



RAPID COMMUNICATION

X26nt-mediated recruitment of eIF4A2 facilitates CCND1 translation to drive endothelial cell cycle progression

Dysregulation of cell cycle control plays a pivotal role in tumor progression. Acting as a crucial regulator of the G1/S phase transition, dysregulation of cyclin D1 (*CCND1*) can disrupt the delicate balance between cell proliferation and quiescence, leading to uncontrolled cell cycle progression.¹ Endoplasmic reticulum stress influences the cell cycle through myriad pathways. During endoplasmic reticulum stress, the inositol-requiring enzyme 1 alpha (*IRE1α*) can be activated, and its endoribonuclease domain cleaves unspliced X-box binding protein 1 (*XBP1u*) mRNA, generating a spliced *XBP1* (*XBP1s*) and a non-coding RNA, whose length is 26 nt (named exosomal 26-nt-long ncRNA, X26nt).^{2,3} Studies have demonstrated that *XBP1s* can induce the expression of pro-angiogenic factors, promoting the formation of new blood vessels.^{4,5} Our previous study demonstrated that gastric cancer exosome-derived X26nt induces tumor-associated angiogenesis via promoting endothelial cell migration targeting vascular endothelial-cadherin.² However, the exact mechanisms by which *XBP1* splicing regulates endothelial cell proliferation are still not fully understood.

When synchronizing human umbilical vein endothelial cells (HUVECs) at the late G1 phase by double thymidine block and then releasing it to re-enter the cell cycle, we observed an increase in the number of cells in the G2/M phase at 6 h and 9 h after release (Fig. S1A). We collected samples at the same indicated time points and performed PCR and Western blot analysis. The results showed that the expression level of the spliced form of *XBP1* (*XBP1s*) was consistently increased at 6 h and 15 h (Fig. 1A, B; Fig. S1B). Vascular endothelial growth factor (VEGF) induced endoplasmic reticulum stress resulting in up-regulation of the cell cycle-related protein *CCND1*. Upon *XBP1* knockdown or

inhibition of *XBP1* splicing (*IRE1α* knockdown), VEGF-induced up-regulation of *CCND1* was eliminated (Fig. S2). Based on this, we hypothesized that *XBP1* splicing could play a crucial role in mediating cell cycle in HUVECs. However, further experiments revealed that over-expression of *XBP1s* (Ad-*XBP1s*) suppressed the expression of *CCND1* and other cell cycle-related proteins in HUVECs (Fig. 1C, D; Fig. S3). These findings indicated that *XBP1s* may not be the key factor regulating cell cycle-related protein *CCND1*. Thus, we focused on X26nt and hypothesized that it could act as a mediator of the cell cycle. Knockdown of *XBP1* significantly reduced cell proliferation, and its X26nt, not *XBP1s*, could restore the proliferation capability (Fig. S4).

Our former studies showed that X26nt could promote the proliferation, migration, and tube formation of endothelial cells.³ To investigate the role of X26nt in the proliferation of HUVECs, we conducted loss-of-function assays by adding X26nt to *XBP1s* knockdown cells and observed that X26nt significantly reversed the cell cycle arrest in the G1/G0 phase induced by *XBP1s* knockdown cells (Fig. 1E; Fig. S5A). We also constructed a mutant S26nt as a negative control for X26nt and examined the protein levels of *CCND1* and specificity protein 1 (SP1) in cytoplasmic and nuclear fractions within S26nt or X26nt overexpression (Fig. 1F). The findings confirmed that X26nt genuinely enhanced the cytoplasmic expression of *CCND1*. The expression of X26nt was down-regulated in Ad-*XBP1s*, which indicated that X26nt was the effective factor in promoting the expression of *CCND1* and cell cycle (Fig. S5B). To explore the mechanism underlying the promotion of *CCND1* by X26nt, we treated HUVECs with actinomycin D and cycloheximide. Actinomycin D can inhibit RNA formation and therefore reflect the efficiency of transcription. Cycloheximide can modulate translation efficiency by inhibiting protein production. The results demonstrated that X26nt could still enhance the expression of *CCND1* after RNA transcription

