Caliciviral protein-based artificial translational activator for mammalian gene circuits with RNA-only delivery

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Synthetic RNA-based gene circuits enable sophisticated gene regulation without the risk of insertional mutagenesis. While various RNA binding proteins have been used for translational repression in gene circuits, the direct translational activation of synthetic mRNAs has not been achieved. Here we develop Caliciviral VPg-based Translational activator (CaVT), which activates the translation of synthetic mRNAs without the canonical 5' cap. The level of translation can be modulated by changing the locations, sequences, and modified nucleosides of CaVT-binding motifs in the target mRNAs, enabling the simultaneous translational activation and repression of different mRNAs with RNA-only delivery. We demonstrate the efficient regulation of apoptosis and genome editing by tuning translation levels with CaVT. In addition, we design programmable CaVT that responds to endogenous microRNAs or small molecules, achieving both cell-state-specific and conditional translational activation from synthetic mRNAs. CaVT will become an important tool in synthetic biology for both biological studies and future therapeutic applications.
Mammalian synthetic biology provides a promising tool for both biological studies and medical applications, as it enables the sophisticated regulation of endogenous and exogenous gene expressions. Various proteins (e.g., transcription factors, receptors, and apoptotic proteins) have been utilized as components of mammalian synthetic biology. Currently, most of these components are delivered to cells as a DNA format, but DNA format risks insertional mutagenesis, which may cause serious problems in therapeutic applications. As an alternative, synthetic messenger RNAs (mRNAs) are promising vectors because they do not cause insertional mutagenesis. In addition, synthetic mRNAs containing modified nucleosides can avoid nucleic acid-mediated induction of inflammation.

Although synthetic mRNAs are useful for mammalian synthetic biology, the available regulatory components for mRNA-based gene circuits (RNA circuits) are much less than those for DNA-based gene circuits (DNA circuits). Especially, while transcriptional activators are important components in DNA circuits, the direct activation of gene expression is difficult in RNA circuits. Rather, in current RNA circuits, the activation of gene expressions has been achieved by the repression of translational repressors by combining multiple RNA-binding proteins (RBPs). However, in such indirect methods, the number of necessary components tend to increase, complicating the system.

In this study, we develop Caliciviral VPG-based Translational activator (CaVT), which enables the direct translational activation of synthetic mRNAs in RNA circuits (Fig. 1). CaVT is composed of MS2 coat protein (MS2CP), which is a motif-specific RBP, and a caliciviral VPG protein, which acts as a substitute 5′-cap structure. CaVT binds to its target RNA motif in the 5′ UTRs of mRNAs without a canonical 5′-cap to directly activate their translation (Fig. 2a). The translational activation level can be modulated by changing the locations, sequences, and modified nucleosides of the target motif, and even translational repression can be achieved. This characteristic of CaVT enables different regulations of multiple mRNAs by a single protein (i.e., while one mRNA is translationally activated, another mRNA is translationally repressed through the RNA–protein interaction). Indeed, we simultaneously activate and repress the translation of proapoptotic and antiapoptotic proteins using bifunctional CaVT, making it possible to perform efficient cell-fate regulation with RNA-only delivery. CaVT-based RNA circuits efficiently regulate genome editing by the activation and repression of Cas9 and anti-CRISPR AcrIIA4 translation, respectively. In addition, we develop microRNA (miRNA)-responsive CaVT-based RNA circuits, in which the translation of two mRNAs are simultaneously upregulated and downregulated by one miRNA, by inserting the target site of the miRNAs into mRNAs that express CaVT. This miRNA-

![Fig. 1 Graphical abstract of translational regulation by CaVT.](https://doi.org/10.1038/s41467-020-15061-x)

CaVT can act as both a translational activator and repressor. While mRNAs containing a weak binding motif are translationally activated by CaVT, mRNAs containing a strong binding motif are translationally repressed by CaVT. Through these translational regulations, CaVT can be used to control reporter expression, apoptosis induction, and genome editing. The implementation of microRNA- or small molecule responsiveness to CaVT enables cell selective or conditional regulation of these biological phenomena.
responsive RNA circuit enables the cell-selective regulation of genome editing. Finally, we achieve small molecule drug-inducible translational activation by combining CaVT and drug-inducible hetero-dimerization domains, in which the translational activation level can be modulated by changing the concentration of the drug. CaVT provides a promising tool for mammalian synthetic biology and will help with the design of more user-friendly, cell-type-specific, and conditional RNA circuits.

