

Generation of a mouse model for studying the role of upregulated RTEL1 activity in tumorigenesis

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Abstract Regulator of telomere length 1 (RTEL1) is a DNA helicase protein that has been demonstrated to be required for the maintenance of telomere length and genomic stability. It has also been found to be essential for DNA homologous recombination during DNA repairing. Human *RTEL1* genomic locus (20q13.3) is frequently amplified in multiple types of human cancers, including hepatocellular carcinoma and gastrointestinal tract tumors, indicating that upregulated RTEL1 activity could be important for tumorigenesis. In this study, we have developed a conditional transgenic mouse model that overexpress mouse *Rtel1* in a Cre-excision manner. By crossing with a ubiquitous Cre mouse line, we further demonstrated that these established *Rtel1* conditional transgenic mice allow to efficiently and highly express a functional *Rtel1* that is able to rescue the embryonic defects of *Rtel1* null mouse allele. Furthermore, we demonstrated that more than 70% transgenic mice that widely overexpress *Rtel1* developed liver tumors that recapitulate many malignant features of human hepatocellular carcinoma (HCC). Our work not only generated a valuable mouse model for determining the role of RTEL1 in the development of cancers, but also provided the first genetic evidence to support that

amplification of *RTEL1*, as observed in several types of human cancers, is tumorigenic.

Keywords RTEL1 · Mouse transgenic · ES cells · Cre · Overexpression · Hepatocellular carcinoma (HCC)

Introduction

RTEL1 is a newly identified DNA helicase that has been shown to be essential for the maintenance of telomere length and genomic stability (Barber et al. 2008; Ding et al. 2004; Youds et al. 2010). It belongs to the DEAH subfamily of the Superfamily 2 (SF2) helicases and is further classified as a member of DinG, Rad3-related DNA helicase with 5′–3′ directionality (Ding et al. 2004; Fairman-Williams et al. 2010). RTEL1 was identified in an effort to search for the essential factors that govern the telomere length diversity in mammalian species. In mice, *Rtel1* gene is located on distal chromosomal 2q region that has previously been shown to be involved in the regulation of mouse species-specific telomere length (Zhu et al. 1998). Mice with *Rtel1* deficiency have global telomere loss and multiple genomic instabilities, such as chromosome fusions and breaks (Ding et al. 2004), demonstrating that RTEL1 has an important role in the regulation of telomere length and genomic stability.

More recently, RTEL1 was also found to be required for DNA homologous recombination (HR)

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(Barber et al. 2008; Youds et al. 2010). HR is one of the major pathways to maintain genomic stability and is involved in the repair of complex DNA damage, including DNA double-strand breaks (DSBs), inter-strand crosslinks and DNA gaps. The function of HR in the recovery of stalled and collapsed replication forks is also critical to faithfully duplicate and segregate the genome to daughter cells. Moreover, HR has also been demonstrated to be essential for accurate chromosome segregation during meiosis (see a recent review by Heyer et al. 2010). One critical step in HR is to form a D-loop structure by the invasion of RAD51-single strand DNA filament into homologous DNA template (Heyer et al. 2010; Holthausen et al. 2010; San Filippo et al. 2008). Within D-loops, the invaded DNA strand serves as a primer for DNA synthesis, and the synthesized DNA strand is subsequently displaced and annealed to the other processed DNA end. This SDSA (synthesis-dependent strand annealing) repair pathway leads to yield a non-crossover repair product that can avoid crossover and thus the possibility of deleterious genome rearrangements (Heyer et al. 2010). Therefore, SDSA is considered as the predominant recombinational repair pathway in somatic cells. RTEL1 was demonstrated to promote SDSA by the disruption of D-loops in vitro (Barber et al. 2008). Consistent with this biochemical activity, depletion of RTEL1 in human cells was found to have a fourfold increase in DSB-mediated intra-chromosomal recombination (Barber et al. 2008), and *C. elegans* with *Rtell* mutation showed significantly increased crossover recombination during meiosis (Barber et al. 2008; Youds et al. 2010). These results strongly indicate that RTEL1 has an important function in anti-recombination. Since telomeres can form a large duplex loop (T-loops) that largely resembles the D-loops during HR (Griffith et al. 1999), this function of RTEL1 could also be essential for the replication of telomeres. In the absence of RTEL1, telomeric T-loops could not be efficiently resolved, leading to large telomeric deletions and other telomere dysfunction phenotypes as observed in *Rtell* null or *Rtell* knockdown cells (Ding et al. 2004; Sfeir et al. 2009).

