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RAPID COMMUNICATION

LncRNA-AP006284.1 promotes prostate cancer cell growth and motility by forming RNA-DNA triplexes and recruiting GNL3/SFPQ complex to facilitate RASSF7 transcription

Long noncoding RNAs (lncRNAs) are known to be involved in transcriptional regulation and their deregulation is associated with the development of human diseases such as cancer.[1,2] LncRNA can directly bind to purine-rich double-stranded DNA sequences through Hoogsteen base pairing to form an RNA-DNA triplex motifs and regulate gene expression.[3–5] However, its regulatory mechanisms and functions in tumors remain unclear. Here, we report that the LMNTD2 antisense RNA 1 (LMNTD2-AS1, also known as AP006284.1) is highly expressed in prostate cancer (PCa) and positively correlated with the expression of its adjacent coding gene N-terminal Ras-association domain family 7 (*RASSF7*). Mechanistically, AP006284.1 tethers to the RASSF7 promoter via RNA-DNA triplexes in *cis*-acting manner, which enhances chromatin accessibility and recruits the transcription factor complex GNL3/SFPQ to activate the expression of RASSF7, a repressor of the Hippo signaling pathway. Consistently, overexpression of either AP006284.1 or RASSF7 inactivated the Hippo signaling and malignant proliferation of PCa cells.

We performed differential expression analysis on the RNA-seq data of PCa tissues and normal tissues in the TCGA database (Fig. S1A). By analysis of expression patterns between differentially expressed lncRNAs and mRNAs, we identified lncRNA AP006284.1 is located upstream of the transcription start site of RASSF7 and has a co-expression relationship (Fig. S1B, C). AP006284.1 and RASSF7 are both highly expressed in cancer tissues in the PCa TCGA database (Fig. S1D). Kaplan–Meier analysis indicated that high AP006284.1 and RASSF7 expression had a worse prognosis in PCa patients, and the prognosis value of AP006284.1 appears much greater than RASSF7 (Fig. S1E). We further verified that AP006284.1 and RASSF7 were highly expressed in PCa tissues and showed a significant positive correlation (Fig. S1F, G). LncATLAS database and nucleo-cytoplasmic separation assay showed that the nuclear localization of AP006284.1 is a common phenomenon in various cancer cell lines (Fig. S1H, I). Furthermore, the changes in AP006284.1 expression could affect the expression of RASSF7 (Fig. 1A, S2A–F), so we speculated that AP006284.1 may be involved in the regulation of RASSF7 expression.

AP006284.1 and RASSF7 could promote PCa cell proliferation and migration, and inhibit apoptosis (Fig. S3A–C). Rescue experiments showed that overexpression of RASSF7 restored the effects caused by AP006284.1 knockdown (Fig. S3D, E), suggesting that AP006284.1 promoted PCa cell proliferation and inhibited apoptosis by positively regulating RASSF7 expression. Moreover, we confirmed that RASSF7 can inhibit YAP phosphorylation and promote nuclear distribution by interacting with ASPP1/2, thereby affecting the Hippo signaling pathway (Fig. S4A–K). Therefore, AP006284.1 may promote PCa cell growth and motility by blocking the Hippo signaling pathway.

We speculated that AP006284.1 could directly bind to GA-rich DNA sequences through Hoogsteen base pairing to form RNA-DNA triplexes, and regulated the expression of neighboring genes in *cis*. We used Triplexator software to predict the possible TFOs (triplex-forming oligonucleotides) of AP006284.1 and search potential TTSs (triplex target sites) within 5 kb upstream of RASSF7 that could form DNA-DNA-RNA triplexes with AP006284.1 (Fig. 1B; Fig. S5A). When we mutated the triplex-forming site with the highest