**Results**

**Fusion of MS2CP and VPg to develop a translational activator.** In the natural life cycle of calicivirus, VPg proteins covalently bind with the 5′-ends of genomic and subgenomic RNAs. Because the covalent bond-formation is associated with transcription by the caliciviral RNA-dependent RNA polymerase, it is difficult to bind VPg proteins with synthetic mRNAs in the same manner. Thus, we utilized a dlFG variant of MS2CP.

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**Legend:**
- **a:** Conventional eukaryotic mRNA
  - **b:** Caliciviral RNA
  - **CaVT (MS2CP-VPg(FCV)):** Caliciviral VPg-based translational activator system
  - **Coding region pAA-Cap**
  - **Translational initiation**
  - **MS2CP**
  - **VPg**
  - **VPG (MS2CP-VPg(NV-GI))**
  - **VPG (MS2CP-VPg(FCV))**
  - **tagRFP (transfection control)**
  - **hmAG1 mRNA without MS2 binding motif**
  - **1×MS2(C)site1-hmAG1 mRNA**

**Graph:**
- **Fold change of hmAG1/tagRFP ratio from reporter mRNA only:**
  - **MS2CP**
  - **MS2CP-VPg(NV-GI)**
  - **CaVT (MS2CP-VPg(FCV))**
  - **0.26-fold**
  - **2.3-fold**
  - **0.33-fold**

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**Table:**
- **Fold change of hmAG1/tagRFP ratio from reporter mRNA only:**
  - **ms22CP**
  - **ms22CP-VPg(NV-GI)**
  - **CaVT (ms22CP-VPg(FCV))**
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**Legend:**
- **+ Indicated protein**
- **Reporter mRNA only**

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Optimization of target mRNAs for CaVT. To investigate the relationship between affinity and translational activation, we next used two MS2CP variants: conventional MS2CP and its V29I mutant, which has high affinity for the MS2-binding motif15. We also designed six different types of hmAG1 mRNA variants (Fig. 3a). These hmAG1 mRNAs contained one of the two MS2-binding motif variants (C or U), in which the C variant has a higher affinity for MS2CP than the U variant in a native nucleoside context17. The locations of these motifs were 5′ end (site1), the center of 5′ UTR (site2), or immediately upstream of the Kozak sequence (site3). In addition, we prepared these hmAG1 mRNA variants to have two different nucleoside compositions: N1-methyl-pseudouridine (N1mΨ) instead of uridine, and pseudouridine (Ψ) and 5-methylcytidine (5mC) instead of uridine and cytidine, respectively. Our recent study suggested that MS2CP could bind to N1mΨ-containing RNAs more strongly than to Ψ/5mC-containing RNAs18. These CaVT and hmAG1 mRNA variants were co-transfected into HeLa cells, and the hmAG1/tagRFP ratio was analyzed to monitor translational activation. Although both MS2CP-VPg(NV-GI) and MS2CP-VPg(FCV) activated hmAG1 translation, MS2CP-VPg(NV-GI) showed high nonspecific translational activation of mRNA without the MS2-binding motif, and MS2CP-mediated binding to the target mRNA decreased its activation level (Fig. 2b–e). In contrast, MS2CP-VPg(FCV) showed low nonspecific translational activation, and the addition of the MS2-binding motif to hmAG1 mRNA resulted in 2.3-fold increase of MS2CP-VPg(FCV)-mediated translational activation (Fig. 2b–e). In contrast, MS2CP or unfused VPg(FCV) protein alone did not show a MS2-binding motif-dependent increase of translational activation (Fig. 2b–e; Supplementary Fig. 1). From these results, we concluded that MS2CP-VPg(FCV) is more suitable for the translational activation of specific target mRNAs. We named this fusion protein Caliciviral VPg-based Translational activator (CaVT) and used it in the following experiments.

To further confirm that the CaVT-mediated increase of translation was caused by a cap-mimicking effect of VPg, we investigated the effect of CaVT on 1xMS2(C)site1-hmAG1 mRNA already capped with translationally active cap analog (Anti-Reverse Cap Analog; ARCA). In the case of ARCA-capped 1xMS2(C)site1-hmAG1 mRNA, the binding of CaVT induced translational repression rather than translational activation (Supplementary Fig. 2), which was a similar effect as VPg-unfused MS2CP16.

