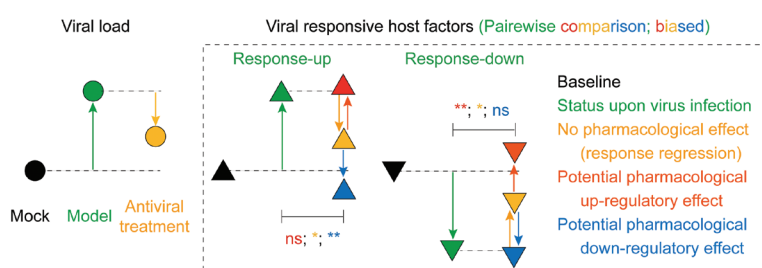


Response regression model as a guiding principle for pharmacological mechanistic investigation of host-targeted antivirals

Graphical abstract



Highlights

- The status of virus-responsive host factors is highly dynamic, according to fluctuations in viral kinetics.
- The status of virus-responsive host factors is prone to regress toward baseline with decreasing viral load after antiviral treatment.
- Additional pharmacological regulation of virus-responsive host factors by antivirals may cause response regression deviation or transcendence.
- A response regression model was developed as a guiding principle to identify the host factors potentially regulated by host-targeted antivirals.

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In brief

For pharmacological mechanistic study of host-targeted antivirals, potential pharmacological regulatory effects on virus-responsive host factors must critically be distinguished from perturbations caused by viral load fluctuation.

Response regression model as a guiding principle for pharmacological mechanistic investigation of host-targeted antivirals

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ABSTRACT

The host response to viral infections largely depends on the viral load intensity, and the fluctuating viral kinetics after antiviral treatment should perturb host responses accordingly. Therefore, conventional comparison of individual host factors between virus-infected models with vs without antiviral treatment usually generates biased results that tend to reflect perturbations due to fluctuating viral kinetics. The extent of this bias is predominately determined by the range of viral load fluctuation. Herein, we raised a response regression model to serve as a guiding principle to precisely explore the pharmacological mechanisms of host-targeted antiviral (HTA) agents. The statuses of virus-responsive host factors were estimated to regress toward baseline in accordance with decreased viral load, whereas host factors with regression deviation or regression transcendence statuses were considered potential targets of the pharmacological effects. In a pilot application, we developed a mathematical method for transcriptomic analysis based on the response regression model, to identify the actual differentially expressed genes potentially affected by HTAs. Our study should substantially facilitate future pharmacological mechanistic study of HTAs. Moreover, our adapted strategy could be further expanded to other multi-omics analyses and diverse infectious diseases.

Keywords: Response regression model, Host-targeted antiviral, Pharmacological mechanistic study, Multi-omics analysis

1. INTRODUCTION

As external stimuli, viral infections can evoke massive and complicated host responses including both protective and pathological processes [1, 2]. The degree of host response usually correlates well with the intensity of the external stimulus. However, in viral infections, the viral kinetics is highly dynamic as the virus replicates in the host, thus conferring continuously fluctuating host stimulation [3]. Accordingly, the host response to viral stimuli fluctuates throughout the time course of infection [4, 5]. The dynamic nature of virus-host interaction substantially complicates mechanistic studies of antiviral agents.

Generally, antiviral agents can be classified into direct-acting antivirals (DAAs) and host-targeted antivirals (HTAs), which target viral and host factors, respectively [6-8]. The mechanism of action of DAAs is straightforward and targets critical viral components, with very little involvement from host factors.

Although an alleviated host response is observed together with decreased viral replication after DAA treatment, this effect simply reflects the decreased intensity of the external stimulus. In contrast, HTAs have regulatory effects on certain host factors and subsequently block viral replication and/or spread. Altered viral kinetics might influence the status of host responses, probably including the HTA's target factors. Therefore, the ultimate status of a host factor after HTA treatment can reasonably be assumed to be comprehensively determined by the actual pharmacological effect and the feedback from altered viral kinetics. However, in pharmacological mechanistic studies, the actual pharmacological effects of HTAs on host factors must critically be distinguished from the disturbances caused by fluctuating viral kinetics.

Multi-omics techniques, including transcriptomics, proteomics, and metabolomics, provide advanced approaches for pharmacological mechanistic studies [9-11]. The multi-omics data obtained from disease models

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with vs without drug treatment are frequently compared to identify potential pharmacological targets; this method is considered reasonable and highly valuable in mechanistic studies of drugs against diverse diseases [12, 13]. Nonetheless, for antivirals, particularly HTAs, this conventional analytic strategy is greatly compromised because of the aforementioned reasons. In this study, we developed an optimized method for analyzing transcriptomic data on viral infection in the presence or absence of antiviral treatments, to identify specific target host factors potentially influenced by the pharmacological effects of HTAs while excluding the likely disturbance effects caused by fluctuating viral kinetics.

2. MATERIALS AND METHODS

2.1 Ethics statement

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Shandong University of Traditional Chinese Medicine (approval SDUTCM20250307564).

2.2 Animal models

Female BALB/c mice (4–6 weeks old, $n=4$ per group) were intranasally inoculated with recombinant influenza A virus (IAV) stably carrying a firefly luciferase (Fluc) gene, termed PR8-Fluc [14], at a dose of 2000 50% tissue culture infectious dose (TCID₅₀)/mouse. At day 2 post infection (p.i.), the mice were subjected to *in vivo* imaging to monitor viral load in real time. Subsequently, the mice were sacrificed, and their lung tissues were collected for transcriptomic sequencing.

For antiviral treatment, the antiviral immune modulator recombinant human interferon- α 2b (Unipul, Hainan, China) was administered intranasally at a dose of 5000 IU/mouse at 12 hours (h) before viral challenge and after viral challenge (IFN group). The DAA agent oseltamivir phosphate (Roche, Basel, Switzerland) was administered orally at 10 mg/kg/day twice per day, starting at 2 h p.i. until the end of the experiment (osel group). Infected mice receiving no treatment were denoted the model group, whereas mock-infected mice served as healthy controls (mock group). In addition, mice inoculated with a much lower dose of the virus (200 TCID₅₀/mouse; M2 group) served as a reference group.

All animals were maintained under specific-pathogen-free conditions, and all possible efforts were made to minimize any suffering and the number of experimental animals.

2.3 *In vivo* imaging

In vivo imaging was performed as previously described [14]. In brief, all mice in each group were anaesthetized at day 2 p.i., and the substrate D-luciferin (PerkinElmer, Waltham, MA, USA) was injected intraperitoneally at

150 mg/kg. At 10 min after substrate administration, images were acquired with a Xenogen IVIS 200 instrument and analyzed in Living Image software (version 4.4).

2.4 Transcriptomic sequencing

Transcriptomic sequencing of lung tissue from each mouse was performed by Novogene, Beijing, China. Briefly, total RNA from each sample was extracted, and RNA integrity was measured with an Agilent 2100 bio-analyzer system. Libraries of amplified RNA were prepared in accordance with the Illumina protocol. The clustering of the index-coded samples was performed with a TruSeq PE Cluster Kit v3-cBot-HS (Illumina). After cluster generation, the library preparations were sequenced, and 150 bp paired-end reads were generated. Reads that contained adapter or poly-N sequences, or had low quality, were removed from the raw data to generate clean reads for further analysis. Paired-end clean reads were aligned to the reference genome with Hisat2 (v2.0.5). The mapped reads of each sample were assembled with StringTie (v1.3.3b). The numbers of reads mapped to each gene were counted with FeatureCounts (v1.5.0). According to the gene length and read counts mapped to the gene, the fragments per kilobase of exon model per million mapped fragments (FPKM) value of each gene was calculated.

The transcriptomic profiles of samples from each group were subjected to pairwise comparison, and the adjusted p value (p_{adj}) and fold change (FC) of each gene from each group of infected mice were calculated. Genes with $p_{adj} < 0.05$ and $|\log_2 FC| > 1$ from pairwise comparison were identified as differentially expressed genes (DEGs) [15]. Specifically, on the basis of the model vs mock group comparison, genes with $p_{adj} < 0.05$ and $|\log_2 FC| > 1$ were denoted virus-responsive genes (VRGs), including both up-regulated VRGs (VRGs-up; $\log_2 FC > 1$) and down-regulated VRGs (VRGs-down; $\log_2 FC < -1$), whereas the others were denoted non-VRGs.

2.5 Response-regression model as a guiding principle to identify DEGs of interest after antiviral treatment, according to transcriptomic data

A response regression model was developed as a guiding principle to identify the DEGs specifically affected by the antiviral treatment. The VRGs-up, VRGs-down, and non-VRGs were analyzed separately.