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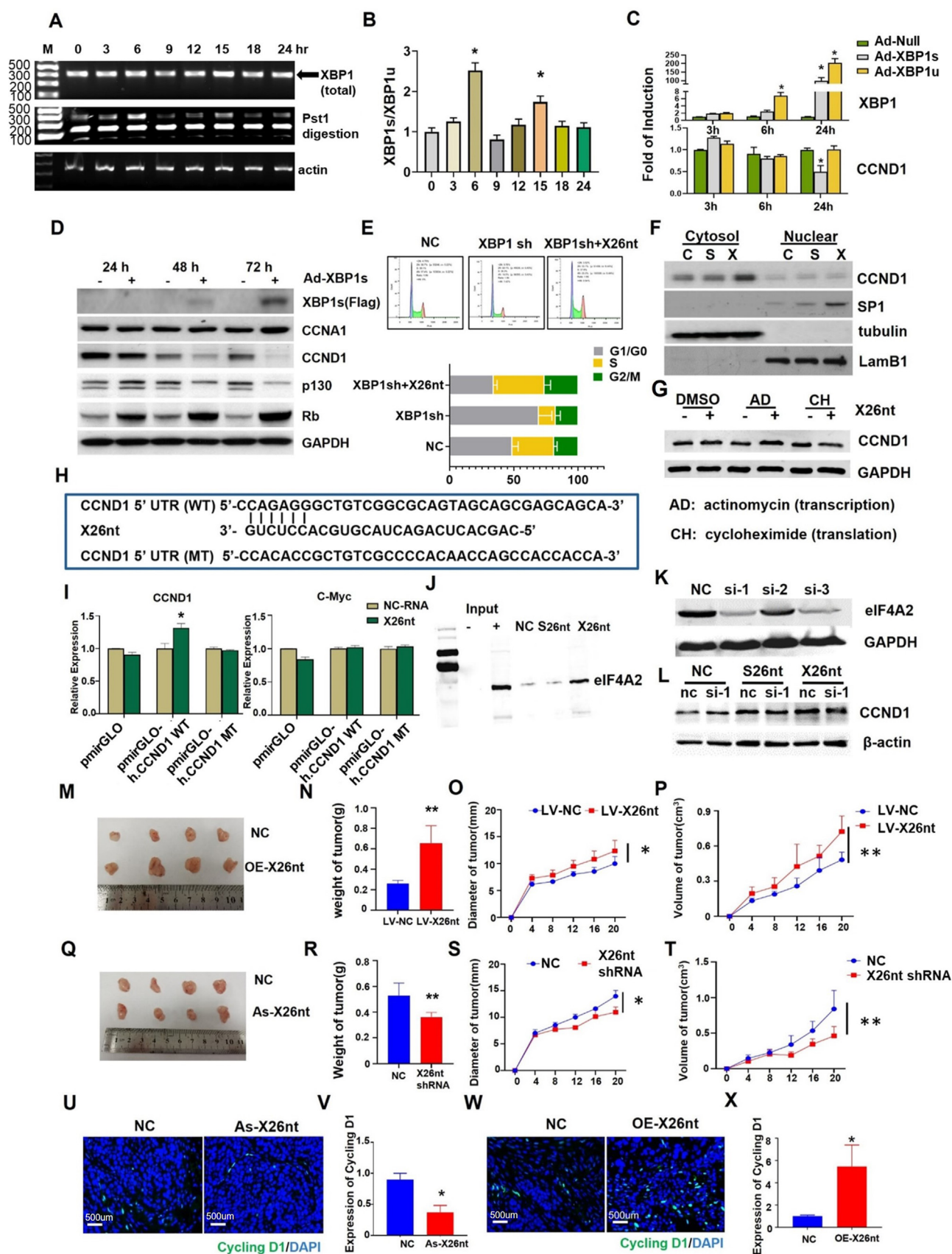


Figure 1 X26 nt can increase *CCND1* through binding to the 5' UTR of *CCND1* mRNA mediated by EIF4A2, facilitate the proliferation of HUVECs, promote tumor angiogenesis, and therefore contribute to tumor progression. (A) HUVECs were synchronized at

was inhibited, but its effect was lost when protein translation was inhibited (Fig. 1G; Fig. S6A). This suggests that X26nt may regulate the translation efficiency of *CCND1*.

