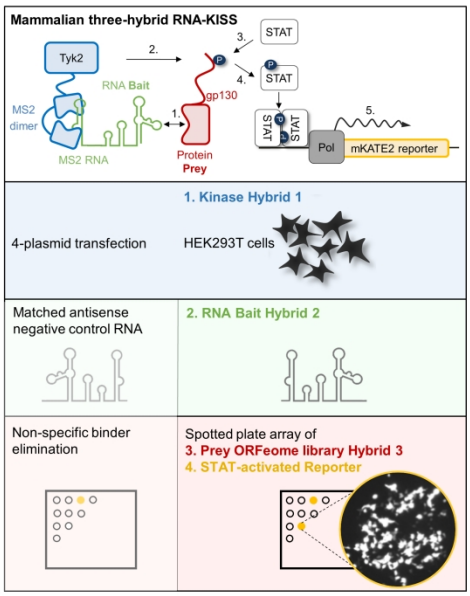


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# The development of RNA-KISS, a mammalian three-hybrid method to detect RNA – protein interactions in living mammalian cells

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KEYWORDS: RNA-protein interaction, detection method, KISS, Dengue flavivirus, DDX6, PACT, mammalian, cell microarray screen

## ABSTRACT

RNA-protein interactions are essential for the regulation of mRNA and non-coding RNA functions and are implicated in many diseases, such as cancer and neurodegenerative disorders. A method that can detect RNA-protein interactions in living mammalian cells on a proteome-wide scale will be an important asset to identify and study these interactions. Here we show that a combination of the mammalian two-hybrid protein-protein detection method KISS (Kinase Substrate Sensor) and the yeast RNA three-hybrid method, utilizing the specific interaction between the MS2 RNA and MS2 coat protein, is capable of detecting RNA-protein interactions in living mammalian cells. For conceptional proof we used the subgenomic flavivirus RNA (sfRNA) of the dengue virus (DENV), a highly structured non-coding RNA derived from the DENV genome known to target host cell proteins involved in innate immunity and antiviral defense, as bait. Using RNA-KISS, we could confirm the previously established interaction between the RNA-binding domain of DDX6 and the DENV sfRNA. Finally, we performed a human proteome-wide screen for DENV sfRNA-binding host factors, identifying several known flavivirus host factors such as DDX6 and PACT, further validating the RNA-KISS method as a robust and high-throughput cell-based RNA-protein interaction screening tool.