(1) For the VRGs-up, the expression level of each gene (for example, gene A) in the lungs of mice in the test group was compared with that in the model and mock groups. A new parameter, the regression index (RI), was introduced to indicate the overall effect of both the pharmacological effects and disturbance effects due to fluctuating viral load on gene expression, defined as follows:

$$RI_{(gene\ A,\ test)} = \frac{\log_2 FC_{(gene\ A,\ test\ vs\ mock)}}{\log_2 FC_{(gene\ A,\ model\ vs\ mock)}} \quad (1)$$

To exclude the possible effects of fluctuating viral load on the expression of each given gene, we included a reference group (M2 or osel group). The estimated status (est.RI) of the indicated VRGs-up in response to the fluctuating viral load in the test group was predicted as follows:

$$Est.RI_{(gene\ A,\ test)} = \frac{Median\ RI_{(VRGs-up,\ test)}}{Median\ RI_{(VRGs-up,\ reference)}} \times RI_{(gene\ A,\ reference)} \quad (2)$$

where the ratio of median $RI_{(VRGs-up,\ test)}$ to median $RI_{(VRGs-up,\ reference)}$ was also designated as k_1 and used in subsequent analysis. The actual pharmacological effect of the antiviral therapeutic in the test group on the indicated gene was normalized by the following:

$$Norm.RI_{(gene\ A,\ test)} = RI_{(gene\ A,\ test)} - Est.RI_{(gene\ A,\ test)} \quad (3)$$

The VRGs with norm.RI values >0 were classified as response regression deviated DEGs, whereas the VRGs with norm.RI values <0 were classified as response regression transcended DEGs. Subsequently, the norm.RIs of each VRG-up for the test group were plotted against the related RI in the reference group. For response regression deviated genes, the DEGs of interest were identified with the following threshold:

$$y = k_2 / (k_1 x + a) \quad (4)$$

For response regression transcended genes, the DEGs of interest were identified with the following threshold:

$$y = k_2 / (k_1 x - k_1 - a) \quad (5)$$

where x indicates the RI of the indicated gene in the reference group, a is a constant introduced to eliminate the bias of sensitivity to pharmacological regulations for different VRGs, and k_2 is a flexible coefficient introduced to adjust the strictness for identifying the DEGs of interest. DEGs with $|norm.RI|$ values $>|threshold|$ were identified as DEGs of interest. In this study, a was forced to be 1, and the final k_2 was set to allow 10% of the total VRGs-up to be identified as hits.

The response regression deviated and transcended DEGs of interest for these VRGs-up were recognized as target genes that were up-regulated and down-regulated, respectively, by the test therapeutic.

(2) For the VRGs-down, the response regression deviation or transcendence was analyzed in the same manner as the VRGs-up, except that the response regression deviated and transcended DEGs of interest for these VRGs-down were recognized as target genes that were down-regulated and up-regulated, respectively, by the test therapeutic.

(3) For the non-VRGs, the transcriptomic profiles were compared between the test group and model group, and the genes with $p_{adj} < 0.05$ and $|\log_2 FC| > 1$ were defined as DEGs of interest.

2.6 Functional enrichment analysis of DEGs

To determine the biological functions or pathways affected by the antiviral therapeutics, we conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the up-regulated DEGs (up-DEGs) and down-regulated DEGs (down-DEGs) of interest, separately, with OmicShare tools [16].

3. RESULTS

3.1 Viral load fluctuation substantially perturbs host transcriptomic profiles

We previously developed a reporter IAV expressing Fluc, termed PR8-Fluc, and demonstrated that the infection and replication of this reporter IAV in mice can be monitored and quantified in real time [14]. In the current study, this reporter virus was used to examine and compare the transcriptomic profiles of mice under the following conditions: (1) mock-infected mice (mock group), (2) mice infected with the virus (2000 TCID₅₀/mouse, model group), (3) mice infected with the virus at a lower dose (200 TCID₅₀/mouse, M2 group), (4) mice infected with the virus and treated with oseltamivir phosphate (osel group), and (5) mice infected with the virus and treated with recombinant IFN- α 2b (IFN group). Briefly, female BALB/c mice were mock or nasally inoculated with various doses of PR8-Fluc, and then subjected to antiviral treatment or left untreated. At day 2 p.i., the viral loads in mice in all groups were monitored with an in vivo imaging technique (Figure 1A), as previously described [14].

As expected, luminescence signals indicative of the viral loads had relatively high intensity in the model group, whereas the M2 group had lower signals consistent with the lower viral dose (Figure 1B). In addition, the markedly diminished infection signals in both the osel and IFN groups suggested antiviral efficacy (Figure 1B). Of note, IFN treatment decreased the infection signals to an intensity similar to that in the M2 group, whereas osel treatment led to much greater signal attenuation, thus suggesting differing efficacy of the two antiviral agents in viral load reduction at the administered doses.

Immediately after imaging acquisition, the mice in all groups were sacrificed, and their lung tissues were subjected to transcriptomic sequencing analysis. To evaluate the effects of viral load fluctuation on host transcriptomic profiles, we compared the data for the infected groups vs the mock group. The DEGs were defined as those with $|\log_2 FC| > 1$ and $p_{adj} < 0.05$. The model vs mock comparison identified 1371 up-DEGs and 541 down-DEGs, thus indicating a relatively strong host

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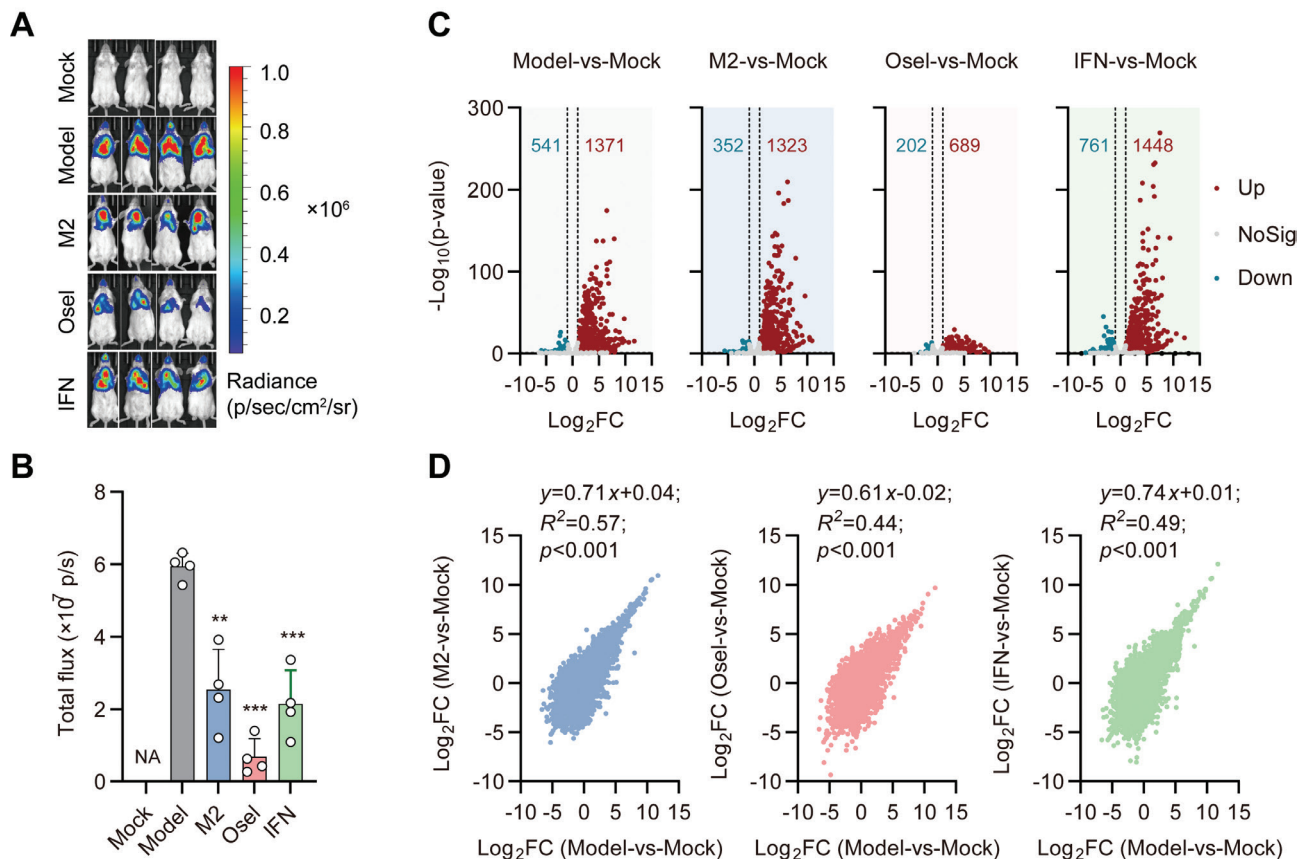


Figure 1 | Perturbance effects of viral load fluctuation on host transcriptomic profiles.

Groups of female BALB/c mice (4–6 weeks, $n=4/\text{group}$) were infected with recombinant influenza A virus for expression of firefly luciferase (PR8-Fluc) at a dose of 2000 TCID₅₀/mouse. Infected mice receiving no treatment served as the model group, whereas those receiving treatment with recombinant interferon- $\alpha 2b$ or oseltamivir phosphate were designated as the IFN and osel groups, respectively. Mice infected with a much lower dose (200 TCID₅₀/mouse) of PR8-Fluc served as a reference group, denoted M2. Mice in the mock group were mock infected and served as healthy controls. At day 2 post infection, the mice in all groups were subjected to live imaging to monitor viral loads in real time. Then the mice were sacrificed, and the lung tissues were subjected to transcriptomic sequencing. **(A)** In vivo bioluminescence imaging photos. **(B)** Analysis of bioluminescence signals from mice in each group. **(C)** Transcriptomic comparison between the virus-infected and mock-infected groups. **(D)** Overall correlation of the FCs for individual host factors in the virus-infected groups vs the mock group.

response to viral infection (**Figure 1C**). In contrast, the M2 vs mock comparison indicated 1323 up-DEGs and 352 down-DEGs; because these numbers were smaller than those from the model vs mock comparison, a slightly weaker host response appeared to have been triggered by the lower viral load in the M2 group. Moreover, only 689 up-DEGs and 202 down-DEGs were identified from the osel vs mock comparison, thus suggesting a much weaker host response, in agreement with the markedly diminished viral load in the osel group. Of note, although the mice in the IFN and M2 groups had similar viral loads, more DEGs were identified in the IFN group (1448 up-DEGs and 761 down-DEGs) than the M2 group or the model group, probably because of additional pharmacological modulatory effects of recombinant IFN- $\alpha 2b$ on host transcriptomic profiles.