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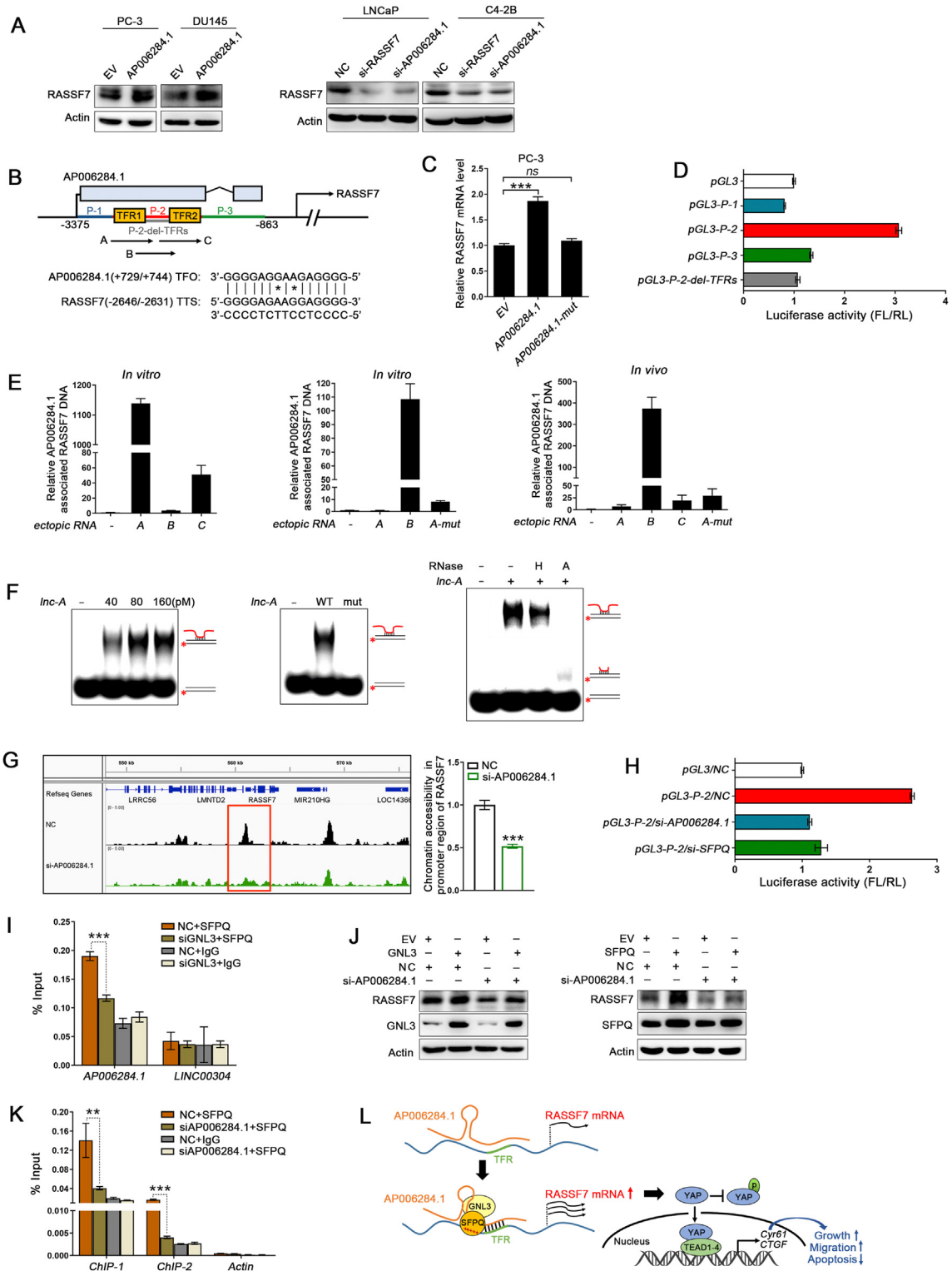