Taken together, several recent studies have established an essential role of RTEL1 DNA helicase in the maintenance of telomere length and genomic stability. Given that telomere dysfunction is dramatically mutagenic and plays an important role in tumor initiation and progression (Maser and DePinho 2002),

RTEL1 deficiency is expected to have a tumorigenic function. However, as indicated by human genetic data that *RTEL1* genomic locus (20q13.3) is frequently amplified in several human cancers (Bai et al. 2000; Katoh et al. 2006; Muleris et al. 1995; Pitti et al. 1998; Wong et al. 1999), upregulated RTEL1 activity could also be important for tumorigenesis. High RTEL1 activity may also induce genomic instability by preventing HR (Uringa et al. 2011), leading to the formation of tumor. As a first approach to determine the role of increased RTEL1 activity in tumorigenesis, in this study, we established a transgenic mouse model that conditionally overexpress mouse *Rtel1*. We further demonstrated that this established mouse model can serve as a valuable genetic tool to assess the tumorigenic function of upregulated RTEL1 activity in vivo. With this mouse model, we showed for the first time that upregulated RTEL1, as observed in human cancers, is tumorigenic and is able to initiate the formation of hepatocellular carcinoma (HCC) in mice.

Methods

Generation of *Rtel1* conditional transgenic mice

The mouse *Rtel1* cDNA with a 3' inserted V5 tag was cloned to a conditional transgenic vector as described previously (Ding et al. 2002). The transgenic vector was verified by restriction enzymes and sequencing.

The transgenic vector was linearized with *SacII* and then electroporated into R1 mouse embryonic stem (ES) cells. The transfected ES cells were selected with G418 (250 $\mu\text{g/ml}$). The G418-resistant ES clones were screened by Southern blot analysis using a *neo* cDNA probe. The genomic DNA was digested with multiple restriction enzymes with unique digestion in the transgenic vector (not in the probe region). Two clones with overall, nonmosaic LacZ expression were used to generate chimeric mice by ES cell \leftrightarrow diploid embryo aggregation. The transmitting chimeric males were crossed with 129S1 inbred and ICR outbred females to produce hemizygous transgenic offsprings, which were then further bred with *EIIa-Cre* line (JAX lab, Bar Harbor/Maine) to delete *loxP* flanked *β -geo* cassette. The resulted mouse allele, termed *pCX-Rtel1-V5*, was maintained in a 129S1/C57B6 background. All mouse experiments were performed in

accordance with procedures approved by the University of Manitoba Animal Care and Use Committee.

A PCR based genotyping method was applied to genotype *pCX-Rtel1-V5* transgenic mice with the primers: forward (5'-TTCGGCTTCTGGCGTGTGACC-3') and reward (5'-CTCGGAGATGTTGTTGCCAGGC-3'). One pair of primers: forward (5'-AGCC TCTGCTAACCATGTTCA-3') and reward (5'-TGG GATAGGTTACGTTGGTGT-3'), were used to genotype *Rtel1* conditional transgenic mice.

Immunohistochemistry

Mice were transcardially perfused with 4% paraformaldehyde in PBS buffer (pH 7.4). Tissues were dissected, and then post-fixed in the same fixative overnight. After a brief washing with PBS, the fixed tissues were embedded in paraffin. 5 µm Paraffin-embedded mouse tissue sections were pretreated with Retrieval solution (Dako) for 20–30 min at 98°. Pretreated slides were blocked with either mouse IgG blocking reagent (Vector Laboratory) or serum free blocking reagent (Dako), and then incubated with primary antibodies overnight at 4°. Primary antibodies used were rabbit anti-V5 (Abcam, dilution 1:1,000), mouse anti-Ki67 (BD Pharmingen, dilution 1:100), mouse anti-β-catenin (BD Pharmingen, dilution 1:200), and rabbit anti-AFP (Dako, dilution 1:500). After washing with TBS with 0.1% Tween20 buffer, sections were incubated with biotinylated anti-rabbit or anti-mouse antibody for 60 min at room temperature. The sections were washed 3 times with TBS-0.1% Tween20 buffer and incubated with avidin-biotin-peroxidase complex (Vector Laboratory), according to the manufacturer's protocol. Color was developed through the use of metal 3,3'-diaminobenzidine tetrachloride for 5 min at room temperature.