Cell-fate regulation by CaVT. The killing of undesired cells by regulating apoptotic protein expression has important applications in synthetic biology19. Cell-killing gene circuits could be used for cancer gene therapies or cell therapies20,21. However, sophisticated apoptosis regulation has remained a challenge due to leakiness of the protein expression and the circuit complexity. Thus, we designed an efficient apoptosis-regulatory system using CaVT-mediated, translational activation and repression of apoptotic proteins. First, we prepared mRNA (without canonical 5′-cap) that contains human Bax (pro-apoptotic protein) gene and a U-variant motif in the middle of its 5′ UTR (1xMS2(U)site2-Bax) (Fig. 5a, left). Co-transfection of CaVT mRNA increased the number of apoptotic cells by 1xMS2(U)site2-Bax, but even in the absence of CaVT, some portion of apoptotic induction was observed (Fig. 5b–d).
Fig. 3 Effects of modified nucleosides, sites, and variants of MS2-binding motif and of variants of MS2CP on translational activation by CaVT. HeLa cells were co-transfected with hmAG1 mRNAs with MS2-binding motif (cap analog: A-cap, modified nucleosides: N1mΨ or mΨ/5mC), tagRFP mRNA, and an mRNA that expresses CaVT or its V29I mutant. The fluorescence was measured by a flow cytometer. a Schematic diagrams of hmAG1 mRNAs with MS2 binding motif. b CaVT (or its V29I mutant)-mediated fold change of the hmAG1/tagRFP ratio in cells transfected with the indicated reporter mRNAs. Means of the hmAG1/tagRFP ratio in each cell expressing both hmAG1 and tagRFP were calculated and normalized by the hmAG1/tagRFP ratio in reporter mRNA only samples. The bar graph shows the average of three independent experiments (mean ± SD). Source data are provided as a Source Data file. c Representative histograms of the hmAG1/tagRFP ratio in cells expressing both hmAG1 and tagRFP. While cells transfected with only reporter mRNAs are shown as red, cells transfected with mRNA that express CaVT or its V29I mutant are shown as cyan and orange, respectively.
When we transfected 1xMS2(U)site2-hmAG1, some leaky expression was observed in the absence of CaVT (Supplementary Figs. 3 and 5). Based on the results of the hmAG1 experiments, we considered the leaky expression of Bax may be the cause of apoptosis in the absence of CaVT. To reduce the apoptotic effect caused by this leaky expression, we next designed mRNA coding an antiapoptotic protein, Bcl-xL22, which directly binds with Bax and inhibits apoptosis. The Bcl-xL mRNA, named 2xScMS2(C)-BclxL, contains two copies of the C variant motif stabilized by the scaffold, which should cause CaVT-mediated translational repression of the flanking coding region. Thus, CaVT should simultaneously activate and repress the translation of 1xMS2(U)site2-Bax and 2xScMS2(C)-BclxL, respectively (Fig. 5a, right). In the absence of CaVT, the co-transfection of 1xMS2(U)site2-Bax and 2xScMS2(C)-BclxL showed no increase of apoptotic cells compared with mRNA-untreated cells. In contrast, the additional co-transfection of CaVT mRNA significantly increased the number of apoptotic cells (Fig. 5b–d). These results indicate that our CaVT-mediated translational regulation system enables sophisticated cell-fate regulation by the simultaneous activation and repression of different mRNAs by a single protein.

CaVT-mediated regulation of genome editing. Next, we aimed to control genome editing with CaVT (Fig. 6a). We first prepared mRNA for the translational activation of Streptococcus pyogenes-derived Cas9 (1xMS2(U)site2-SpCas9). Cas9 is a target-programmable nuclease that makes a complex with a guide RNA

![Fig. 4 CaVT-mediated translational repression of the mRNA containing the strong binding motif.](image-url)
that contains a sequence complementary to its target DNA. Cas9 is widely used for genome editing such as gene knockout or knock-in, because the cleavage site can be programmed by designing guide RNA sequences. To check the CaVT-mediated translational activation of Cas9, we co-transfected 1xMS2(U)site2-SpCas9 mRNA and a single-guide RNA (sgRNA) that targets EGFP gene into EGFP-expressing HeLa cells (HeLa-EGFP) (Fig. 6a, top). As in the case of Bax, co-transfection of CaVT increased the knockout of EGFP (approximately 60% of cells were EGFP negative), but some portion of EGFP knockout was observed in the absence of CaVT (approximately 40% of cells were EGFP negative), which is significantly higher than in non-treated cells (Fig. 6b, c). Therefore, we prepared mRNA encoding an anti-CRISPR protein, AcrIIA4, which binds with Cas9 to inhibit Cas9 DNA recognition and endonuclease activity. The AcrIIA4-coding mRNA, named 2xScMS2(C)-AcrIIA4, has
the identical motif as 2xScMS2(C)-BclxL, which enables CaVT-mediated translational repression of AcrIIA4 (Fig. 6a, bottom). When co-transfected with CaVT mRNA, cells transfected with 1xMS2(U)site2-SpCas9 and 2xScMS2(C)-AcrIIA4 mRNAs showed high EGFP knockout efficiency comparable to the positive control, in which cells were transfected with Cas9 mRNA with translationally active cap analog (ARCA). On the other hand, in the absence of CaVT, the EGFP negative ratio of mRNAs co-transfected with 1xMS2(U)site2-SpCas9 and 2xScMS2(C)-AcrIIA4 was comparable to that of non-treated cells (Fig. 6b, c). These results show that CaVT enables the efficient regulation of genome editing with simultaneous upregulation and down-regulation of Cas9 and AcrIIA4.