To better evaluate the overall relationship between the host response and viral load, we separately plotted the $\log_2\text{FC}$ of each gene from the M2/osel/IFN vs mock comparisons, against that from model vs mock comparison. The $\log_2\text{FC}$ values of individual genes from the M2 vs mock, osel vs mock, or IFN vs mock comparisons positively correlated with those from the model vs mock comparison, with slope values <1 indicative of weaker host responses (**Figure 1D**). Moreover, as the M2 and IFN groups showed similar viral loads, the slopes were nearly identical for the “M2 vs mock comparison” (0.71) and “IFN vs mock comparison” (0.74), whereas “osel vs mock comparison” exhibited a much smaller slope (0.61), in line with the much lower viral loads in the osel group. These data clearly demonstrated that the changes in host transcriptomic profiles in response

to viral infection depend greatly on the intensity of viral load.

3.2 Conventional transcriptomic comparison between virus-infected models with or without antiviral treatment generates biased DEGs

The transcriptomic profiles of a disease model with or without therapeutic treatment were subjected to pairwise comparison to identify DEGs that might be potential targets of the pharmacological effects. We also sought to determine whether this conventional analytic strategy was suitable for the pharmacological mechanistic study of antiviral treatments. To this end, we separately compared the transcriptomic profiles of the M2, osel, and IFN groups vs the model group, thus identifying 2/6, 132/172, and 99/93 up/down-DEGs, respectively (Figure 2A). This finding contradicted the knowledge that osel is a DAA that should pose little pharmacological effect on host factors, whereas IFN is an HTA that blocks viral replication by regulating the expression of a cascade of host genes.

Next, we assessed the responsiveness of the DEGs identified through conventional transcriptomic comparison between the M2/osel/IFN and model groups with IAV infection. The up-DEGs were more readily down-regulated in response to IAV infection, whereas the down-DEGs were prone to up-regulation after IAV infection (Figure 2B). These biased DEGs therefore principally reflected the weakened host response as a result of decreased viral load.

To better understand the biological implications of these biased DEGs, we separately plotted the overall $\log_2FC$ values of individual host factors from the M2/osel/IFN vs model comparisons against those from the model vs mock comparison. The $\log_2FC$ values of individual genes from the M2 vs model, osel vs model, and IFN vs model comparisons negatively correlated with those from the model vs mock comparison (Figure 2C). "M2 vs model comparison" and "IFN vs model comparison" had nearly identical slope values of -0.39 and -0.38 , respectively, whereas "osel vs model comparison" had a smaller slope (-0.52), in agreement with the relatively lower viral loads and weaker host responses in the M2 and IFN groups, and the much lower viral loads and much weaker host responses in the osel group, than those in the model group.

Together, our data suggested that conventional transcriptomic analysis by pairwise comparison between infected models with or without therapeutic treatment generated substantially biased DEGs, thus compromising its use for pharmacological mechanistic investigation of antiviral agents, particularly HTAs.

3.3 Expression of viral responsive genes tends to regress toward baseline with decreasing viral load

To specifically identify the host factors potentially affected by the pharmacological effects of an antiviral agent, the perturbations in the host transcriptomic profile due to

fluctuating viral kinetics should be excluded. Of note, the sensitivity of individual genes to the perturbation effect of viral load fluctuation substantially varies depending on their responsiveness to viral infection. We speculated that the expression levels of VRGs would highly correlate with viral load intensity, whereas those of non-VRGs would remain at baseline regardless of the presence of viral infection and any viral load fluctuation.

We therefore divided the host genes into VRGs and non-VRGs, primarily according to the transcriptomic comparison between the model and mock groups. Briefly, the 1371 up-DEGs and 541 down-DEGs identified from the model vs mock comparison were defined as VRGs-up and VRGs-down, respectively, whereas the others were classified as non-VRGs (Figure 3A). The expression of each non-VRG in all four infected groups (model/M2/osel/IFN) was separately compared with that in the mock group, and the $\log_2FC$ values of individual non-VRGs were determined (Figure 3B). As expected, the overall $\log_2FC$ values of the non-VRGs in each comparison were scattered around 0, thus suggesting the homeostasis status of these genes regardless of the presence of viral infection or viral load fluctuation.

In contrast, the expression of VRGs-up tended to be lower (Figure 3C), whereas the expression of VRGs-down tended to be higher (Figure 3D), in the M2/osel/IFN groups than the model group, in accordance with lower viral load and weakened host response. To summarize, the response intensities of both VRGs-up and VRGs-down tended to regress toward baseline as the viral load decreased, and the degree of response regression was determined by the extent of the viral load decrease.

Therefore, for non-VRGs, conventional transcriptomic analysis could rationally be used to conduct pairwise comparisons between infected models with or without therapeutic treatment, to identify potential targets of the pharmacological effects of antiviral agents (Figure 3E). However, for VRGs, an adapted transcriptomic analysis strategy was necessary to distinguish the specific pharmacological effects from the perturbation effects caused by viral load fluctuations (Figure 3E).

3.4 Development of a method to distinguish specific pharmacological effects from perturbation effects of fluctuating viral kinetics on virus-responsive genes

We attempted to develop a mathematical method to distinguish the pharmacological effects of antivirals on VRGs, in the presence of perturbations due to fluctuating viral kinetics. First, an RI defined by equation (1) was introduced to measure the extent of response regression for the VRGs. An RI value of 1 indicated no regression, whereas an RI value of 0 indicated complete regression to baseline. The RI of each VRG in the M2, osel, and IFN groups, according to the diminished viral load, was then calculated separately. As expected, the overall RIs of either VRGs-up or VRGs-down in all three groups tended to decrease toward 0, thus suggesting a

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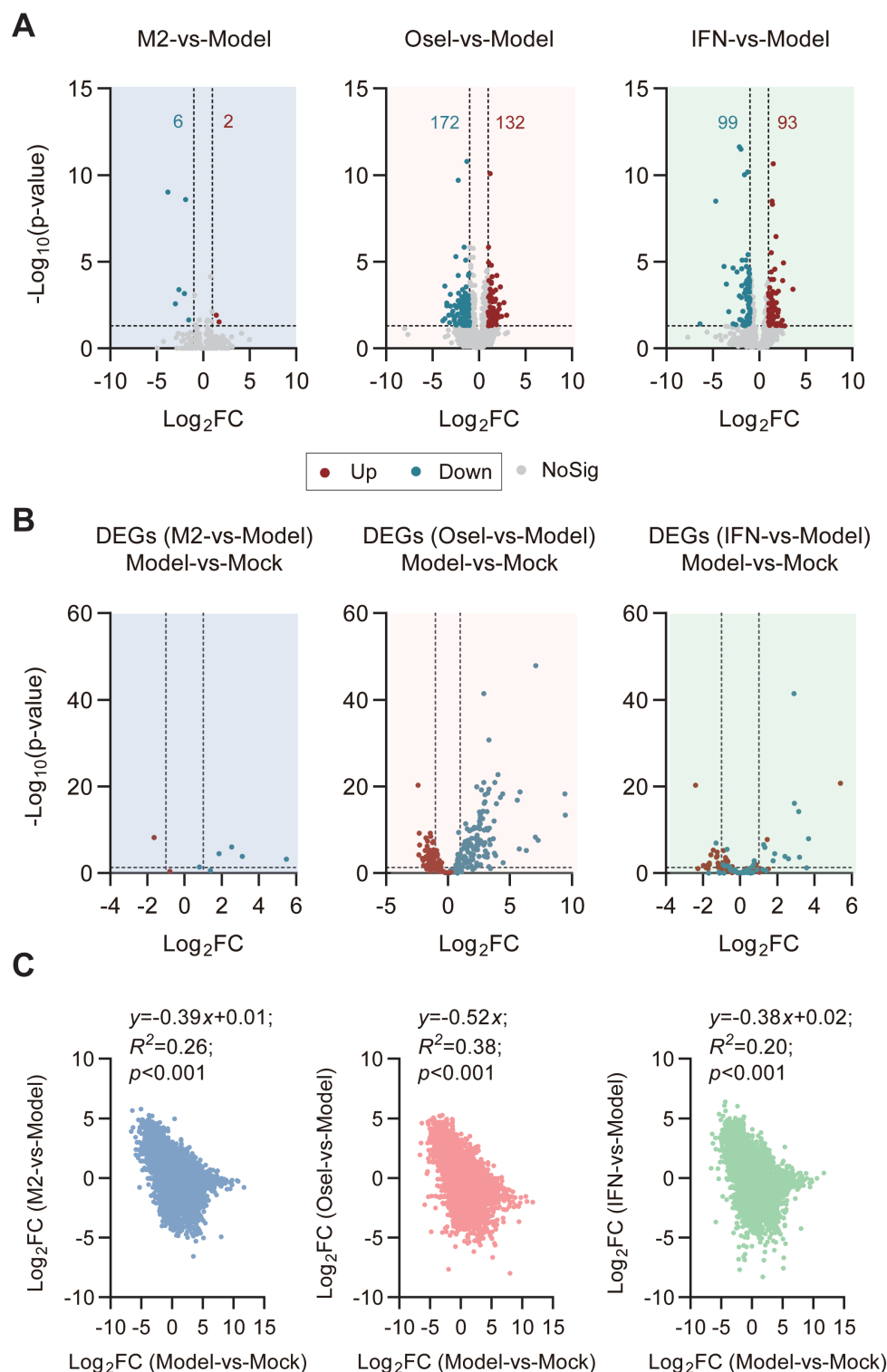


Figure 2 | Biased identification of differentially expressed genes (DEGs) via conventional pairwise transcriptomic comparison.