Western blot analysis

For western blots, mouse liver and lung tissues were homogenized with ice-cold RIPA buffer. 30 µg lysate was separated by 8% SDS-PAGE and transferred to nitrocellulose membrane, which was blocked with 5% de-fatted milk and incubated with mouse anti-V5 antibody (dilution 1:3,000, Invitrogen). Protein was detected using the enhanced chemiluminescence system (Amersham Life Science).

Results and discussion

To develop a mouse model that conditionally overexpress mouse *Rtel1*, we applied a transgenic strategy as described previously (Ding et al. 2002). Briefly, a strong and ubiquitous promoter, *pCAGGS* (a chicken β-actin promoter with the upstream CMV enhancer) (Niwa et al. 1991), was used to drive the expression of a *loxP* flanked β-*geo* cassette which is followed by mouse *Rtel1* cDNA. The *Rtel1* expression can be turned on only after Cre-mediated excision of *loxP* flanked β-*geo* (Ding et al. 2002) (Fig. 1a). To facilitate the detection of *Rtel1* protein in transgenic mice, a V5 tag was inserted 3' downstream of the last coding codon of *Rtel1* cDNA. This addition will not affect the cellular function of *Rtel1* as demonstrated by the previous studies (Barber et al. 2008; Ding et al. 2004). The transgenic vector (Fig. 1a) was electroporated into R1 ES cells. Clones with single copy insertion were identified by Southern hybridization (Fig. 1b).

We applied the ES cell ↔ diploid aggregation assay to create chimeras from two independently targeted ES clones. Both clones gave germline transmission of transgenic allele. The transmitting chimeric males were crossed with 129S1 inbred and ICR outbred females to produce hemizygous transgenic offspring that appeared normal and were fertile. Without Cre recombinase-mediated excision, these transgenic mice only expressed LacZ detected by X-gal staining (data not shown). To determine *Rtel1* expression upon Cre-mediated excision, we crossed *Rtel1* conditional transgenic mice with *EIIa-Cre* transgenic mice (a ubiquitous Cre transgenic line). The resulted mouse allele, termed *pCX-Rtel1-V5*, was postnatal viable and fertile. We further applied immunohistochemistry (IHC) to determine the expression of *Rtel1-V5* protein in *pCX-Rtel1-V5* adult mice with anti-V5 antibody. As shown in Fig. 2, strong nuclear-localized *Rtel1-V5* protein was found to be widely presented in most tissues, such as liver, lung, heart, kidney, intestine and pancreas. We also applied western blot to demonstrate that a 150 kd full length *Rtel1-V5* protein was expressed in *pCX-Rtel1-V5* mouse tissues (Fig. 2g). This highly and widely expression of *Rtel1-V5* was consistently observed in two independent conditional transgenic lines upon *EIIa-Cre*-mediated excision, demonstrating that our established *Rtel1* conditional transgenic mice allow to efficiently overexpress *Rtel1* in vivo.