Cell-selective regulation by miRNA-responsive CaVT. We next investigated whether CaVT-based RNA circuits could detect endogenous signals and produce desired outputs in a cell-type-specific manner. We chose mRNAs as a representative marker, because there are various miRNAs and their activities depend on the cell type. MiRNAs are small (about 22 nt) noncoding RNAs that regulate the translation of mRNAs through mRNA degradation or translational repression. MiRNAs make complexes with Argonaute proteins (e.g., Ago2) and cleave or translationally repress mRNAs containing sequences partially or perfectly complementary to the miRNAs.

To achieve cellular state-dependent translational activation and repression in RNA circuits, we focused on miRNA-responsive mRNAs that we had previously used to sort or visualize specific cell types. Thus, we designed CaVT mRNA that contains a complementary sequence to miR-21-5p or miR-302a-5p, two miRNAs highly expressed in HeLa and human iPSc cells (hiPSCs, 201B7 strain), respectively. Because endogenous miR-302a-5p activity is very low in HeLa cells, when co-transfected with the apoptosis-inducing circuit composed of 1xMS2(U)site2-Bax and 2xScMS2(C)-BclxL (cap analog: ARCA), and CaVT mRNA. For the positive control, 1xMS2(U)site2-Bax mRNA (cap analog: ARCA) was transfected. All mRNAs contained N1ΨG. One day after the transfection, the cells were stained and analyzed by a flow cytometer. The bar graph shows the average of four independent experiments (mean ± SD). The GFP expression was comparable to the positive control by ANOVA with Dunnett multiple comparison test (two-sided). Exact P values are shown in Supplementary Table 1. Source data are provided as a Source Data file.

Next, we investigated whether miRNA-responsive CaVT can be used for cell-selective genome editing. For this purpose, EGFP-expressing stable cell lines of HeLa cells and hiPScs were co-transfected with a genome editing-circuit composed of 1xMS2(U)site2-SpCas9, 2xScMS2(C)-AcrIIA4, EGFP-targeting sgRNA, and a miRNA-responsive or conventional CaVT mRNA (Fig. 7c). As in the case of the apoptosis-inducing circuit, in HeLa cells, EGFP-knockout induced by miR-302a-5p-responsive CaVT was as efficient as that by conventional CaVT. In contrast, the efficiency of miR-21-5p-responsive CaVT to induce EGFP-knockout was very low and at a level similar to non-treated cells (Fig. 7d top; Supplementary Fig. 10a). These results are consistent with the low miR-302a-5p activity and high miR-21-5p activity in HeLa cells. In hiPSc cells, which have high miR-302a-5p and moderate miR-21-5p expression, the EGFP-knockout efficiencies induced by miR-302a-5p and miR-21-5p-responsive CaVT were approximately 1/4- and 1/2-fold that of conventional CaVT, respectively (Fig. 7d bottom; Supplementary Fig. 10b). These results indicated that CaVT can be a useful component for RNA circuits to achieve cell-selective regulation.