We conducted further investigations to understand the mechanism underlying X26nt interacting with *CCND1*. Using bioinformatics tools, we predicted the binding site of X26nt on 5' UTR of *CCND1* mRNA (Fig. 1H). Subsequently, a dual-luciferase reporter assay demonstrated that X26nt significantly enhanced the activity of firefly luciferase reporter containing the wild-type 5' UTR of *CCND1* mRNA. Furthermore, this effect was abolished when the predicted binding site in 5' UTR was mutated. Interestingly, the expression level of *C-MYC* was not affected by either X26nt or mutated 5' UTR of *CCND1* mRNA (Fig. 1I). To further validate the direct protein target of X26nt in proliferating endothelial cells, we synthesized biotinylated X26nt (Bio-X26nt), incubated it with VEGF-treated endothelial cells, and performed affinity purification using streptavidin beads. Mass spectrometry analysis demonstrated that eukaryotic translation initiation factor 4A2 (EIF4A2) exhibited a higher score in the VEGF-treated group, indicating its potential involvement in X26nt-mediated effects. Importantly, EIF4A2 is known to play a critical role in protein translation. This observation was further substantiated by Western blot analysis, which displayed the formation of a complex involving X26nt and EIF4A2 (Fig. 1J; Fig. S7). To substantiate that X26nt could trigger *CCND1* via its interaction with EIF4A2, we utilized siRNA to suppress EIF4A2 (Fig. 1K). The findings revealed that the capacity of X26nt to enhance *CCND1* expression was nullified when EIF4A2 was suppressed, corroborating our initial hypothesis (Fig. 1L; Fig. S6B).

Our former research also found that X26nt was significantly higher in gastric cancer serum and tissues, and X26nt accelerated tumor angiogenesis.³ Furthermore, we found that the levels of X26nt were remarkably higher in gastric

cancer cells than in HUVECs (Fig. S8). To ascertain the role of X26nt in promoting tumor growth, a tumor-implanted model was constructed by subcutaneously injecting loss-of-function (via shRNA lentivirus-mediated knockdown of X26nt) and gain-of-function (via adenoviral transfer of X26nt) BGC-823 cells (human gastric adenocarcinoma cell line) into the back of the armpit of nude mice. Analysis of changes in tumor weight and volume trends revealed that X26nt overexpression promoted tumor growth (Fig. 1M–P), while its knockdown had the opposite effect (Fig. 1Q–T). Additionally, we noted that X26nt knockdown resulted in a decrease in *CCND1*, while inversely, X26nt overexpression led to an increase in *CCND1* (Fig. 1U–X). Consequently, we concluded that X26nt could increase *CCND1* expression and promote tumor growth *in vivo*.

Our former study also revealed that X26nt could inhibit the expression of vascular endothelial-cadherin and enhance the migration and tube formation abilities of endothelial cells.³ Our subsequent results confirmed that loss of X26nt induced cell cycle arrest in the G1/G0 phase. Our discovery showed that X26nt could enhance the translation efficiency of *CCND1* by binding to the 5' UTR of *CCND1* mRNA, with the involvement of EIF4A2. This could promote the cell cycle progression of HUVECs in the late G1/S phase. *In vivo* experiments further confirmed that X26nt accelerated the expression of *CCND1* and tumor growth. As far as we are concerned, there is no evidence showing that the increasing level of X26nt has a direct effect on the cell cycle. In our prior research, we did observe that the levels of X26nt were significantly elevated in the serum from gastric cancer patients compared with normal serum. Hence, we propose that the overexpressed X26nt might be secreted via gastric cancer cell-derived exosomes and subsequently internalized by HUVECs.³ This could lead to the proliferation and migration of endothelial cells,