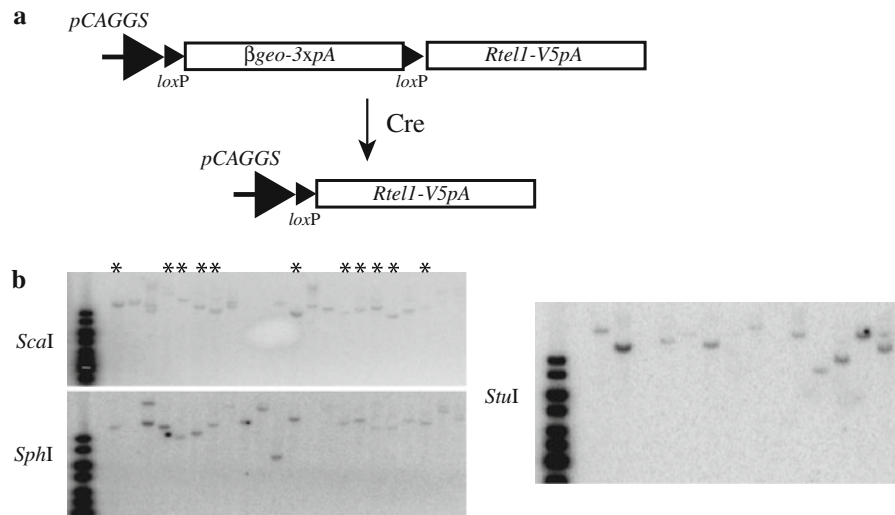


Fig. 1 A transgenic approach for establishing *Rtell* conditional transgenic mice. **a** Schematic representation of transgenic vector. *pCAGGS* promoter (the fusion of a chicken β -actin promoter with upstream CMV enhancer) is followed by a *loxP* flanked β -*geo* with three SV40 polyadenylation signals (pA) and a mouse *Rtell-V5* transgene. Prior to Cre excision, this transgenic vector expresses only β -*geo*. The *Rtell-V5* expression is turned on after Cre-mediated excision. **b** Southern blot analysis of transgene integration in R1 ES cells using a *neo*

cDNA probe. The genomic DNA was digested with restriction enzymes with unique digestion in the transgenic vector (not in the probe region), i.e.. *ScaI* and *SphI*. The potential ES clones with a single copy and single site integration of the transgene (labeled by asterisks) was further analyzed by another restriction enzyme with unique digestion, *StuI*. All these diagnostic digests were consistent with a single copy and single site integration of the transgene

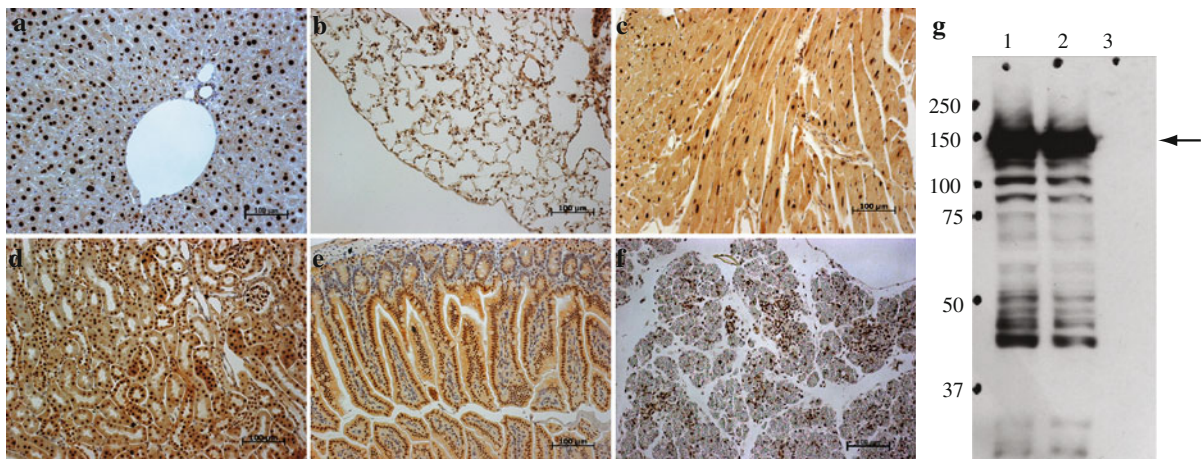


Fig. 2 Characterization of *Rtell-V5* protein expression in *pCX-Rtell-V5* mice. **a–f** IHC with anti-V5 antibody on mouse tissues collected from 2 months old *pCX-Rtell-V5* mice. **a** liver; **b** lung; **c** heart; **d** kidney; **e** intestine; **f** pancreas. **g** western blot with anti-V5 antibody on mouse tissue lysates harvested from

pCX-Rtell-V5 mice. 1 liver; 2 intestine; 3 liver from wild-type control. Arrow indicates a major detected protein band with 150 Kd that matches with the predicted molecular weight of full length *Rtell-V5* protein

To demonstrate that *Rtell-V5* protein expressed from *pCX-Rtell-V5* transgenic mice is functional, we bred *pCX-Rtell-V5* mice with *Rtell* null mouse allele. We previously showed that *Rtell*^{-/-} mice were

embryonic lethal, and died between E9.5 and E10.5 with multiple developmental defects that are characterized by neural tube defect (NTD), bulbous allantois, pericardial effusion and the deficiency of trophoblasts