Drug-inducible translational activation by split CaVT. Drug-controllable gene-expression systems can modulate gene expressions at suitable levels and time windows. Drug-regulatable artificial transcriptional activators (e.g., tetracycline-inducible transactivator) have been widely used for DNA circuits, but they cannot be applied to RNA circuits. To construct a drug-regulatable CaVT applicable to conditional gene regulation in RNA circuits, we utilized the drug-responsive DmrA–DmrC hetero-dimerization system (a variant of the rapamycin-responsive FKBP–FRB hetero-dimerization system), in which DmrA and DmrC bind to each other in the presence of A/C heterodimerizer. We fused MS2CP (or its V29I mutant) and VPG(FCV) with DmrA and DmrC, respectively, to make VPG (FCV) interact with target mRNAs only in the presence of the dimerizer (Fig. 8a). Then, we co-transfected twelve variants of target mRNAs (the six constructs shown in Fig. 3a with two modified nucleoside compositions (N1ΨG or Ψ5mC)), MS2CP-1xDmrA mRNA, and DmrC–VPG(FCV) mRNA into HeLa cells, and incubated the cells in the presence or absence of the dimerizer. Translational activation of the target mRNAs was observed in all combinations, but the most suitable combination of nucleoside modifications and motif variants was N1ΨG-containing mRNA with a U-variant (Fig. 8b, c; Supplementary Figs. 11 and 12), which is consistent with the results of conventional CaVT. These results indicate that the dimerizer-mediated translational activation was induced by MS2CP-1xDmrA-DmrC–VPG(FCV) interaction-dependent mechanism, we also added the dimerizer.
Dimerizer-mediated translational activation was observed only in the presence of both MS2CP-1xDmrA and DmrC-VPg(FCV) (Supplementary Fig. 13). To check the dose-dependency of the drug-regulatable CaVT, we incubated cells in a medium with various concentrations of the dimerizer after the co-transfection of 1xMS2(U)site1-hmAG1, MS2CP-1xDmrA, and DmrC-VPg(FCV) mRNAs. As shown in Fig. 8d, e and Supplementary Fig. 14, a dose-dependent increase in the translation was observed. Finally, we captured time-lapse images to investigate the response time of the drug-regulatable CaVT. As early as 3 h after the dimerizer addition, an increase of hmAG1 fluorescence was observed (Fig. 8f; Supplementary Videos 1 and 2). Together, these results indicate that we could achieve conditional translational activation of the drug-regulatable Cas9.

![Diagram of CaVT](image)

**Diagram:**
- **CaVT**  
  - Translational activation: A-Cap → Cas9 → pA → EGFP knock-out
  - Translational repression: A-Cap → Cas9 → pA → ARCA → AcrIIA4

**Graph:**
- **EGFP negative population (%)**
  - Non-treated
  - No sgRNA (negative control)
  - Cas9 mRNA with ARCA (positive control)
  - 1xMS2(U)site2-Cas9
  - 2xScMS2(C)-AcrIIA4 (0.5 ng)
  - 1xMS2(U)site2-Cas9
  - 2xScMS2(C)-AcrIIA4 (1 ng)

**Table:**

| Condition                                      | Count |
|-----------------------------------------------|-------|
| Non-treated                                   | 150   |
| No sgRNA (negative control)                   | 100   |
| Cas9 mRNA with ARCA (positive control)        | 50    |
| 1xMS2(U)site2-Cas9                            | 150   |
| 2xScMS2(C)-AcrIIA4 (0.5 ng)                   | 100   |
| 1xMS2(U)site2-Cas9                            | 50    |
| 2xScMS2(C)-AcrIIA4 (1 ng)                     | 50    |

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translational activation from synthetic mRNAs with our drug-regulatable CaVT system.

**Discussion**

In the present study, we developed the novel translational activator, CaVT, which functions in RNA-based mammalian synthetic circuits. To date, several RBPs such as L7Ae, MS2CP, U1A, Lin28A, and TetR have been used to develop RNA circuits.\(^9\)–\(^11\),\(^13\) However, while the translational repression of mRNAs has been achieved by these RBPs, direct translational activation in mammalian cells is rather difficult. Thus, the “repression of translational repressors” approach has been used for indirect translational activation.\(^9\)–\(^11\) However, this layered approach makes the design of circuits complicated. In addition, the approach increases the components of the circuits, which increases the burden to cells. Therefore, the development of a method to directly activate mRNA translation is needed. We have previously developed L7Ae-mediated “ON switch”, in which L7Ae-binding to mRNA prevents nonsense-mediated mRNA decay (NMD) to upregulate expression.\(^38\) However, because nuclear pre-mRNA processing is necessary for NMD, mRNAs for NMD-based ON switches need to be transcribed in the nucleus. This limitation makes it difficult to use NMD-based ON switches in synthetic RNA circuits. In contrast, all components of our CaVT-mediated translational activation system can be transfected as synthetic mRNAs. Because synthetic mRNA does not cause insertional mutagenesis, it should be safer than DNA delivery, making it a promising method for medical applications such as gene therapies and regenerative medicines. Therefore, a translational activator compatible with RNA-only delivery will expand the use of synthetic gene circuits in clinical applications.