(A) Conventional transcriptomic analysis comparing transcriptomic profiles of the M2 vs model, osel vs model, and IFN vs model groups separately. (B) The DEGs identified by M2 vs model, osel vs model, and IFN vs model comparisons separately, were selected. The transcriptomic profiles of these DEGs in the model and mock groups were then pairwise compared. (C) Overall correlation of the FCs for individual host factors for M2 vs model, osel vs model, and IFN vs model comparisons and the model vs mock comparison.

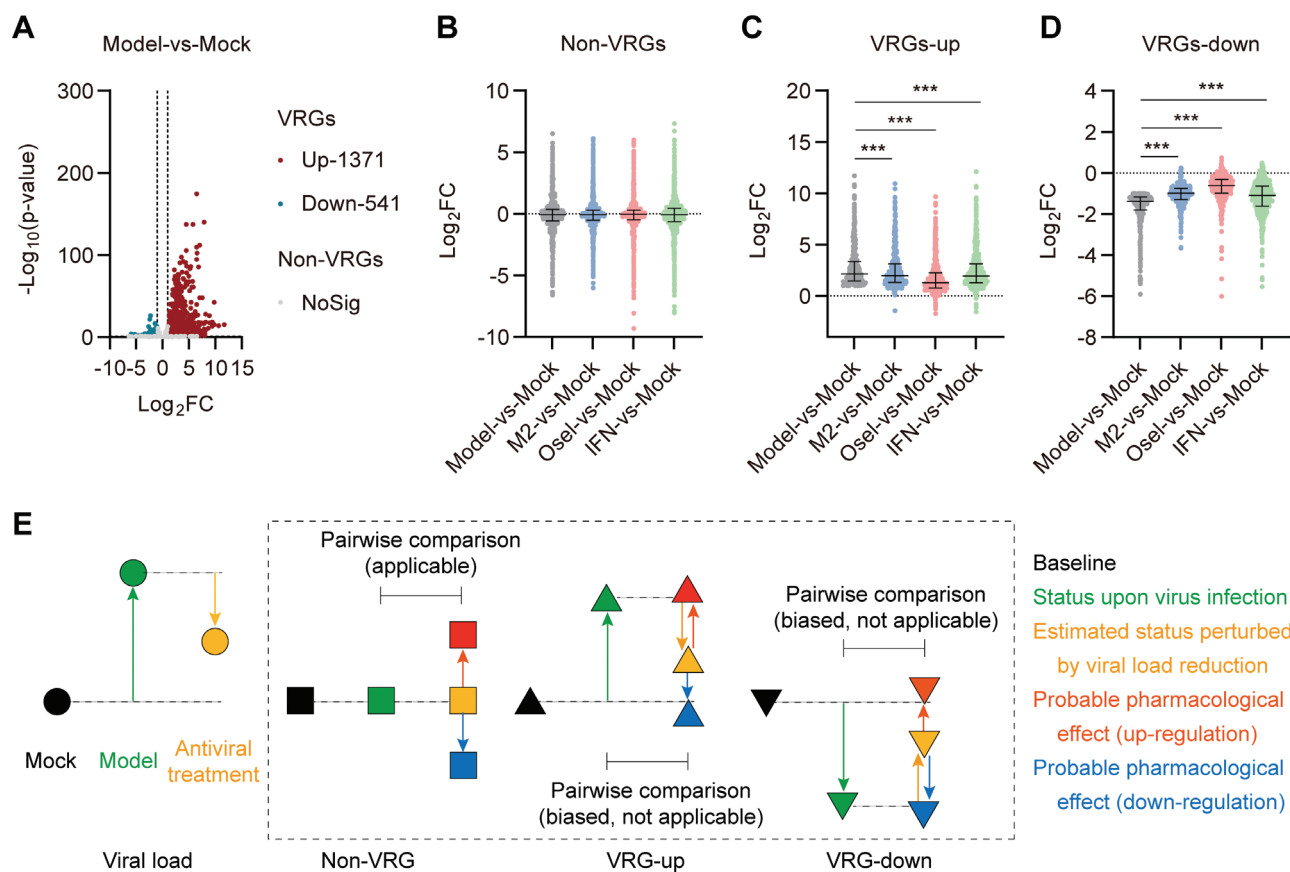


Figure 3 | Tendency of virus-responsive factors to regress toward baseline with decreasing viral load.

(A) Comparison of the transcriptomic profiles between model and mock groups, identifying 1371 up-regulated virus-responsive genes (VRGs-up), 541 down-regulated VRGs (VRGs-down), and non-VRGs. (B) The $\log_2FC$ values of non-VRGs for virus-infected vs mock groups. (C) The $\log_2FC$ values of VRGs-up for virus-infected vs mock groups. (D) The $\log_2FC$ values of VRGs-down for virus-infected vs mock groups. (E) Varying statuses of non-VRGs, VRGs-up, and VRGs-down after viral infection in the absence or presence of potential pharmacological regulation and/or perturbation effects caused by decreased viral load. ***, $p < 0.001$, Student's t test.

marked response regression in line with the decreased viral load (Figure 4A). Of note, the median RIs for these VRGs-up or VRGs-down had similar values in the M2 and IFN groups, whereas the values in the osel group were much smaller, thus suggesting a correlation between the regression extent of VRGs and the degree of viral load decrease. Next, we conducted a pilot study focused on the potential pharmacological effects of recombinant IFN- $\alpha 2b$ on VRGs-up. The transcriptomic profile in the M2 group served as a reference for estimation of the status of response regression for each VRG-up according to the diminished viral load after IFN treatment, because the mice in the M2 group had a lower viral load but received no pharmacological treatment. The RI value of each VRG-up in the IFN and M2 groups was plotted (Figure 4B), and the estimated RI-IFN (est. RI-IFN) for each VRG-up in response to diminished viral load was calculated with the equation $y = k_1 x$, where y and x represent the est. RI-IFN and RI-M2 of the indicated VRG-up, respectively, and k_1 is a constant defined

as the ratio between the median RI-IFN and median RI-M2 among all VRGs-up.

Subsequently, the RI-IFN value of each VRG-up was normalized as the difference between the actual and estimated RI-IFN values with the equation $y = y_0 - k_1 x_0$, where y indicates the normalized RI-IFN (norm. RI-IFN), and y_0 and x_0 indicate the original RI-IFN and RI-M2 for individual VRGs-up, respectively. We assumed that the norm. RI-IFN reflected the pharmacological effect of IFN- $\alpha 2b$ on the indicated VRG-up; positive or negative values indicated response regression deviation or transcendence, respectively; and the absolute value represented the extent of deviation/transcendence.

The norm. RI-IFN for each VRG-up was subsequently plotted against RI-M2 (Figure 4C). Notably, the genes with RI-M2 values approaching 0 tended to be response regression deviated, whereas VRGs with RI-M2 values approaching 1 were more sensitive to the response regression transcendence regulation. This phenomenon can be explained by the varying sensitivities of VRGs-up

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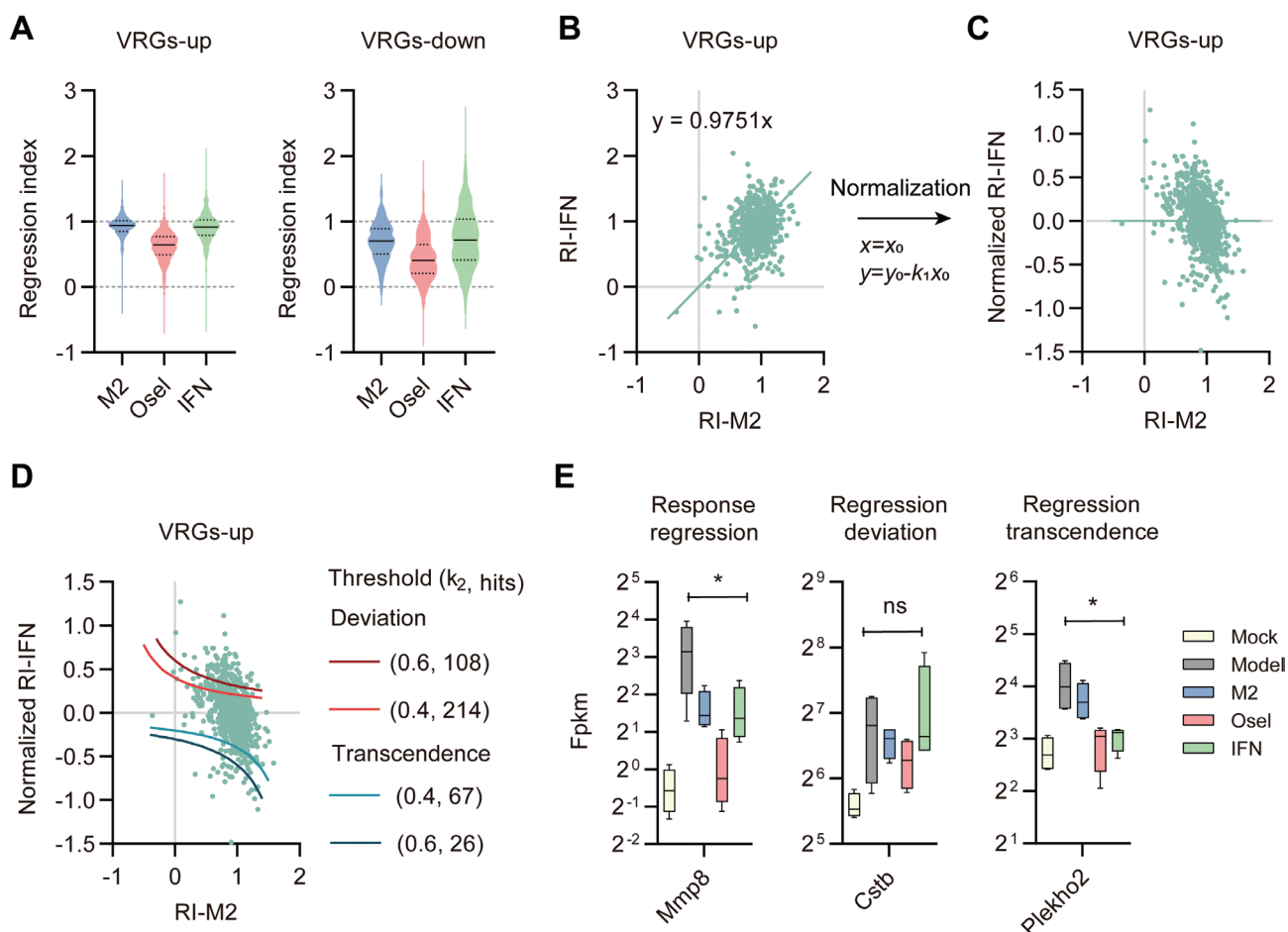


Figure 4 | Development of a method to identify response regression deviated or transcended VRGs.