Figure 1 LncRNA-AP006284.1 forms RNA-DNA triplexes and recruits the GNL3/SFPQ complex to promote RASSF7 transcription. **(A)** Western blotting was performed to evaluate the effect of AP006284.1 on RASSF7 protein expression in PCa cells. **(B)** Schematic representation of the RASSF7 locus. The arrow marks the TSS of RASSF7, and AP006284.1 is located upstream of the transcription start site of RASSF7. The boxes represent potential triplex-forming regions (TFRs) (TFR1, -2646/-2631; TFR2, -1905/-1891). The arrowed lines illustrate derivatives of AP006284.1 (A, −2741/-2587; B, −2197/-2057; C, −2002/-1848) that were transcribed *in vitro* to capture the RASSF7 promoter. The highest-scoring TFO-TTS-forming sequence is indicated. **(C)** PC-3 cells were transfected with the AP006284.1 or AP006284.1 mutant plasmid, and RT-qPCR was used to detect the changes in RASSF7 mRNA levels. **(D)** The promoter activity of different fragments of RASSF7 (P-1, -3375/-2742; P-2, -2741/-1848; P-3, -1847/-864; P-2-del-TFRs,

score in lncRNA, the lncRNA failed to upregulate the expression of RASSF7 in PC-3 cells (Fig. 1C). Dual-luciferase experiments also showed that the RASSF7 promoter fragment containing TFR1/2 (triplex-forming region) promoted transcription of the luciferase reporter, whereas deletion of TFR in the region abolished the luciferase expression, suggesting the importance of the triplex forming in transcriptional activation (Fig. 1D).

To verify the triplexes forming, we constructed the A, B, C and A-mut fragments of AP006284.1 labeled with biotin to perform triplex pulldown assay, we found that fragments A and C formed complexes with the promoter region of RASSF7, with the exception of fragment B. While the amount of target DNA enriched by the TFO mutant RNA fragments (A-mut) was significantly reduced (Fig. 1E; Fig. S5B). We also monitored the formation of triplexes through electrophoretic mobility shift assays (EMSAs). The results showed that the amount of formed DNA-RNA triplexes increased with an increasing RNA molar concentration and triplexes were undetected after TFO mutation, a shorter complex fragment was observed upon treatment with RNase A (Fig. 1F). In addition, the dissociation constant suggest that the triplex complex had a certain degree of stability (Fig. S5C, D). These results provided strong evidence that AP006284.1 formed an RNA-DNA triplex structure in the RASSF7 promoter region.

Furthermore, we employed ATAC-seq to explore the effect of AP006284.1 on chromatin accessibility. ATAC-seq peaks and ATAC-qPCR assays showed that knocking down AP006284.1 reduced chromatin accessibility within RASSF7 regulatory regions (Fig. 1G). We speculated that the RNA-DNA triplex structure could promote RASSF7 expression through chromatin remodeling.

To study whether AP006284.1 could also recruit proteins to promote transcription, we analyzed AP006284.1 binding proteins by RNA pulldown and selected candidate proteins for further verification (Fig. S6A—C). The results confirmed that the binding of GNL3 to AP006284.1 was specific, GNL3 significantly affected the expression of RASSF7 but did not affect AP006284.1 (Fig. S6D—F).

Considering that GNL3 is not a transcription factor, it may act as a transcriptional cofactor. Co-IP and RNA pulldown assays verified that transcription factor SFPQ can interact with GNL3 and AP006284.1, and SFPQ promoted the expression of RASSF7 (Fig. S6G—J). Furthermore, dual-luciferase experiments showed that knocking down lncRNA-AP006284.1 or SFPQ significantly weakened the transcriptional activity of the RASSF7 promoter (Fig. 1H), indicating that AP006284.1 and SFPQ were critical for the transcriptional activity of the RASSF7 promoter region.