## INTRODUCTION

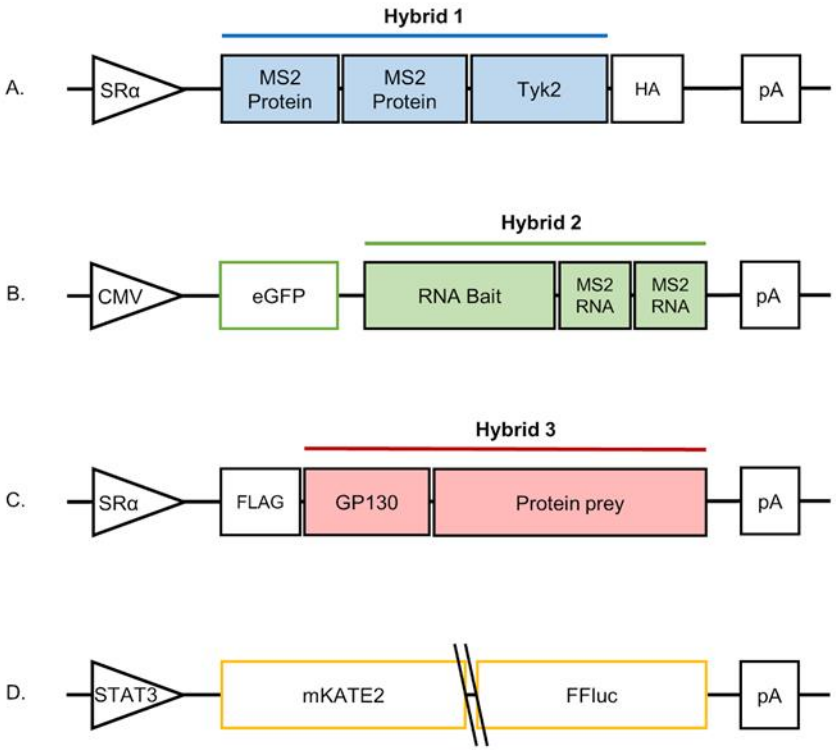
RNA-protein interactions are important for cellular homeostasis; its perturbation may cause cellular dysfunction and lead to disease. It is estimated that roughly 5% of the human proteome consists of RNA-binding proteins (RBPs).[1,2] Recently, next generation sequencing technologies have identified several classes of non-coding RNAs (ncRNAs), including thousands of long non-coding RNAs (lncRNAs). For most of the ncRNAs the function is not yet identified,[3,4] yet many of them have been linked to various important pathologies such as cancer, neurodegenerative disorders or cardiovascular diseases.[5,6]. Next to these cellular ncRNAs, also RNA and DNA viruses make use of RNA molecules for their replication cycle and to produce the structural proteins necessary to form novel virions. Moreover, recent reports show that many viruses significantly alter the RNA landscape in cells by interfering with mRNA splicing, mRNA nuclear export, mRNA capping and decapping.[7] In particular, all members of the large family of the medically important flaviviruses [8], [9] such as the dengue virus (DENV), yellow fever virus or Zika virus produce a unique stable subgenomic flavivirus RNA (sfRNA) derived from the 3' noncoding region of their RNA genomes.[10] This non-coding viral RNA serves as an essential virulence factor, perturbs cellular RNA homeostasis,[11] and can by interacting with cellular host proteins inhibit, amongst others, innate immunity.[12,13,14] Likewise, disease symptoms caused by different flaviviruses show a wide clinical spectrum and include viral encephalitis, viral hemorrhagic fever, multi-organ failure and shock, as well as embryonic malformations such as microcephaly. Though urgently needed, no efficient vaccines (with the exception of the yellow fever and Japanese encephalitis virus) nor drugs exist to prevent or treat flavivirus infections, partially due to a fundamental gap in understanding of the molecular basis how these viruses cause disease, including on relevant cellular targets of their virulence factor sfRNA.

Similar to the 'guilt-by-association' principle for protein-protein interactions,[15] the identification of specific RNA-protein interactions may hint to possible functions of a particular RNA. Generally, RNA-protein detection methods can be divided into two classes: RNA-centric and protein-centric. In the RNA-centric approaches the focus is on a particular RNA of interest and the identification of proteins that can bind to it, whereas in the protein-centric approaches one or more RNAs are interrogated against a protein of interest. Examples of the earliest methods that were developed to detect RNA-protein interactions are RNA EMSA (electrophoretic mobility shift assay), RNA pulldown, oligonucleotide-targeted RNase H protection assay and FISH co-localization. Later on more high-throughput methods were developed like RNA-affinity purification methods followed by mass spectrometry analysis (RAP-MS) and RNA screening with protein libraries. For an overview of different methods, including computational methods, we refer to Xu *et al*.[16], and Ramanathan *et al*.[17]

As the authors state in the Xu et al. paper[16] the major challenge for defining proteins that interact with lncRNAs is that the RNA-centric methods are still not well suited for exploring low abundance transcripts, such as lncRNAs. Biochemical approaches (pull-down of RBP from cell lysates) are indeed mostly limited to the study of highly abundant and ubiquitous proteins.[14,18] Alternatively, the yeast three-hybrid (Y3H) method circumvents this limitation by strongly overexpressing both the RNA and protein of interest exogenously in yeast reporter cells in appropriate RNA and proteins display scaffolds (hybrids) in a controlled binary fashion.[19, 20] Our novel developed RNA-KISS method still profits from the benefits of Y3H systems, but offers the additional advantage of testing RNA-protein interactions in the more relevant environment of a living mammalian cell, allowing *e.g.* for correct post-translational modifications. Moreover, the detection of the RNA-protein interaction in RNA-KISS is localization independent whereas in the

original Y3H the functional interaction has to take place in the nucleus. Additionally, a separation of the interaction and read-out zone into different cellular compartments can limit the detection of false positives. To further suppress false positives, stringent controls are used to allow for the correction of the signals thus obtained for the background.