(A) Regression indexes (RIs) of VRGs-up and VRGs-down in the M2, osel, and IFN groups compared with the model and mock groups. (B) Plot of RIs of each VRG-up in the IFN and M2 groups. The regression line was set according to the equation $y = k_1 x$; where k_1 is the ratio of the median RI value for VRGs-up in the IFN group to that in the M2 group. (C) RI of each VRG-up in the IFN group, normalized and plotted against the related RI in the M2 group. (D) Variable thresholds were set to identify the response regression deviation and transience for the VRGs. The coefficient k_2 was introduced to adjust the strictness of thresholding. (E) Expression patterns of typical VRGs-up with proper response regression, regression deviation, and regression transience in all groups. * and ns indicate genes identified as DEG or non-DEG through conventional pairwise comparison, respectively.

to viral load fluctuation, leaving individual VRGs-up with different spaces for detection of potential pharmacological up- or down-regulatory effects. To eliminate this intrinsic bias, we set variable thresholds with equations $y = k_2 / (k_1 x + a)$ and $y = -k_2 / (k_1 x - a k - 1)$ to screen genes with response regression deviation and transience, respectively. The constant parameter a was introduced to offset the bias due to the varying sensitivity among genes, whereas k_2 was flexible and was used to adjust the strictness of screening (Figure 4D). To simplify the mathematical optimization, we forced the parameter a to equal 1, and adjusted k_2 to include the top 10% deviated or transcended genes among all VRGs-up as DEGs of interest.

The typical VRGs-up with proper response regression, regression deviation, or regression transience are

shown in Figure 4E. As expected, all these genes were markedly up-regulated in the model group, and the lower dose of infection in the M2 group induced weaker up-regulation. In addition, osel treatment further decreased the expression of all these genes, in agreement with the markedly lower viral load in the osel group. However, after IFN treatment, the expression of these VRGs varied. Compared with that in the model group, (1) the expression of the *Mmp8* gene in the IFN group was lower and similar to that in the M2 group, in agreement with the similar viral load decrease in the IFN and M2 groups, thus suggesting proper response regression; (2) the expression of the *Cstb* gene in the IFN group did not change with the diminished viral load but remained at a similar level to that in model mice,

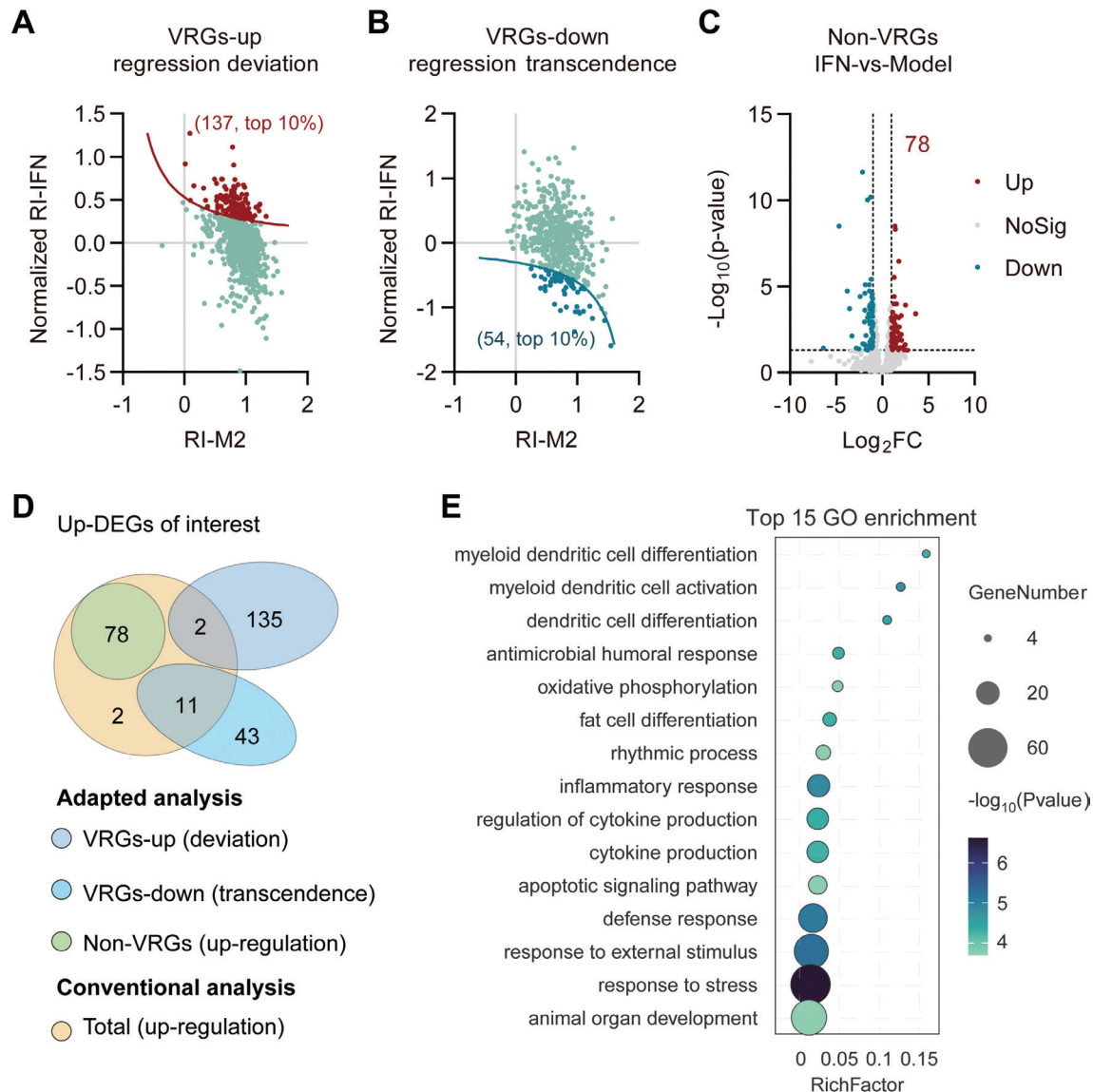


Figure 5 | Feasibility of the adapted transcriptomic analysis strategy.

(A) The top 10% response regression deviated genes among the total VRGs-up were selected as up-DEGs of interest, which were expected to be specifically up-regulated by IFN treatment. (B) The RI-IFN of each VRG-down was normalized with M2 as a reference and plotted against RI-M2. The top 10% response regression transcended VRGs-down were selected as up-DEGs of interest for IFN treatment. (C) Conventional transcriptomic comparison for non-VRGs in mice between the IFN and model groups. (D) Venn diagram of the DEGs for IFN treatment identified with the adapted analysis strategy and the conventional transcriptomic comparison between the IFN and model groups. (E) GO enrichment analysis with the up-DEGs of interest for IFN treatment. The top 15 enriched GO terms are displayed.

thus suggesting a response regression deviation; (3) the expression of the *Plekho2* gene in the IFN group was down-regulated to a level exceeding that in the M2 group and comparable to that in the osel group, thus suggesting response regression transcendence. We speculated that the response regression deviated and transcended VRGs-up were probably up- or down-regulated, respectively, by the pharmacological effects of recombinant IFN- α 2b.

Mmp8 was identified as a DEG from the conventional model vs IFN pairwise comparison but was excluded as false positive with the adapted analysis strategy guided by the response regression model. In contrast, the *Cstb* gene was identified as a non-DEG from the conventional model vs IFN pairwise comparison but was identified with the adapted method as a hit that was potentially up-regulated by the indicated antiviral agent. The novel transcriptomic analysis method demonstrated distinctive

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advantages over the conventional direct pairwise strategy in the accuracy of DEG identification.

3.5 Validation of feasibility of the adapted transcriptomic analysis strategy

To validate the feasibility of our adapted transcriptomic analysis strategy, we focused on the transcriptomic profile alterations in the lungs of infected mice after IFN- α 2b treatment. Because we anticipated that recombinant IFN- α 2b induces a cascade of antiviral proteins, we focused on genes potentially upregulated by recombinant IFN- α 2b. A total of 137 (top 10%) response regression deviated genes among the 1371 total VRGs-up were identified as up-DEGs of interest (Figure 5A). In contrast, the VRGs-down with response regression transcendence were expected to be up-regulated by antiviral treatments. The RIs-IFN of the VRGs-down were normalized and plotted against the reference RIs-M2 (Figure 5B), and 54 (top 10%) response regression transcended genes among the total 541 VRGs-down were selected as up-DEGs of interest after IFN- α 2b treatment. Furthermore, direct IFN vs model comparison of the expression profiles of non-VRGs uncovered an additional 78 up-DEGs potentially influenced by recombinant IFN- α 2b (Figure 5C). Collectively, with the adapted analysis strategy, we identified 269 up-DEGs of interest as targets potentially influenced by the pharmacological effects of recombinant IFN- α 2b.