Different from FCV-derived VPg (VPg(FCV)), norovirus GI (NV-GI)-derived VPg (VPg(NV-GI)) showed high nonspecific translational activation (Fig. 2b–e; Supplementary Fig. 1). The MS2-independent translational activation may be caused by the direct binding of VPg to the G(5′)ppp(5′)A (a cap analog used to prepare A-capped mRNAs), because norovirus VPg can bind to nucleotide triphosphates directly.\(^40\) FCV and norovirus VPg also differ in their interactions with translation-related endogenous proteins. For example, norovirus VPg directly interacts with eIF3, eIF4E, eIF4G, and polyA-binding protein (PABP), and its function is insensitive to eIF4E depletion.\(^41\)–\(^43\) In contrast, VPg(FCV) directly interacts with eIF4E, but not eIF4G or PABP, and it needs eIF4E-eIF4G interaction for translational activation.\(^41\),\(^44\) Although VPg(NV-GI) showed higher translational activation than VPg(FCV) (Fig. 2b–e; Supplementary Fig. 1), we selected VPg(FCV) as a component of CaVT, because MS2CP-VPg(NV-GI) exhibited relatively high nonspecific translational activation (Fig. 2b–e). For example, the drug-inducible translational activation system (Fig. 8) requires that VPg only activates translation in an MS2 binding-dependent manner. In addition, norovirus VPg inhibits both Cap- and IRES-dependent translation,\(^42\) while VPg(FCV) does not affect that kind of translation.\(^44\) Thus, VPg (FCV) seems to have less effect on non-target gene expressions than VPg(NV-GI) and is thus more suitable to construct CaVT for mammalian gene circuits.

When we varied the insertion sites of the MS2-binding motif in the 5′ UTR, site2 (the center of the 5′ UTR) showed the highest translational fold change by CaVT (Fig. 3b; Supplementary Fig. 4a). One possible explanation for the low basal expression of the site2-mRNAs is that the structure of the 5′ UTR contributes to VPg-independent translation. In the site1 and site3 constructs, the MS2-binding motif was simply added to the edge of the 5′ UTR. On the other hand, in the site2 construct, the MS2 binding motif interrupted the original 5′ UTR, which may affect the 5′ UTR structure and translation level.

Modified nucleosides such as N1mΨ, Ψ, and 5mC have been widely used for mRNA transfection to prevent immune responses and increase translations.\(^3\)–\(^5\),\(^45\) Recently, we found that such nucleoside modifications also affect the affinities of RBPs including MS2CP.\(^18\) Because the binding of RBPs near the 5′ terminus of mRNAs decreases translation\(^16\) and because RBP affinities to the mRNAs correlate with translational repression efficiency,\(^16\) we can estimate the relative affinity of MS2CP to each target mRNA with different nucleosides based on MS2CP-1xDmrA-mediated translational repression in the absence of A/C heterodimerizer (the condition in which DmrC-VPg(FCV) cannot interact with the target mRNAs, Fig. 8b). From the data of Fig. 8b (site1-mRNAs), we assume that the C-variant with N1mΨ has the highest affinity to MS2CP, followed by the U variant with Ψ/5mC, the U variant with N1mΨ, and the C variant with Ψ/5mC. Low-affinity combinations such as the U variant with N1mΨ and the C variant with Ψ/5mC showed higher translational activation (Fig. 3b; Supplementary Fig. 4a). One possible explanation for these observations is that the translational level is determined by the balance between VPg(FCV)-mediated activation and MS2CP-mediated repression, and that too strong binding to MS2CP could enhance the latter.

When we used site1-mRNAs as targets, CaVT V29I high-affinity mutant consistently showed lower translational activation than normal CaVT (Fig. 3b, c; Supplementary Fig. 4). As explained above, because the binding of RBPs such as MS2CP near the 5′ terminus of mRNAs repress translation,\(^16\) it is possible that translational repression by strong binding at site1 impairs VPg(FCV)-mediated translational activation. In most cases, CaVT and its V29I mutant showed similar effects on site2 and site3 mRNAs. However, in the case of C-variant with N1mΨ, the highest affinity motif, V29I mutant consistently showed lower translational activation than normal CaVT (Fig. 3b, c and Supplementary Fig. 4). These results suggested that strong binding can repress translation even when the binding sites are not near the 5′ terminus.