To be able to detect RNA-protein interactions we use the same principle to bridge hybrid 1 and 2 as in the Y3H method,[19 20] namely the high-affinity binding of the bacteriophage MS2 coat protein to its genomic RNA stemloop packaging signal, and implemented this in the KISS method.[21] The KISS method is based on type I cytokine receptor signaling. The tyrosine kinase and kinase-like domain of Tyk2 (further referred to as Tyk2) are fused to a protein of interest and a possible interaction protein partner to a part of the glycoprotein 130 (gp130) receptor chain. When both proteins interact, Tyk2 is able to phosphorylate the tyrosine residues present in the gp130 tail creating STAT (signal transducer and activator of transcription) recruitment sites. Endogenous STAT molecules will bind the phosphorylated gp130 chain and will in turn be phosphorylated by Tyk2, upon which these activated STATs will translocate to the nucleus and activate transcription of a STAT responsive reporter gene. A typical reporter gene is a luciferase encoding gene or a gene encoding a fluorescent protein or growth selection marker. For RNA-KISS we fuse Tyk2 to a dimer of the MS2 coat protein and the RNA of interest is fused to a MS2 RNA stemloop. The RNA interacting protein candidates are, as in the classic KISS approach, fused to a part of the gp130 chain, further referred to as prey. The MS2 binding RNA that is coupled to the RNA of interest (bait) will bind to the MS2 coat protein that is fused to Tyk2. When a prey interacts with the RNA of interest, the gp130 moiety will come in close proximity to Tyk2. This leads to phosphorylation of the gp130 chain, recruitment and activation of endogenous STAT molecules and ultimately to the activation of a STAT-responsive reporter gene of interest (see Figure 1).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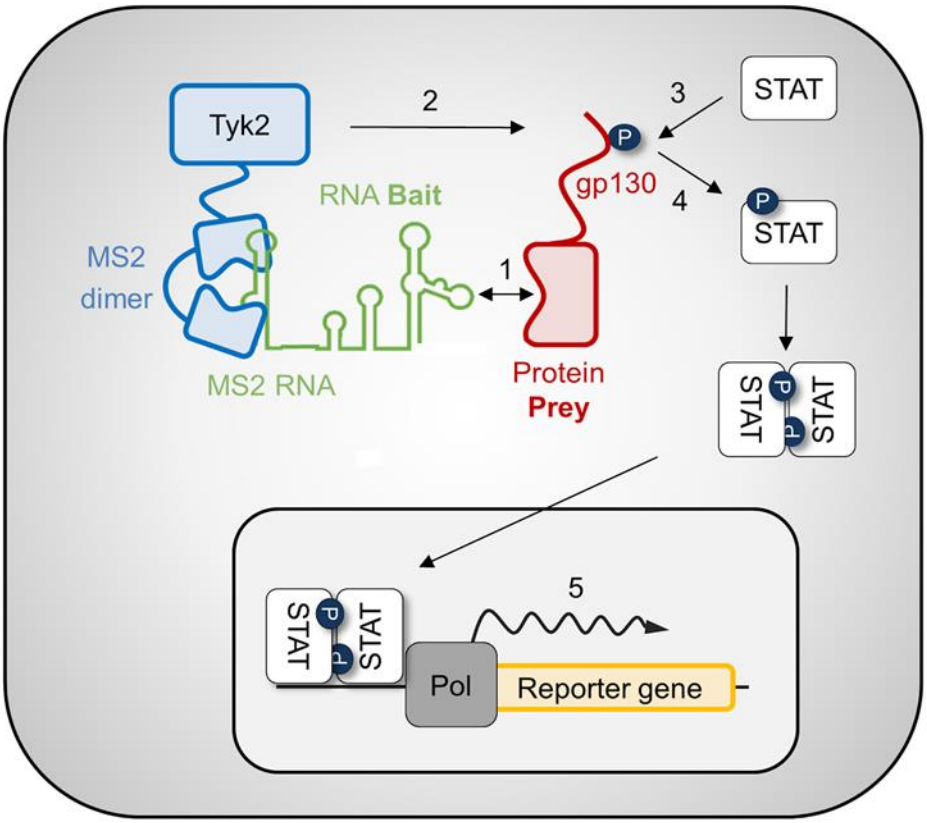
Tyk2
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STAT
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Reporter gene