the late G1 phase by double thymidine block, and then released to re-enter the cell cycle. Cells were harvested at the time indicated after release. PCR analysis of *XBP1* (total) and *XBP1s* expression after release at different times ($n = 3$). (B) Quantitative analysis *XBP1s/XBP1u* rate of (A). (C) HUVECs were infected with Ad-*XBP1s* and Ad-*XBP1u*, and *XBP1* and *CCND1* expression were analyzed by PCR. Ad-null was introduced as a control. (D) Western blots of *XBP1s*, *CCNA1*, *CCND1*, P130, RB, and E2F2 of Ad-*XBP1s*-infected-HUVECs were collected at 24 h, 48 h, and 72 h. GAPDH was introduced as the loading control. (E) Flow cytometry analysis showed the cell cycle distribution of HUVECs transfected with negative control (NC) or *XBP1* shRNA, and cells transfected with *XBP1* shRNA cocultured with X26nt. Bar graphs show the percentages of HUVECs in the G0/G1, S, and G2/M phases. (F) Western blot analysis of cytosol and nuclear proteins collected from HUVECs cocultured with negative control, S26nt, or X26nt. TUBULIN and LAMB1 were introduced as the loading controls. (G) The protein expression of *CCND1* with the treatment of actinomycin (AD) and cycloheximide (CH) in HUVECs cocultured with or without X26nt. (H) The schematic showing potential X26nt binding sites in the *CCND1* mRNA 5' UTR. (I) The luciferase assay of HUVECs co-transfected firefly luciferase reporter plasmid containing either wild-type (pmirGLO-h. *CCND1* WT) or mutant *CCND1* mRNA 5' UTR with pmirGLO-h. *CCND1* mRNA cocultured with X26nt or NC-RNA. * $p < 0.05$. (J) Western blots of EIF4A2 in cells cocultured with NC, S26nt, and X26nt. (K) Immunoblots of EIF4A2 in HUVECs transfected with three different si-EIF4A2 plasmids. The scrambled EIF4A2 inhibitors were transfected as the control (nc). (L) Western blot analysis of *CCND1* in nc and si-1 HUVECs co-cultured with S26nt and X26nt. β -ACTIN was introduced as the loading control. (M–P) The formed tumors from BGC-823 cells transfected with X26nt-overexpressing (OE-X26nt) lentivirus and control lentivirus (Control) were isolated and compared. Analysis of tumor diameter, volume, and weight in each group is shown. (Q–T) The BGC-823 cells were subcutaneously injected into the BALB/c nude mice to create a tumor-implanted model. When the tumor appeared, X26nt antisense RNA (As-X26nt) plasmid and mock control (NC) were injected into the tumor every other day before the mice were sacrificed. Analysis of tumor diameter volume and weight in each group is shown. (U–X) Immunofluorescence staining of *CCND1*/DAPI in tumors treated with X26nt asRNA plasmid and NC, as well as X26nt OE lentivirus and control. * $p < 0.05$ and ** $p < 0.01$ versus the control. Columns, mean (B–G, I–L: $n = 3$; M–X, $n = 4$); bars, standard deviations. The data presented are representative or average of three independent experiments. X26nt, exosomal 26-nt-long ncRNA; *CCND1*, cyclin D1; EIF4A2, eukaryotic translation initiation factor 4A2; *XBP1*, X-box binding protein 1; *XBP1s*, spliced X-box binding protein 1; *CCNA1*, cyclin A1; E2F2, E2F transcription factor 2; LAMB1, laminin subunit beta 1.

thereby encouraging tumor angiogenesis (Fig. S9). Ultimately, X26nt may be a weapon for tumor cells to modify the tumor microenvironment, and it may serve as a potential target for tumor therapy.

CRediT authorship contribution statement

Xinghe Zhao: Visualization, Investigation. **Xiaocui Chen:** Methodology, Investigation, Formal analysis, Data curation. **Zheyi Chen:** Methodology, Investigation, Formal analysis, Data curation. **Lisong Shen:** Writing – review & editing, Writing – original draft, Resources, Funding acquisition, Formal analysis, Conceptualization. **Lingfang Zeng:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Junyao Yang:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Ethics declaration

The animal study protocol was approved by the Institutional Review Board of the Laboratory Animal Ethical and Welfare Committee Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (approval number: XHEC-F-2024-002, date of approval: 2024-01-30).

Conflict of interests

The authors declared no conflict of interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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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.2025.101667>.

References

1. Bustany S, Cahu J, Guardiola P, Sola B. Cyclin D1 sensitizes myeloma cells to endoplasmic reticulum stress-mediated apoptosis by activating the unfolded protein response pathway. *BMC Cancer*. 2015;15:262.
2. Yang J, Xu J, Danniel M, et al. The interaction between XBP1 and ENOS contributes to endothelial cell migration. *Exp Cell Res*. 2018;363(2):262–270.
3. Chen X, Zhang S, Du K, et al. Gastric cancer-secreted exosomal X26nt increases angiogenesis and vascular permeability by targeting VE-cadherin. *Cancer Sci*. 2021;112(5):1839–1852.
4. Martin D, Li Y, Yang J, et al. Unspliced X-box-binding protein 1 (XBP1) protects endothelial cells from oxidative stress through interaction with histone deacetylase 3. *J Biol Chem*. 2014;289(44):30625–30634.
5. Zeng L, Xiao Q, Chen M, et al. Vascular endothelial cell growth-activated XBP1 splicing in endothelial cells is crucial for angiogenesis. *Circulation*. 2013;127(16):1712–1722.

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