REVIEW

Development of a novel RNA-programmable artificial transactivator able to upregulate endogenous genes ad libitum

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Here we provide a concise overview of a new platform we recently developed for transactivating endogenous genes ad libitum. It relies on a binary design, including an RNA cofactor in charge of recognizing the target gene, and a polypeptidic apofactor stimulating transcription. Compared to similar CRISPR-based devices, our artificial transactivators are seven-folds smaller and elicit a lower, however robust and biologically effective, expression gain. Remarkably, they only work in cells which already transcribe the gene of interest. These properties make our novel platform an appealing potential tool for restoring normal expression levels of haploinsufficient genes upon generalized delivery.

Keywords: artificial transactivator; NMHV; CRISPR

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In recent years, several attempts have been made to develop new strategies to perturbate endogenous gene expression levels. Small activating miRNA/siRNA-like RNAs (saRNAs) were demonstrated to be an effective tool to achieve this goal [1-5]. On the other hand, artificial enzymes able to recognize arbitrarily selected genes and transactivate them have been also recently described. They include Zinc Finger- (ZF-), TransActivator Like Element- (TALE-) and RNA-programmable, Clustered Regularly Interspaced Short Palindromic Repeats- (CRISPR-) type transactivators [6-11].

In the paper highlighted here [12], we describe a novel, small and non-CRISPR transactivator prototype we recently developed. It is composed by a polypeptidic moiety, NMHV, which stimulates gene transcription, and a non coding RNA domain, which drives the whole complex to the gene of interest (GOI). NMHV includes two SV40-T protein nuclear localization signals (NLS), an RNA binding domain (RBD) corresponding to the bacteriophage MS2 coat protein [13], a monomeric A influenza virus hemagglutinin epitope (HA) and three VP16 transactivating domains from the herpes simplex virus 1. In its original formulation, the ncRNA co-factor included an hexameric MS2 coat protein stem&loop sequence [14, 15] (MF6) and a gene-specific, 120-180-bases long RNA bait (Figure 1a).