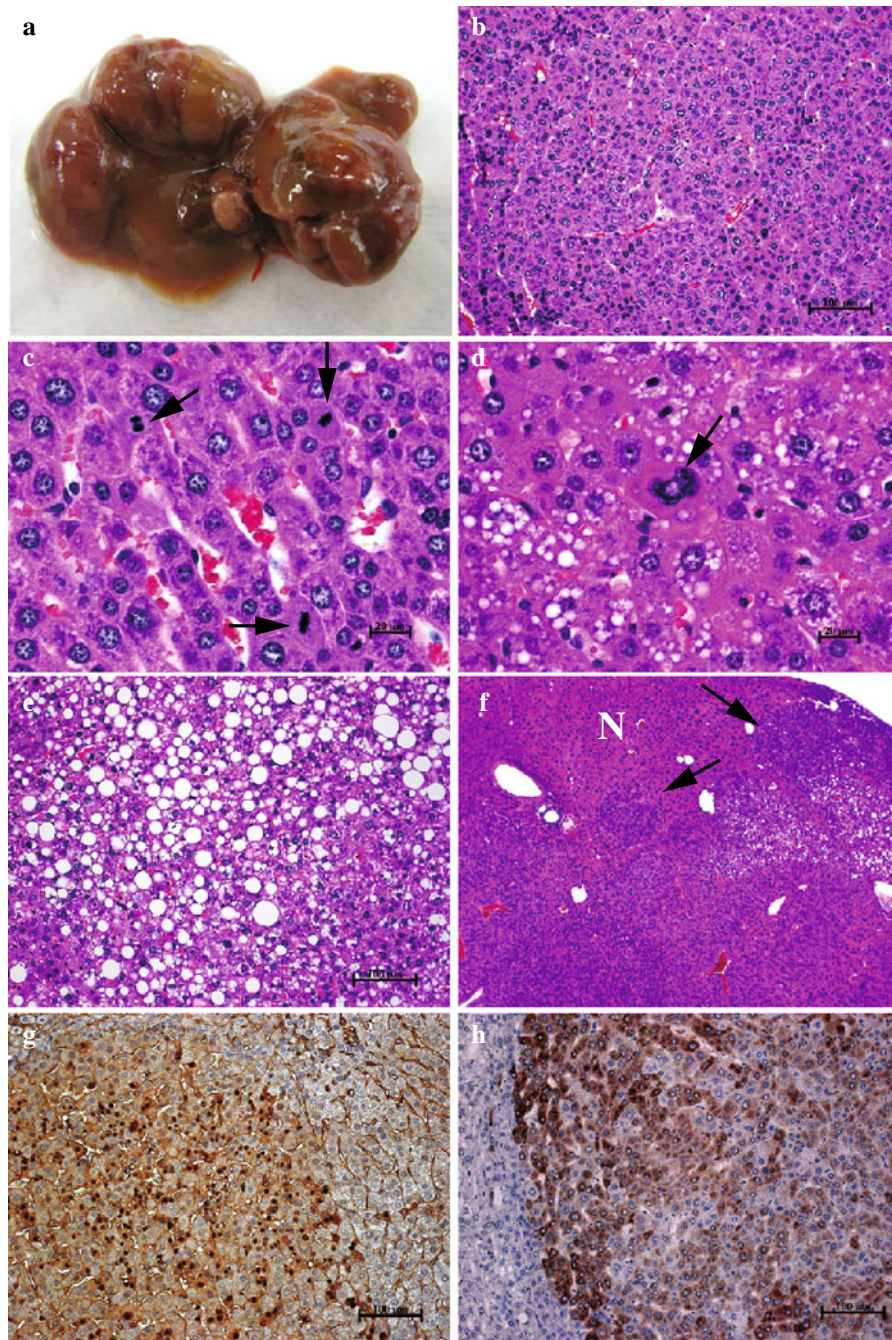


Fig. 3 Development of liver tumors in *pCX-Rtel1-V5* mice. **a** Representative image of liver from a 12-month old *pCX-Rtel1-V5* mouse. Note the widespread tumor nodules throughout the liver. **b–f** Histological characterization of liver tumors from *pCX-Rtel1-V5* mice reveals characteristics of hepatocellular carcinoma. **b** shows a trabecular growth of multiple layers of tumor cells. **c** shows increased mitotic figures (*arrows* indicate)