Venn diagram analysis was performed to visualize the distinction between the up-DEGs identified with the adapted strategy vs conventional transcriptomic analysis from the IFN vs model comparison. Except for the non-VRGs, only 2 of 137 response regression deviated VRGs-up and 11 of 54 response regression transcended VRGs-down were identified as DEGs through conventional analysis (Figure 5D). In addition, two VRGs identified as DEGs from the conventional IFN vs model comparison were excluded as false positives with the adapted strategy (Figure 5D).

To further examine the reliability of the adapted analysis strategy in identifying host factors affected by the pharmacological effects of recombinant IFN- α 2b, we subjected the 269 total up-DEGs of interest to GO enrichment analysis to determine their potential functions (top 15 enriched biological processes in Figure 5E). Interestingly, the pleiotropic regulatory effects of IFN- α 2b on most of these enriched biological processes have been well documented, including (myeloid) DC differentiation/myeloid DC activation [17] and subsequent antimicrobial humoral response [18, 19], oxidative phosphorylation [20], fat cell differentiation [21], rhythmic process [22, 23], defense response/response to stress (or external stimulus)/inflammatory response [24-26], (regulation of) cytokine production [24, 27], and apoptotic signaling pathway [28, 29].

Therefore, we developed a novel feasible method for transcriptomic analysis to identify host factors potentially influenced by the pharmacological effects of HTAs.

3.6 Oseltamivir is a sub-optimal reference because of potent off-target effects

In anti-influenza pharmacodynamic studies, oseltamivir is frequently used as a positive control. Because oseltamivir is a well-known DAA presumed to have little effect on host transcriptomic profiles, we asked whether oseltamivir might be a suitable reference to identify target genes potentially influenced by HTAs. Using the adapted strategy described above, we separately normalized the RI-IFN values of VRGs-up and VRGs-down to related RI-oseltamivir values, then selected the top 10% response regression deviated VRGs-up (number=137, Figure 6A) and transcended VRGs-down (number=54, Figure 6B) as up-DEGs of interest for IFN treatment. Unexpectedly, the up-DEGs of interest of IFN identified with oseltamivir or M2 as a reference were highly distinctive, thus generating only 44 (approximately 32%) and 15 (approximately 27%) common hits from VRGs-up and VRGs-down, respectively (Figure 6C).

To unravel the possible reasons underlying this low overlap rate, we compared the detailed expression patterns of the controversial up-DEGs of interest with either M2 or oseltamivir as a reference. Typically, two VRGs-up, *Spon2* and *Gm32633*, were specifically identified as up-DEGs of interest with the M2 but not the oseltamivir group as a reference (Figure 6D). According to the response regression model, both the *Spon2* and *Gm32633* genes were up-regulated after viral infection in the model group, whereas the lower dose of infection in the M2 group induced weaker up-regulation. However, the expression of the two VRGs-up did not regress toward baseline as the viral load decreased after oseltamivir or IFN treatment, thus suggesting a similar response regression deviation. In contrast, the VRGs-up *Mafb* and *F3* were specifically identified as up-DEGs of interest with the oseltamivir but not the M2 group as a reference (Figure 6E). Both genes showed similar degrees of response regression toward baseline in the M2 and IFN groups, in accordance with the decreased viral load. However, with oseltamivir treatment, the expression levels nearly returned to baseline, thus suggesting potent response regression transcendence. These findings suggested that oseltamivir might have off-target effects on hosts, and caution is necessary in mechanistic studies of novel anti-influenza HTAs using oseltamivir as a reference.

3.7 Exploration of potential secondary pharmacological effects of oseltamivir

Next, we attempted to explore the off-target effects of oseltamivir through adapted transcriptomic analysis with M2 as a reference. Because no preset direction preference existed for the off-target regulatory effects on host factors for oseltamivir, we selected both response regression deviated and transcended hits from these VRGs-up and VRGs-down, separately (Figure 7A and B), as well as the up- and down-regulated DEGs from non-VRGs (Figure 7C). Collectively, 267 up- and 231 down-DEGs of interest were identified as targets potentially

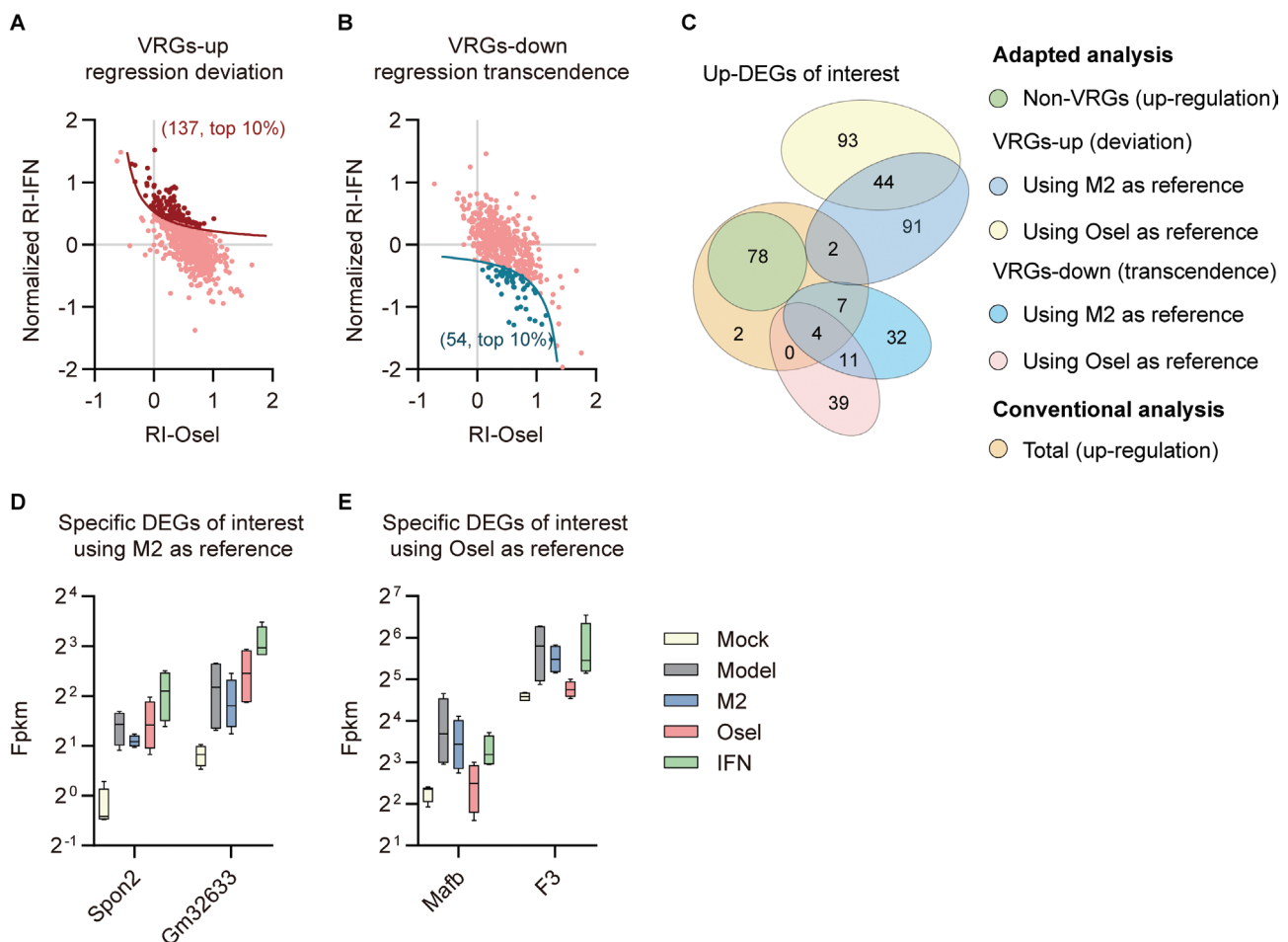


Figure 6 | Identification of up-regulated DEGs of interest for IFN treatment, with osel as a reference.

(A) The RI-IFN of each VRG-up was normalized with the osel group as a reference, and the top 10% response regression deviated genes were selected as up-DEGs of interest for IFN treatment. (B) The RI-IFN of each VRG-down was normalized with the osel group as a reference, and the top 10% response regression transcended genes were selected as up-DEGs of interest for IFN treatment. (C) Venn diagram of the up-DEGs of interest identified with M2 and osel as references, separately. (D) Expression patterns of two typical up-DEGs of interest specifically identified with M2 as a reference. (E) Expression patterns of two typical up-DEGs of interest specifically identified with osel as a reference.

influenced by osel treatment (Figure 7D). Notably, 33 of the 132 up-DEGs and 80 of the 172 down-DEGs identified through direct osel vs model comparison were ruled out as false positives with the adapted method. In addition, conventional transcriptomic analysis of the direct osel vs model comparison was unable to recognize these response regression deviated VRGs-up and VRGs-down as DEGs.

To better understand the nature of the overlapping or controversial DEGs of interest identified from VRGs through adapted or conventional transcriptomic analysis, we assessed the expression patterns of typical up-DEGs of interest for osel treatment. Of note, the response regression deviated VRGs-up rarely exhibited significant differential expression from the osel vs model comparison (Figure 7E), whereas the expression patterns for VRGs-down substantially varied. For instance, Figure

7F shows two typical VRGs-down that were identified as DEGs through conventional transcriptomic analysis but excluded as false positives through the adapted strategy. Notably, the expression pattern for the two genes among groups perfectly matched the viral load fluctuation. However, although a substantial portion of those response regression transcended VRGs-down were also detected as up-DEGs in the osel vs model comparison (Figure 7G), the remainder were not detected in conventional transcriptomic analysis, probably because of varying sensitivity of these genes to viral load fluctuation (Figure 7H).