Translational activation by CaVT or MS2CP-VPg(NV-GI) became sluggish in cells with high transfection efficiency (i.e., cells with high tagRFP expression) when we used target mRNAs.
with an MS2 binding motif at site1 (Fig. 2d). This effect may be caused by a similar mechanism as the low translational activation of target mRNAs when using a high-affinity motif. Namely, if the concentration of CaVT or MS2CP-Vpg(NV-GI) is high, these proteins may constantly bind target mRNAs to inhibit ribosome-scanning. We also achieved drug-inducible translational activation by combining CaVT and drug-inducible hetero-dimerization domains (Fig. 8; Supplementary Figs. 11–14; Videos 1 and 2). Because inducible translation systems enable setting the expression level and time at suitable ranges for therapies, they will be an important component for future gene therapies. Although a
drug-regulatable translational repressor was recently reported, to our knowledge, ours is the first report of a drug-regulatable translational activator for RNA circuits. Furthermore, because the domains of drug-regulatable CaVT are independent modules, the DmrA and DmrC used in this study can be exchanged with any other inducible hetero-dimerization domain. Therefore, we can develop other regulatable CaVT according to the application. This feature is also suitable for the simultaneous use of multiple orthogonal CaVT regulated by different signals.

One important feature of CaVT is that the single protein enables different levels of translational activation and even translational repression by modulating the locations, sequences, and modified nucleosides of its binding motif. Utilizing this feature, we demonstrated efficient regulation of apoptosis by the simultaneous translational activation of a pro-apoptotic protein and repression of an anti-apoptotic protein. Like apoptosis, there are many biological phenomena that are regulated by the balance of promoting and repressive factors. For example, cellular reprogramming and differentiation are regulated by the balance of transcription factors that contribute to the pluripotent and differentiated states. One of our future goals is regulating complex cell-fate control by RNA circuits. Saxena et al. reported a lineage control by RNA circuits. SAXEN et al.17 reported a lineage control by RNA circuits.

The half-life of synthetic mRNAs is short, which is a suitable feature for applications that need only short-term transgene expression (e.g., purification of specific cells), because the expression burden is low. However, some biological phenomena including reprogramming or differentiation need long-term gene expression. In such cases, the repeated incorporation of mRNAs into the cell is needed. Another approach is the use of RNA replicons that are replicated and maintained long-term in mammalian cells. While mRNAs expressed from RNA replicons have active 5′-cap structures, the target mRNAs of CaVT should not. Therefore, if we combine RNA replicons and CaVT, additional modifications to remove the 5′-cap may be needed. For example, the addition of self-cleaving ribozymes at the 5′-ends may be helpful.

In summary, we have developed a novel translational regulator composed of an RBP and caliciviral VPG protein. This translational regulator, named CaVT, can be used for both translational activation and repression. Furthermore, it can be designed to exert miRNA-responsive, cell-selective gene regulation.
changed once every 2 days, and passage was performed once every 8 days using 0.5× TrypLE Select (Thermo Fisher Scientific) and cell scrapers. At the times of the cell seeding, plates were coated with iMatrix-511 silk (Nippi, Incorporated, Tokyo, Japan). More details are described in a previous report52.

Reporter mRNA transfection and flow cytometry. Totally, 5 × 10⁴ HeLa cells were seeded onto 24-well plates, and 1 day later they were transfected with the indicated amount of mRNAs using 1 μL/well of Lipofectamine MessengerMAX (Thermo Fisher Scientific). One day after that, the cells were harvested using 200 μL/well of Trypsin/EDTA and suspended in 500 μL/well of DMEM. Then, the fluorescence was measured by a BD Accuri C6 with CFlow Plus software (BD Biosciences, San Jose, USA). The excitation wavelength was 488 nm for both tagRFP and hmAG1. Bandpass filters were 585/40 nm for tagRFP and 530/30 nm (90% attenuation) for hmAG1. Visualization of the data and calculation of the means of the hmAG1/tagRFP ratio in each cell expressing both hmAG1 and tagRFP were performed by FlowJo 7.6.5 (BD Biosciences). Microsoft Excel for Office 365 and Jupyter Notebook were used to depict bar graphs. The values of the fluorescence compensation (0.7–0.9% for 530/
30–585/40 nm and 36–38% for 585/40–530/30 nm) were determined based on the fluorescence of cells transfected with either hmAG1 or tagRFP. Details of the transfection conditions are shown in the Supplementary Methods.