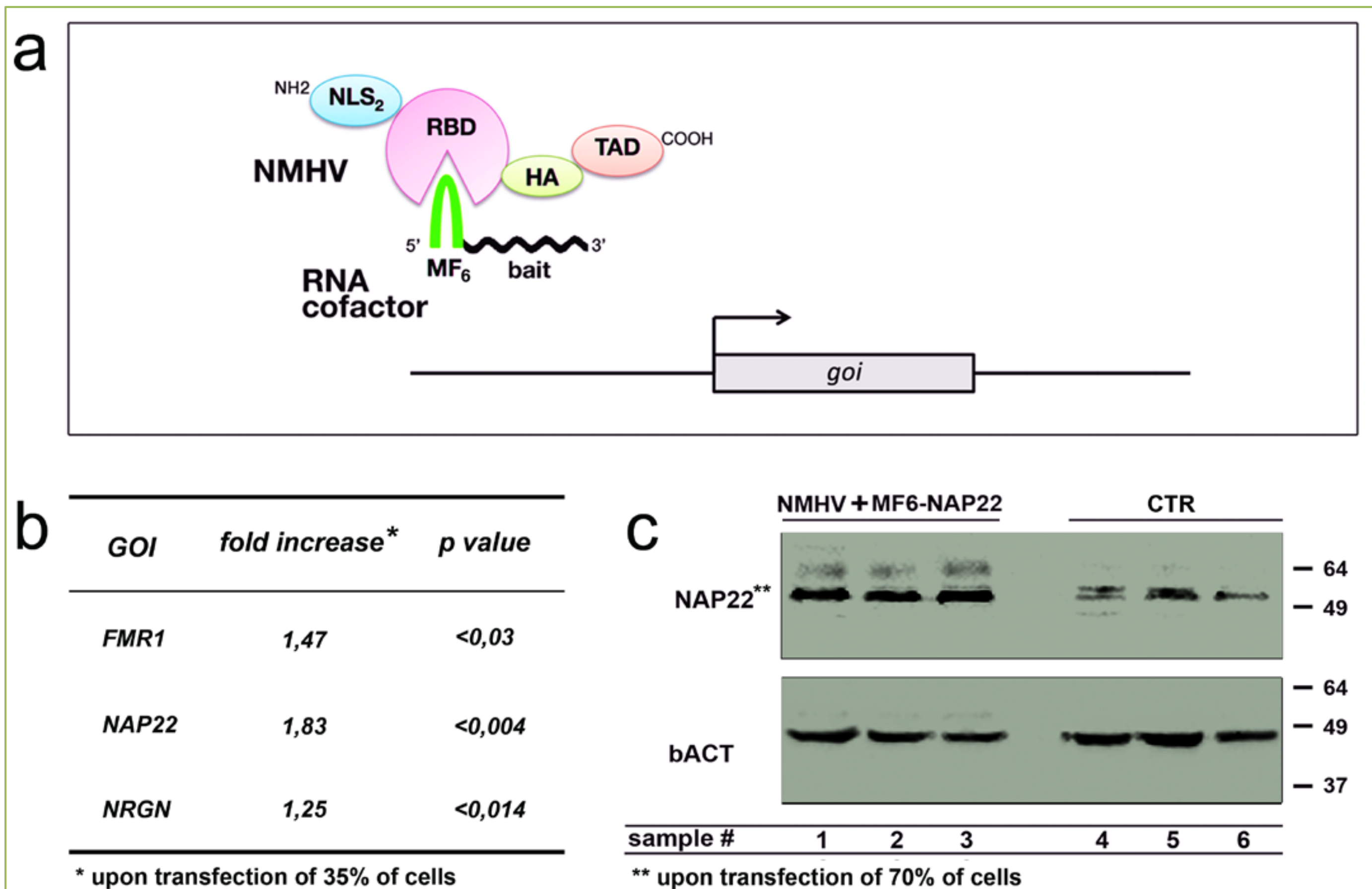

Figure 1. Functional evaluation of NMHV transactivators in HEK293T cells. (**a**) Schematics of the NMHV apo-activator and its RNA cofactor. NLS2, nuclear localization signal 2x; RBD, MS2 RNA-binding domain; HA, hemoagglutinin epitope; TAD, VP16-related transactivator domain, 3x; MF6, MS2-high affinity, stem-and-loop finger, 6x; "bait", short, target gene specific, RNA tag; GOI, gene of interest. (**b**) mRNA expression gains elicited in HEK293T cells by NMHV enzymes programmed to target FMR1, NAP22 and NRGN. Data double-normalized against GAPDH and control-transfected samples. (**c**) Western blotting of NAP22 in HEK293T cells after transfection by NMHV/MF6-NAP22 or CTR (control).

We firstly tested this device on HEK293T cells. We programmed it to recognize three genes, FMR1, NAP22 and NRGN, expressed at high level in this line. When we delivered our device to cells, these GOIs were upregulated by 47%, 83% and 25%, respectively (Figure 1b). Moreover, when we challenged cells with the NMHV/MF6-NAP22 pair, the NAP22 protein was upregulated by 5.77 folds (Figure 1c). Interestingly, our transactivator upregulated each GOI in a specific way, since neither structurally similar genes nor genes flanking the intended transcription unit were affected at all. Moreover, chromatin immunoprecipitation assays showed that, apparently, RNA polymerase II (RNA polII) progression along the transcription unit was facilitated by our device.

To assess the capability of our artificial transactivator to elicit an appreciable biological effect, we assayed it on two genes exerting a fine control of key neuronogenetic parameters, Emx2 and Foxg1. We designed ncRNA cofactors targeting Emx2 and Foxg1 loci. We employed a monomeric (MF1) version of the original hexameric (MF6) ncRNA cofactor, since it resulted more stable in lentiviral vectors needed for this assay. We tested our device in murine dorsal telencephalic precursors at embryonic day 12.5 (E12.5). Compared to controls, delivery of NMHV and MF1-Emx2 or NMHV and MF1-Foxg1 upregulated the two target genes by 16% and 23%, respectively (Figure 2a,d). Despite the small expression gain, this led to a pronounced reduction of the neuronal output, as suggested by the dramatic decrease of cells expressing the early post mitotic marker Tubb3 (Figure 2 b,c and e,f). This is not surprising to us, since it has been previously demonstrated that even a small increase in Emx2 levels robustly promotes pallial precursors proliferation and, as a consequence, reduces their differentiation to neurons [4, 16, 17].