Subsequently, we performed KEGG enrichment analysis of the up- and down-DEGs of interest, separately, to further identify the biological functions or pathways influenced by osel. The top 15 pathways enriched in the up- and down-DEGs of interest for osel treatment are

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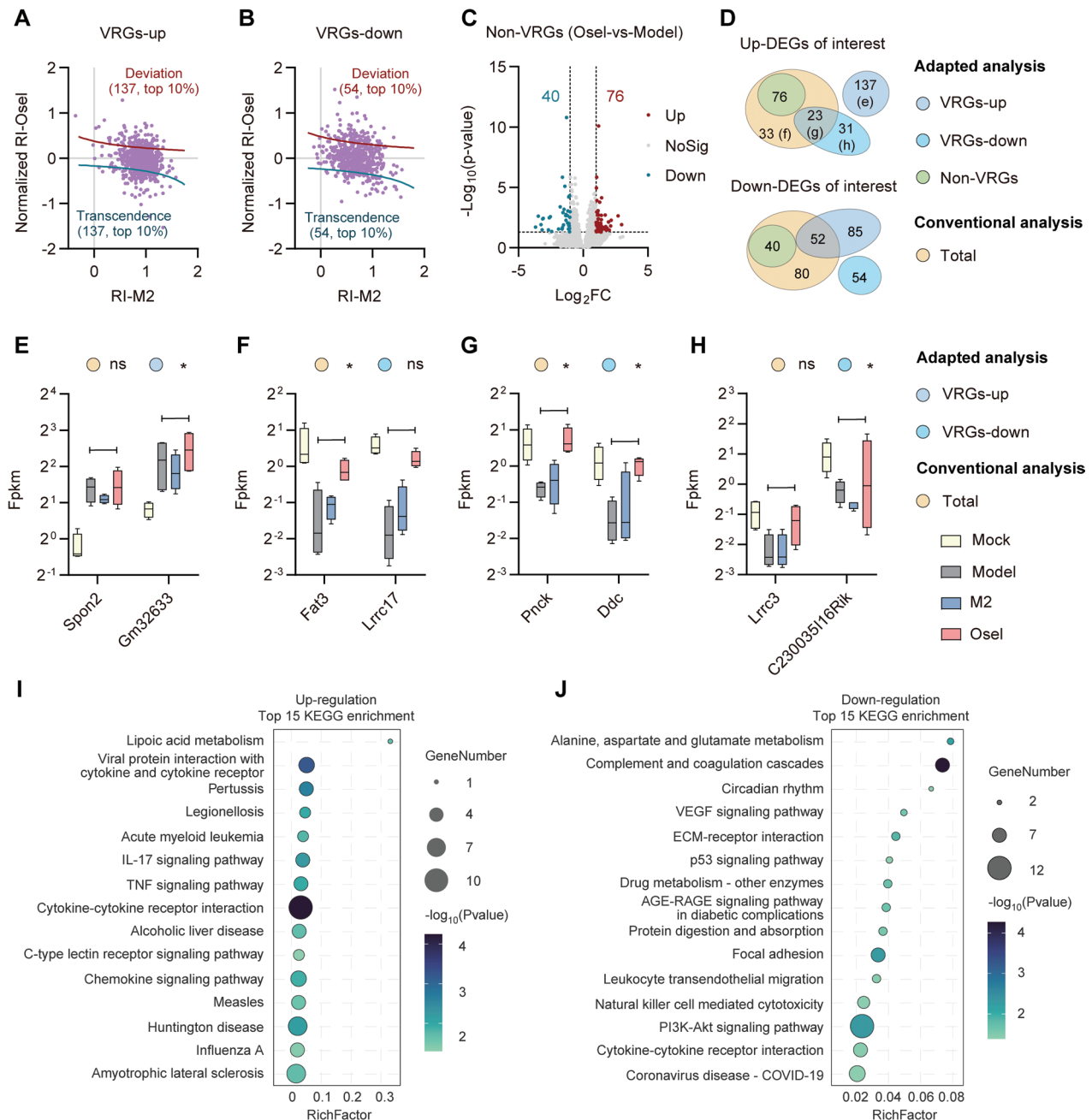


Figure 7 | Identification of host factors specifically influenced by osel treatment and contributing to off-target effects.

(A) The RI-osel of each VRG-up was normalized with M2 as a reference, and the top 10% response regression deviated or transcended genes were selected as up- or down-DEGs of interest for osel treatment, respectively. (B) The RI-osel of each VRG-down was normalized with M2 as a reference, and the top 10% response regression deviated or transcended genes were selected as down- or up-DEGs of interest for osel treatment, respectively. (C) Comparison of the transcriptomic profiles of non-VRGs between the osel vs model groups. (D) Venn diagram of the up- and down-DEGs of interest identified with adapted or conventional analysis methods. (E) Expression patterns of two typical response regression deviated VRGs-up identified as up-DEGs of interest with the adapted but not the conventional analysis strategy. (F) Expression patterns of two typical VRGs-down with proper response regression that were identified as up-DEGs with the conventional but not the adapted analysis strategy. (G) Expression patterns of two typical response regression transcended VRGs-down identified as up-DEGs of interest with both the adapted and conventional analysis strategies. (H) Expression patterns of two typical response regression deviated VRGs-down identified as up-DEGs of interest with the adapted but not the conventional analysis strategy. * and ns indicate that the target gene was and was not identified as a DEG of interest with the indicated analysis strategy, respectively. (I, J) KEGG enrichment analysis of the total 267 up-DEGs of interest (I) or 231 down-DEGs of interest (J) after osel treatment. The top 15 enriched pathways are displayed.

shown in **Figure 7I and 7J**, respectively. Interestingly, among these enriched KEGG pathways, pharmacological effects on Huntington disease and the VEGF signaling pathway have been reported [30–32]. In the future, further validation of the potential pharmacological effects of osel on other enriched KEGG pathways would be valuable. These results further demonstrated the feasibility of the adapted transcriptomic analysis strategy for identification of target genes potentially influenced by antiviral treatments.

4. DISCUSSION

In this study, we sought to solve a critical problem in which viral load fluctuation during the time course of infection markedly perturbs the host response, which can be readily detected through diverse techniques. After antiviral treatment, as viral load decreases, the host responses tend to regress toward baseline. These suppressed host responses together with diminished viral load have been widely used as indicators of antiviral efficacy. However, in pharmacological mechanistic studies of antiviral agents, particularly HTAs, the perturbation effects of fluctuating viral kinetics on the host response are a major obstacle. The host launches cascades of antiviral responses after viral infection, to restrict or eliminate the invading pathogens [1], and these virus-responsive factors may be targets of HTAs. After HTA treatment, the host responses might be affected by the pharmacological effects, perturbations due to fluctuating viral kinetics, or both (**Figure 8**).

Although pairwise transcriptomic comparison of disease models with vs without treatment is valuable to explore the pharmacological mechanisms of diverse therapeutics, this pairwise analysis strategy is substantially limited for HTAs. Our results clearly demonstrated that conventional transcriptomic analysis between groups with vs without antiviral treatment can lead to biased identification of DEGs reflecting the perturbation of host transcriptomic profiles due to fluctuating viral kinetics. Of note, the extent of bias largely depends on the degree of viral load fluctuation.

To distinguish the pharmacological effect of HTAs from the disturbances caused by fluctuating viral kinetics, we proposed using a response regression model as a guiding principle. In transcriptomic analysis, the host factors were divided into non-VRGs and VRGs according to responsiveness to viral infection. Because the non-VRGs were anticipated to remain in homeostasis regardless of the presence of viral infection, the DEGs could reasonably be identified from non-VRGs by conventional transcriptomic comparison as potential targets of the pharmacological effects (**Figure 8A**). However, the status of VRGs was estimated to regress toward baseline in accordance with the diminished viral load after antiviral treatment. If HTAs have additional regulatory effects on individual VRGs, the estimated response regression status should be deviated or transcended. Of note, the

response regression deviation and transcendence indicated up-regulation and down-regulation of VRGs-up, respectively, by the HTA (**Figure 8B**). In contrast, up-regulation and down-regulation of VRGs-down by the HTA should result in response regression transcendence and deviation, respectively (**Figure 8C**).

We additionally developed a practical mathematical method to estimate the status of individual host factors that respond to viral load fluctuation and should be excluded from consideration in pharmacological mechanistic studies of HTAs. A novel parameter, RI, was introduced to measure the extent of response regression for individual VRGs after antiviral treatment. In addition, a reference group receiving a lower infection dose was used to estimate the status of individual VRGs in response to fluctuating viral kinetics. Subsequently, the difference between the actual and estimated RI values of each VRG was calculated (norm.RI) to indicate the potential pharmacological effect of antiviral treatment. Because a positive or negative norm.RI value indicated response regression deviation or transcendence, respectively, the absolute value represented the extent of regulation.

Recombinant IFN- α 2b has been widely used as an HTA that enhances host innate immunity [33, 34]. To provide a proof of concept, we identified the target genes potentially influenced by IFN- α 2b by using the adapted transcriptomic analysis strategy. With this method, numerous VRGs that were identified as non-DEGs through conventional analysis. A substantial proportion of DEGs identified through conventional transcriptomic comparison were excluded as false positives. Notably, enrichment analysis of the total up-DEGs of interest with recombinant IFN- α 2b treatment illustrated that most enriched biological functions have been reported to be readily influenced by type I IFNs, thus demonstrating the accuracy and feasibility of our adapted transcriptomic analysis strategy for pharmacological mechanistic study of HTAs.