To validate the regulatory relationship between lncRNAs, the GNL3/SFPQ complex and RASSF7, we first confirmed the interaction of AP006284.1 with GNL3 and SFPQ by RNA immunoprecipitation (RIP) (Fig. S6K). However, SFPQ binding to AP006284.1 was weakened when GNL3 was knocked down, indicating that the association of SFPQ and AP006284.1 might depend on GNL3 (Fig. 1I; Fig. S6L). Knocking down lncRNA-AP006284.1 could restore the promoting effect of high expression of GNL3 or SFPQ on the expression of RASSF7 (Fig. 1J; Fig. S6M, N). In addition, we predicted the possible binding sites of SFPQ in the RASSF7 promoter through the GTRD website (Fig. S6O). Chromatin immunoprecipitation (ChIP) assays suggested that the amplified sequences of the ChIP-1 and ChIP-8 primers might contain SFPQ binding sites (Fig. S6P). Interestingly, we found that the SFPQ binding site was a GA-rich sequence and was close to the triplex formation sites (Fig. S6Q). As a result, we speculated that the formation of a triplex might promote the binding of SFPQ in the RASSF7 promoter and activate the transcription of RASSF7. Consistent with this point, ChIP results showed that the association of SFPQ with the promoter region of RASSF7 was reduced after AP006284.1 knockdown, suggesting that the AP006284.1/GNL3/SFPQ complex is important in transcriptional regulation of RASSF7 (Fig. 1K).

In this study, we delved into the mechanism by which lncRNA triplex formation regulates gene expression and unveiled an undocumented PCa oncogenic mechanism. We hypothesize that AP006284.1 forms RNA-DNA triplexes with the RASSF7 promoter, which enhances chromatin accessi-

—2631/-1848) was measured using dual-luciferase reporter assays. The empty pGL3 vector was set to 1. FL, firefly luciferase; RL, Renilla luciferase. **(E)** AP006284.1 binds to RASSF7 via Hoogsteen base pairing. The DNA fragments containing TFRs (—2741/-1848) were incubated with the biotin-labeled RNA fragments A, B, C, A-mut *in vitro*, or transfect RNA fragments into LNCaP cells. Specific primers (—2551F/-2442R) were used to detect RNA-associated DNA by qPCR. **(F)** We incubated 1 pmol double-stranded 5′FAM-labeled oligonucleotide comprising TFR1 (—2621/-2666) with a molar excess (40-, 80-, and 160-fold) of RNA-A or 80-fold molar excess of RNA-A-mut at 37 °C for 1 h, treated with 0.5 U RNase H or with 0.5 ng RNase A for 30 min at room temperature, the formation of RNA-DNA triplexes was monitored by EMSA. **(G)** Chromatin accessibility at the RASSF7 locus was assessed using the Integrative Genomics Viewer. The red box indicates the chromatin peak with decreased accessibility in response to AP006284.1 knockdown (left). qPCR assays were also to evaluate changes in chromatin accessibility at RASSF7 loci (right). **(H)** Dual-luciferase assay detects the transcriptional activity of the promoter region of RASSF7 after knocking down lncRNA-AP006284.1 or SFPQ in C4—2B cells. **(I)** RIP assays were performed in C4—2B cells to detect the interaction between GNL3/SFPQ and AP006284.1. IgG was used as a control, LINC00304 used as a negative control. The result shows the percentage of input RNA. **(J)** C4—2B cells were cotransfected with plasmid and siRNA for the recovery assay, and the expression of RASSF7 was examined by Western blotting. **(K)** ChIP-PCR was used to detect the effect of AP006284 on SFPQ binding in the promoter region of RASSF7. IgG was used as a control. The result shows the percentage of input DNA. **(L)** Model illustrating the function of AP006284.1.

bility and recruits the transcription factor complex GNL3/SFPQ to positively regulate the expression of RASSF7. In turn, it inhibits YAP phosphorylation, promotes nuclear entry, initiates the expression of downstream target genes in the Hippo signaling pathway, thereby promoting PCa cell proliferation and motility (Fig. 1L).

Author contributions

Conceptualization, Yali Lu and Yao Li; methodology, Yali Lu and Yan Lin; software, Xiaoyang Zhang and Zhe Kong; validation, Yali Lu and Jun Yan; formal analysis, Yali Lu; investigation, Yali Lu and Jun Yan; resources, Yan Lin; data curation, Yali Lu, Yao Li and Shimin Zhao; writing—original draft preparation, Yali Lu; writing—review and editing, Yao Li and Shimin Zhao; visualization, Chenji Wang; supervision, Yao Li and Shimin Zhao; project administration, Yan Huang; funding acquisition, Yao Li and Lu Zhang. All authors have read and agreed to the published version of the manuscript.

Conflict of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.04.005.

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