Even in Emx2 and Foxg1 cases, no upregulation was observed for (1) potential off-target genes sharing extensive homologies with the intended target gene, (2) other genes active in pallial precursors (and, as such, susceptible to

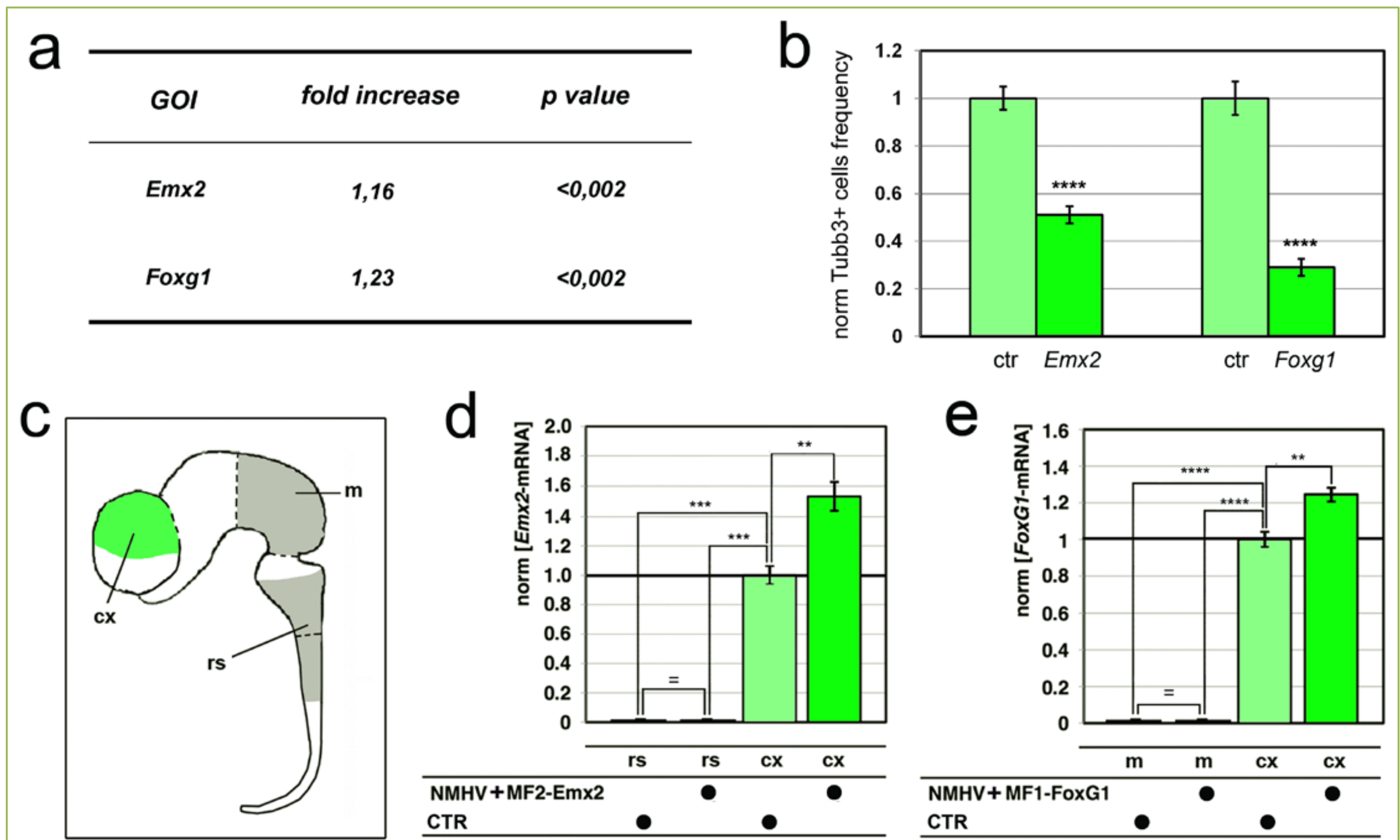

Figure 2. Functional evaluation of NMHV transactivators in murine embryonic neural precursors. (**a**) mRNA expression gains elicited in pallial cells by NMHV enzymes programmed to target Emx2 and Foxg1. Data double-normalized against Gapdh and control-transduced samples. (**b**) Downregulation of the Tubb3$^+$ neuronal differentiating fraction in cultures of pallial precursors transduced with NMHV enzymes transactivating Emx2 and Foxg1. ctr, control. **(c)** Schematics of the murine E10.5 neural tube. cx, cerebral cortex; m, mesencephalon; rs, rhombo-spinal tract. **(d,e)** Unchanged Emx2- and Foxg1-mRNA levels in E10.5 rhombo-spinal and mesencephalic precursors, transduced by NMHV/MF2-Emx2 and NMHV/MF1-Foxg1 pairs, repectively. Transactivation of the two genes in E12.5 pallial precursors transduced with the same pairs are shown, as positive controls. Data double-normalized against Gapdh and control-transduced samples (CTR). **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

exogenous transactivating complexes) as well as (3) genes flanking the GOI locus. Interestingly, when we challenged precursors derived from regions that did not express Emx2 and Foxg1 (the rhombospinal tract and mesencephalon, respectively), no gene upregulation was elicited at all. This suggested that our device might require a chromatin conformation “prone” to gene transcription, in order to evoke an appreciable gene stimulation. This makes our prototype an appealing potential tool for "clean" rescue of gene haploinsufficiencies, upon widespread delivery.

Finally, we worked on NMHV optimization. On one hand we tried to ameliorate the the apofactor-cofactor stability. In this respect, we observed substantial benefits when we increased the number of MS2 coat protein stem&loop sequences in the ncRNA cofactor. When we replaced the monomeric MF1-Emx2 cofactor with a dimeric MF2-Emx2 one, Emx2 was upregulated by +40%. Moreover, when we modulated the apofactor-cofacto ratio, we further increased GOI transactivation.