**WST-1 assay.** Totally, 1 x 10^4 HeLa cells were seeded onto 96-well plates, and 1 day later they were transfected with the indicated amounts of mRNAs using 0.25 μL/well of Lipofectamine MessengerMAX. One day later, medium containing cell proliferation reagent WST-1 (Roche Diagnostics KK) was prepared (1:10 final dilution) and used to replace the medium of the transfected cells. After incubation for 1 h at 37°C, absorbance wavelengths of 440 and 620 nm were measured by a microplate reader (Infinite M1000, Tecan Japan Co., Ltd., Kanagawa, Japan). Microsoft Excel for Office 365 and Jupyter Notebook were used to depict bar graphs. Statistical analysis was performed using R 3.6.1. Details of the transfection conditions are shown in the Supplementary Methods.

**Apoptosis assay by Annexin V and SYTOX red staining.** Totally, 5 x 10^4 HeLa cells were seeded onto 24-well plates, and 1 day later they were transfected with the indicated amounts of mRNAs using 1 μL/well of Lipofectamine MessengerMAX. One day later, the supernatants of each well were collected, and the cells were harvested using 200 μL/well of accutase (Nacalai Tesque). The harvested cells were suspended in the collected supernatants of each well and centrifuged at 200g for 5 min. The precipitated cells were stained with Annexin V, Alexa Fluor 488 conjugate (Thermo Fisher Scientific), and SYTOX Red (Thermo Fisher Scientific) for 30 min at room temperature. Then, 200 μL of PBS was added to each sample, and the fluorescence was measured by BD Accuri C6. The excitation wavelength was 488 nm for Alexa Fluor 488 conjugate and 640 nm for SYTOX Red. The bandpass filters were 533/30 nm for Alexa Fluor 488 conjugate and 675/25 nm for SYTOX Red. The obtained data were analyzed by FlowJo 7.6.5. Microsoft Excel for Office 365 and Jupyter Notebook were used to depict bar graphs. Statistical analysis was performed using R 3.6.1. Details of the transfection conditions are shown in the Supplementary Methods.

**EGFP knockout assay of HeLa cells.** Totally, 5 x 10^4 HeLa-EGFP cells were seeded onto 24-well plates, and 1 day later they were transfected with the indicated amounts of mRNAs and an EGFP-targeting sgRNA using 1 μL/well of Lipofectamine MessengerMAX. One day later, the cells were passaged. Five days after the transfection, the fluorescence was measured by BD Accuri C6. The excitation wavelength and the bandpass filter were 488 and 533/30 nm, respectively. The obtained data were analyzed by FlowJo 7.6.5. Microsoft Excel for Office 365 and Jupyter Notebook were used to depict bar graphs. Statistical analysis was performed using R 3.6.1. Details of the transfection conditions are shown in the Supplementary Methods.

**EGFP knockout assay of iPS cells.** Totally, 5 x 10^4 201B7-EGFP cells were seeded onto 24-well plates, and 1 day later they were transfected with the indicated amounts of mRNAs and an EGFP-targeting sgRNA using 1 μL/well of Lipofectamine MessengerMAX. Five hours after the transfection, the medium was removed, and 400 or 500 μL of fresh medium was added. Then the plate was moved into a Biotestation CT (Nikon, Tokyo, Japan). Two hours later, 100 μL of medium containing 2500 nM/A/C heterodimerizer (Takara Bio) was added to a well containing 400 μL of medium (final concentration = 500 nM). After the addition of A/C heterodimerizer, the time-lapse images were captured using the Biotestation CT (objective lens: 10×) every 30 min. The filter set for fluorescence imaging was 472/520 nm. Exposure times were 4 and 400 ms for bright field and fluorescence imaging, respectively. The contrast of all fluorescent images were identicaly adjusted by ImageJ. Details of the transfection conditions are shown in the Supplementary Methods.

**Data availability**

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. The source data underlying Figs. 2b, 3b, 4b, 5b, c, 6b, 7b, d, 8b, and Supplementary Figs. 1a, 2a, 2b, and 8 are provided as a Source Data file. If additional data are required, they are available from the corresponding author upon reasonable request.

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Author contributions

H.N. and H.S. devised the experimental design and wrote the manuscript. H.N. conceived the concept and carried out the experiments and data analysis.

Competing interests

Kyoto University holds patents regarding microRNA-responsive systems (WO2015105172A1, WO2015141827A1, US10378070B2). In addition, Kyoto University has filed a patent application regarding the translational regulators (IP2020015891). H.N. and H.S. are the inventors of record listed on the patents.

Additional information

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