**Figure 1. Schematic representation of the RNA-KISS method and the necessary components.** RNA-KISS is a mammalian RNA three-hybrid approach set up by co-transfecting 4 plasmids in a mammalian cell expressing: (i) Tyk2 kinase fused to a MS2 coat dimer (A, hybrid 1), (ii) Bait RNA of interest fused to two MS2 RNA stemloops (B, hybrid 2), (iii) Prey protein of interest fused to a gp130 chain (C, hybrid 3) and (iv) a fluorescent mKATE2 or firefly-luciferase (FFluc) reporter gene under control of a STAT-responsive promoter (D). GFP, FLAG- and HA-tags allow to easily confirm expression of all three hybrids using Western blot analysis. (1) When a prey protein expressed as fusion to gp130 binds to the bait RNA under study the gp130 moiety gets in close proximity to the Tyk2 kinase. (2) This bridging lead to phosphorylation of the gp130 chain, followed by the recruitment of endogenous STAT molecules. (4) Upon phosphorylation of the STAT molecules by Tyk2 they dimerise and translocate to the nucleus, (5) ultimately leading to the expression of a fluorescent (mKate2) or luminescent (FFluc) reporter under control of a STAT-responsive promoter, as an easily detectable and measurable proxy for the original RNA bait-protein prey interaction.

As proof of principle for our novel RNA-KISS method we used the sfRNA of the dengue virus type 2 (DENV2) and tested its binding to the cellular DDX6 (DEAD-box helicase 6) protein. DDX6 is an essential P-body protein involved in cellular RNA turnover and has previously been shown to bind to the secondary RNA dumbbell structures in the dengue 3'UTR/sfRNA.[14,22] Furthermore, we reveal that the DDX6 - DENV sfRNA interaction in our RNA-KISS method is indeed dependent on the RNA-binding domain of DDX6.

To be able to screen for novel human bait-interacting proteins, we previously developed a cell microarray high-throughput protein-protein interaction detection platform, based on an arrayed, close to proteome-wide collection of preys.[23] Since RNA-KISS uses the same prey configuration as KISS, the cell microarray platform is directly suitable for a RNA-KISS screen. This approach was performed with the DENV2 sfRNA, with the antisense DENV2 RNA as background control, identifying the known DDX6 prey, as well as novel previously uncharacterized ones, including PACT,[24] a stress-regulated activator of both PKR and RIG-I.[25, 26]

## EXPERIMENTAL PROCEDURES

### Plasmids and Cloning Procedures

To generate pMet7-MS2dimer-Tyk2(AA589-1187)-HA-tag the MS2 coat protein was cloned in the previously described KISS pMet7 bait plasmid[21] by PCR on YBZ-1 yeast cells containing the MS2dimer in its genome[20] using the following primers: 5' GGCCAATTGATGGCTTCTAACTTTACTCAG-3' and 5' GCCCTCGAGAAGTAGAGGCCGGAGTTTGC-3' The by the restriction enzymes MfeI and XhoI digested PCR fragment was ligated in the KISS pMet7 bait plasmid digested by EcoRI and SalI. The MS2 dimer was obtained by PCR on this new pMet7-MS2-Tyk2-HA plasmid using the primers 5'- ACCATTCCAATTTTCGCCAC-3' and 5'- TAACAATAAGCTCGCAGTCG-3' and after EcoRI digestion it was ligated in the EcoRI digested pMet7-MS2-Tyk2-HA plasmid.

