyme (**f**), or oxidation-β-elimination reactions (**g**) were performed. Northern blotting was performed with antisense probe. CIP = calf intestinal phosphatase. PNK = polynucleotide kinase. ATP-indicates samples lacking ATP.
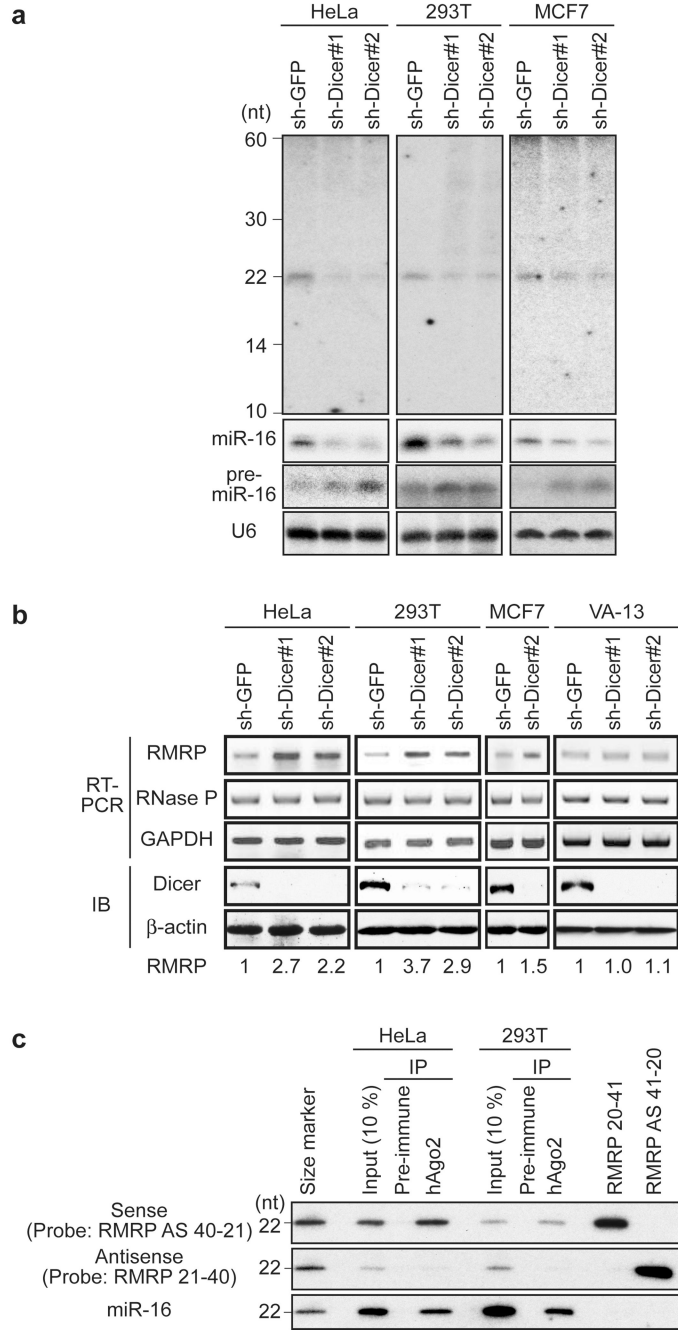
Figure 5. Production of *RMRP*-derived endogenous siRNAs depends on Dicer
a, Effect of suppressing Dicer on *RMRP*-derived small RNAs. Northern blotting was performed to detect [1] small RNAs using the antisense strand of *RMRP* as a probe in the indicated cells expressing control shRNA (sh-GFP) or *Dicer*-specific shRNAs (sh-Dicer #1 and sh-Dicer #2), [2] pre-miR-16 and mature miR-16 using a miR-16 specific probe, and [3] U6 RNA. See Supplementary Fig. 16. **b**, RT-PCR for total *RMRP* from cell lines expressing control shRNA or *Dicer*-specific shRNAs. The relative intensity of *RMRP* is noted at the bottom of the panel. **c**, *RMRP*-derived small RNAs are associated with Ago2. Human Ago2

immune complexes were isolated using anti-hAgo2-specific antisera or pre-immune sera, and small RNAs were detected by Northern blotting. Blotting of oligonucleotides (RMRP 20–41 and RMRP AS 41-20) is also shown.