in tumor cells. **d** shows the multinucleated tumor cells (*arrow*). **e** shows the cellular pleomorphism with loss of cytoplasmic staining. **f** shows the invasion of nodular tumor cells (*arrows* indicate) to normal liver region (N). **g** IHC staining with anti- β -catenin antibody reveals nuclear accumulation of β -catenin in most tumor cells. **h** IHC staining with anti-AFP antibody. Some regions of tumor cells showed strong positive for AFP

(Ding et al. 2004; Wu et al. 2007). All these phenotypes were not observed in *Rtel*^{-/-}/*pCX-Rtel1-V5* E10.5 embryos. *Rtel*^{-/-}/*pCX-Rtel1-V5* mice were postnatally viable, and showed similar behaviors as other wild-type littermates. This clearly indicates that expressed *Rtel1-V5* protein in *pCX-Rtel1-V5* transgenic mice has the same cellular function as native *Rtel1*, which can completely rescue the developmental defects of *Rtel1* null mutants.

Lastly, we applied *pCX-Rtel1-V5* transgenic mice to determine whether upregulated *Rtel1* activity could induce the formation of tumors in vivo. For this purpose, we monitored a cohort of 22 mice (12 *pCX-Rtel1-V5* mice and 10 wild type age matched littermates) with a normal diet for a one-year period. At 12 months old, 8 out of 12 *pCX-Rtel1-V5* mice showed weakness and decreased body weight as compared to wild-type littermates. Post-mortem examination revealed these *pCX-Rtel1-V5* mice developed numerous discrete nodules throughout the livers (Fig. 3a). Upon histological analyses, these nodules were found to display many pathological characteristics of HCC, which include trabecular growth of multiple layers of tumor cells (Fig. 3b), tumor cells with high mitotic figures (Fig. 3c), cellular pleiomorphism with multinucleated cells (Fig. 3d) or with loss of cytoplasmic staining (Fig. 3e), and the invasion of nodular tumor cells into the surrounding parenchyma (Fig. 3f). Similar to some of human HCC, liver tumors from *pCX-Rtel1-V5* mice was also found to have increased β -catenin activity (as reflected by the nuclear accumulation of β -catenin in tumor cells) (Fig. 3g). Some regions of liver tumors from *pCX-Rtel1-V5* mice also showed strong positive for α -fetoprotein (AFP) (Fig. 3h), a diagnostic and prognostic marker in human HCC. This liver tumor phenotype was consistently present in two independent *pCX-Rtel1-V5* transgenic lines. None of wild-type control mice developed liver tumor at this developmental stage. All these indicate that overexpression of *Rtel1* in mouse hepatocytes can induce the formation of liver tumors that recapitulate many malignant features of HCC.

In summary, we have generated a conditional mouse model that highly and efficiently expresses *Rtel1* upon Cre-mediated excision. We further confirmed that *Rtel1-V5* protein expressed in this mouse model is functional. More importantly, we demonstrated that this established mouse model can serve as a valuable genetic tool to assess the tumorigenic

function of upregulated RTEL1 activity in vivo. With this model, we showed that increased expression of *Rtel1* in mouse hepatocytes induced the development of HCC (Fig. 3). This finding is consistent with human genetic data that amplification of RTEL1 genomic locus is not only a common genetic alteration in HCC, but also closely associated with its malignancy and progression (Guan et al. 2000; Katoh et al. 2006; Niketghad et al. 2001; Taniguchi et al. 2010; Wong et al. 1999). Therefore, our data indicate that upregulated RTEL1 activity could be a new genetic factor involved in liver carcinogenesis. Future mechanistic studies are required to provide insight into the nature of the genomic instability in these tumors.

Given that *RTEL1* gene is also frequently amplified in other human cancers, such as gastrointestinal tract tumors and breast cancers (Bai et al. 2000; Muleris et al. 1995; Pitti et al. 1998), our established *Rtel1* transgenic mouse model, which allows to overexpress *Rtel1* in a variety of cells or tissues, can also be applied to determine the role of RTEL1 in the development of these human cancers.

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