sfRNA bait constructs were generated by cloning dengue virus serotype 2 New Guinea strain C (Genbank AF038403) nt.10296-10723 into a unique XhoI restriction site of the yeast three-hybrid vector p3HR2[20] which expresses the bait RNA in a stable scaffold (GC-clamp) and as 5'-terminal fusion to a tandem repeat of two MS2 RNA stemloops, yielding two types of clones with the sense (DENV sfRNA) and antisense RNA (asDENV sfRNA), respectively. Both RNA-expression constructs were digested by a BamHI - EcoRI digest and ligated in a BglII – EcoRI digested pEGFP.C1 plasmid (Clontech).

The prey library was generated using the ORFeome8.1 library[27] and ORFeome Collaboration entry clones[28], kindly provided by the CCSB (Center for Cancer Systems Biology, Dana-Farber Cancer Institute, Boston, US). In total 14,816 single-colony, fully sequenced, full size human open reading frames (ORFs) were C-terminally fused to the Flag-tagged gp130 moiety (pMG1) as described before[21,23]. Additionally, 3134 entry clones from the ORFeome8.1 library, that according to their

GO annotation contain a transmembrane domain, were N-terminally fused to the Flag-tagged gp130 moiety through an LR reaction (Invitrogen) resulting in a 18K prey collection. The human DDX6 prey derives from the GDEh81093F04 (BC065007.1; gi: 40675583) entry clone and human PACT from the GDEh81054D07 (BC009470.1; gi: 14495716) entry clone[27]. The DDX6delRBD prey was generated by deleting the C-terminal RNA binding domain of DDX6 and introducing a stop codon after the following amino acid sequence: SVQKFMNSHLQKPYEINL. This was done by performing a full plasmid PCR on the DDX6 prey plasmid using the following primers: 5'-GGCCATCAGTCGACTTACAGGTTAATCTCATAGGGT-3' and 5'-CTGTAAGTCGACTGATGGCCGCACTAGAGA-3', SalI digestion and self-ligation. The negative control prey plasmid expressing only the Flag-tagged gp130 moiety[29], the STAT3-dependent firefly luciferase reporter pXP2d2-rPAP1-luciferase[30], and the fluorescent pXP2d2-rPAP1-mKate2 reporter[23] were as described before.

**Cell culture**

HEK293T cells were cultured in Dulbecco's modified Eagle's medium containing 4500 mg/L glucose supplemented with 10% fetal calf serum, and incubated at 37 °C, 8% CO2. During an experiment 50 µg/mL gentamycin was added to the cells.

**Luciferase-based RNA-KISS assay**

HEK293T cells were co-transfected with 4 plasmids, namely (1) pMet7-MS2dimer-Tyk2(AA589-1187)-HA-tag, (2) the pEGFP.C1-sfRNA plasmid of interest (DENV or asDENV), (3) the prey plasmid of interest (DDX6, PACT or negative control prey) and (4) the STAT-responsive pXP2d2-rPAPI-firefly-luciferase reporter gene using a standard calcium phosphate transfection method, as

described earlier.[30] Luciferase activity was measured 48 h after transfection using the Luciferase Assay System kit (Promega) on an Envision luminometer (PerkinElmer). Each experiment was done with 3 technical replicates and at least 3 independent biological experiments were performed. The data shown is a representative experiment. To calculate the corrected luciferase induction the obtained bioluminescence signals are corrected for the bioluminescence signal obtained by the asDENV sfRNA or corrected for the bioluminescence signal obtained with the negative control prey. The lowest obtained ratio value is the corrected luciferase induction.