On the other hand we validated shorter ncRNA cofactors, replacing the original 120-180-bases long baits with 60-mers and showing that the latter ones still sustained GOI upregulation. Interestingly, when we mutagenized 30% of the 60-bases long NAP22 bait, we fully suppressed gene upregulation. When the mutagenesis rate was lowered to 15%, uninterrupted homologous RNA strings of at least 18 bases were needed to get gene transactivation.

In summary: (1) we have built an artificial transactivator able to specifically stimulate expression of endogenous genes ad libitum. By this tool, we upregulated five genes, in cell lines as well as in primary cultures of murine pallial precursors. (2) Our artificial transactivator specifically interacted with target gene chromatin in an RNA cofactor-dependent way; however its activity was restricted to cells where the target gene is normally transcribed. (3)

Albeit small, gene upregulation was sufficient to inhibit neuronal differentiation. (4) High homology between the ncRNA bait and the target gene was required to get gene transactivation.

Conflicting interests

The authors have declared that no competing interests exist.

Acknowledgements

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Abbreviations

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; GOI: gene of interest; HA: A influenza virus hemagglutinin epitope; MF1: MS2 bacteriophage coat protein stem&loop sequence; MF6: (MS2 bacteriophage coat protein stem&loop sequence)$_6$; NMHV: NLS_2-RBD-HA-$VP16_3$; NLS: SV40-T protein nuclear localization signal; RBD: RNA binding domain from the MS2 bacteriophage coat protein; TALE: TransActivator Like Element; VP16: herpes simplex virus 1 viral protein 16 transactivating domain; ZF: Zinc Finger.

Author contributions

CF and EG performed the work referred to in this highlight; CF and AM wrote this highlight.

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