Notably, the reference data used to exclude the host perturbations due to viral load fluctuations play a key role in the adapted analysis method. We initially used a group of animals with a lower dose of infection as a reference, thus generating a set of transcriptomic data purely reflecting the perturbations in host responses because of fluctuating viral kinetics. However, in practice, in anti-influenza mechanistic studies, the approved DAA osel is frequently used as a positive control. We demonstrated that osel was not an ideal reference for adapted transcriptomic analysis under the guidance of the response regression model, because of its considerable off-target effects on the host, and its potential to produce extra false positives and negatives.

The off-target effects of osel are increasingly being explored. For example, Singh et al. have demonstrated the neuro-shielding property of osel and repurposed osel to mitigate polyQ-induced toxicity in Huntington disease [30]. Furthermore, because growth factors

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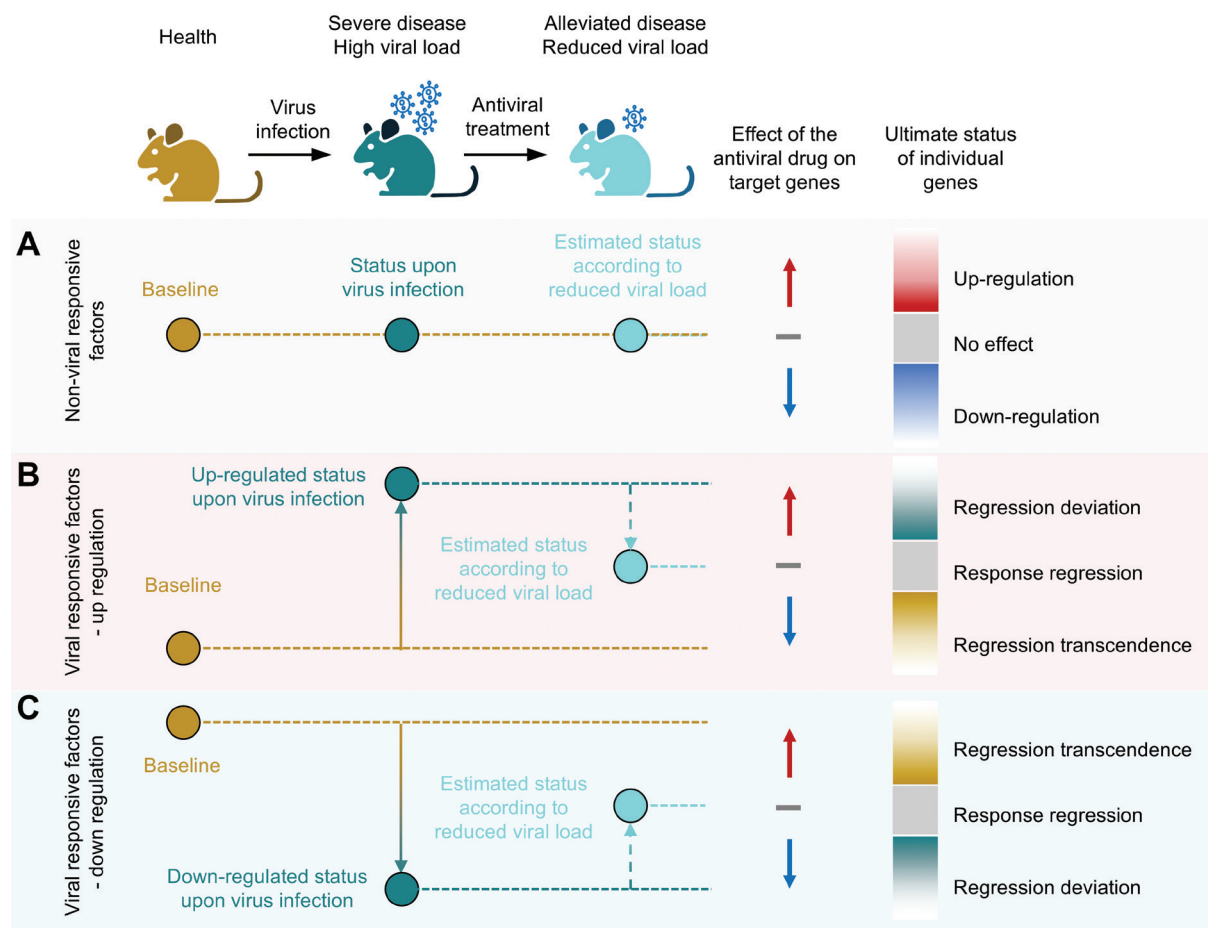


Figure 8 | Representative diagram of the response regression model.

In response to viral infections, a proportion of host factors are up- or down-regulated. The extent of regulation is expected to highly correlate with the intensity of the viral stimulus, whereas the others may be non-virus-responsive. After antiviral treatment, the viral load usually decreases. The status of non-virus-responsive factors can reasonably be assumed to maintain homeostasis during the time course of infection, whereas virus-responsive host factors should tend to regress toward baseline in accordance with decreasing viral load. To identify the host factors specifically influenced by an antiviral treatment, the disturbance caused by fluctuating viral load should be excluded. **(A)** For non-virus-responsive factors, the target can be identified by comparing the status of the indicated factor in virus-infected samples with vs without antiviral treatment. **(B)** For factors up-regulated in response to viral infection, the status is expected to regress toward baseline in accordance with the decreased viral load after antiviral treatment; a deviated response regression may be detected if the treatment has a specific up-regulatory effect on the indicated host factor, whereas response regression transcendence reflects a pharmacological down-regulatory effect. **(C)** For down regulated virus-responsive factors, the response regression deviated and transcended status after antiviral treatment in comparison with the estimated response regression status is indicative of specific down- and up-regulation of the indicated host factor by the antiviral treatment, respectively.

(including vascular endothelial growth factor) and their receptor tyrosine kinases (RTKs) have become promising candidate targets for cancer therapy, the role of mammalian neuraminidase-1 in complex with matrix metalloproteinase-9 and G protein-coupled receptor tethering to the RTKs has been identified as a major target in tumorigenesis [31]. Osel, a well-known neuraminidase inhibitor, has been demonstrated to attenuate RTK phosphorylation, thus impeding human pancreatic cancer growth and metastatic spread in heterotopic xenograft mouse models [32]. Interestingly, both Huntington

disease and the VEGF signaling pathway were found to be enriched in KEGG analysis of the DEGs of interest for osel treatment identified with the adapted transcriptomic analysis strategy, thus further indicating the feasibility of using the response regression model as a guiding principle for the mechanistic study of HTAs.

Indeed, although the response regression model was demonstrated to be valuable in guiding mechanistic study of HTAs, the detailed analysis protocol has several limitations and warrants further optimization in the future. First, the RIs of VRGs and the est.RIs in

response to fluctuating viral kinetics were calculated with simplified equations, which require verification in larger datasets in the future. More fitting equations could be used, if necessary. In addition, because we proposed the thresholds to screen response regression deviated and transcended VRGs with formulas $y=k_2/(k_1x+a)$ and $y=-k_2/(k_1x-ak-1)$, respectively, the constant parameter a , which was introduced to offset the bias of gene sensitivity to viral load fluctuation, was forced to equal 1 in this study. These threshold equations as well as the related parameters also require careful re-evaluation and probably optimization, to further increase the accuracy and feasibility of this approach. Second, the response curve of individual host factors to viral infection might substantially differ. Some innate antiviral cytokines such as IFN-Is are well known to be induced immediately after viral infection and are likely to peak in parallel with, or even before, the peak viral load [35]. However, adaptive immune-related host factors, such as neutralizing antibodies, might be elevated after the peak viral load [35]. Because the adapted transcriptomic analysis strategy used the single-point viral load as a key parameter determining the host response, the current method could reasonably be assumed to be suitable only for data collected in early infection stages, i.e., before or around the peak viral load. In the future, further improving the robustness, generalization, and adaptation capability of the adapted analysis strategy should be of interest. For example, the curve of viral load kinetics could be monitored in real time, and the area under the curve indicative of accumulated viral stimuli could be used to replace the single-point viral load as the key parameter for estimating the theoretical status of the host response.

Of particular note, although the present study was performed on the basis of a virus-infected animal model and focused mainly on transcriptomic analysis, the response regression model might serve as a guiding principle in both in vitro and in vivo models of viral or other microbial infections, as well as phenotypic evaluation and other multi-omics data analysis. Because conventional pairwise comparisons of infectious models with vs without therapeutic treatment might inevitably have generated biased results in previous studies, we call for a careful re-evaluation of pharmacological mechanistic studies of host-targeted antiviral/antimicrobial agents, particularly those of complex antiviral/antimicrobial traditional medicines.

In summary, in the present study, we raised a response regression model as a guiding principle for pharmacological mechanistic studies of HTAs. In addition, we raised an adapted mathematical method to identify specific target host factors potentially influenced by the indicated HTAs, while excluding potential perturbations due to fluctuating viral kinetics. Our study should greatly facilitate mechanistic investigation of antiviral agents as well as novel antiviral development in the future.

DATA AVAILABILITY STATEMENT

The data are available from the corresponding author on reasonable request.

ETHICS STATEMENT

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Shandong University of Traditional Chinese Medicine (approval SDUTC-M20250307564).

AUTHOR CONTRIBUTIONS

Ruikun Du conceptualized the project and research design. Kerui Wu, Zinuo Chen, Yan Ran, and Lingyuan Fan performed the experiments. Ruikun Du, Kerui Wu, Chenglong Chen, and Qinghua Cui contributed to data collection and analysis. Ruikun Du wrote the manuscript. Ruikun Du and Qinghua Cui provided grant support and supervised the study. All authors read and approved the submitted manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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