**Fluorescent-based RNA-KISS cell microarray screen**

The cell microarray prey plates were generated as described in Lievens *et al*.[23] Briefly, prey plasmid DNA and the fluorescent pXP2d2-rPAP1-mKate2 reporter are mixed with a reverse lipid-based transfection reagent, cell adhesion molecules and stabilizing components, and printed on a microtiter-sized polystyrene plate containing 4 rectangular wells using an 2470 arrayer (Aushon Biosystems). Each well on the same plate contains the same 1692 different prey plasmids and 36 controls. To be able to screen the complete 18K prey library 11 different 4-wells plates are utilized. To serve as background control of each 4-well plate one well is seeded with HEK293T cells transfected with the pMet7-MS2dimer-Tyk2(AA589-1187)-HA-tag, and pEGFP.C1-asDENV sfRNA plasmid using polyethyleneimine. The three other wells are seeded with HEK293T cells transfected with the pMet7-MS2dimer-Tyk2(AA589-1187)-HA-tag, and pEGFP.C1-DENV sfRNA plasmid. 72 hours after transfection plates were imaged and fluorescence intensity of each individual spot was determined using an CellCelector microarray scanner (Automated Lab Solutions). Fluorescence intensity data were processed as described before[23] utilizing the MAPPI-

DAT analysis tool[31]. As threshold to score a prey positive the Q-value has to be below 0.2 and the averaged particle count at least 3.

**Western blot analysis**

Cellular lysate, after determination of the luciferase activity, was mixed with Laemlli Loading buffer (5x: 155mM Tris-HCl pH 6.8, 5% SDS, 20% glycerol, 0.025% Bromphenol blue, 1.8 M β-mercaptoethanol). After boiling, samples were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare), and probed with rat-anti-HA (Roche), rabbit-anti-eGFP (Invitrogen), mouse anti-Flag (Sigma) and mouse anti-β-actin (Sigma). For detection a combination of the following secondary antibodies was used: anti-rat-Alexa-Fluor680 (Life technologies), anti-rabbit-Dylight680 (Invitrogen), and anti-mouse-Dylight800 (Invitrogen). Western blot analysis was performed using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

## Results and Discussion

### Development of the RNA-KISS method

In analogy with the original RNA Y3H method[20] we fused the kinase and kinase-like domain of Tyk2 C-terminally to a dimer of the MS2 coat protein. The sequence encoding for the sfRNA of DENV2 was cloned in a mammalian expression vector that simultaneously expresses the enhanced fluorescent green fluorescent protein (eGFP) to easily verify the expression of our bait RNA construct. As matched negative control to correct for background a similar plasmid containing the antisense DENV2 sfRNA (asDENV) was generated (irrelevant bait). The DDX6 ORF was fused C-terminally to the Flag-tagged gp130 part to function as prey. As prey background control a plasmid encoding only the Flag-tagged gp130 moiety was used (negative control prey). HEK293T cells were co-transfected with 4 plasmids, namely (1) a plasmid encoding the MS2dimer-Tyk2-HA fusion (Figure 1, construct A), (2) the eGFP-sfRNA plasmid of interest (DENV or asDENV) (Figure 1, construct B), (3) the prey plasmid of interest (DDX6 or negative control prey) (Figure 1, construct C) and (4) a STAT-responsive luciferase reporter gene (Figure 1, construct D).

### Detection of the interaction of DDX6 with the DENV sfRNA is dependent on its RNA-binding domain

For proof of principle we used the DDX6 prey and tested if we could detect its interaction with the DENV sfRNA. As negative controls we used the asDENV sfRNA and the negative control prey. As extra functional control we investigated if the signal would be lost if the 183aa C-terminal RNA binding domain of DDX6 was deleted (DDX6delRBD).[32] Since we anticipated that too low or too high levels of sfRNA could lead to false negatives or positives, we chose to transfect a range of

different amounts of eGFP-sfRNA plasmid DNA. Too low expression levels may result in luciferase signals below the background threshold and too high expression levels can result in false positive signals. Figure 2 reveals the obtained bioluminescence signals corrected for the bioluminescence signal obtained by the asDENV sfRNA or corrected for the bioluminescence signal obtained with the negative control prey. The lowest obtained ratio value is shown in Figure 2. Raw data for the averaged bioluminescence readouts are shown in Figure S1.

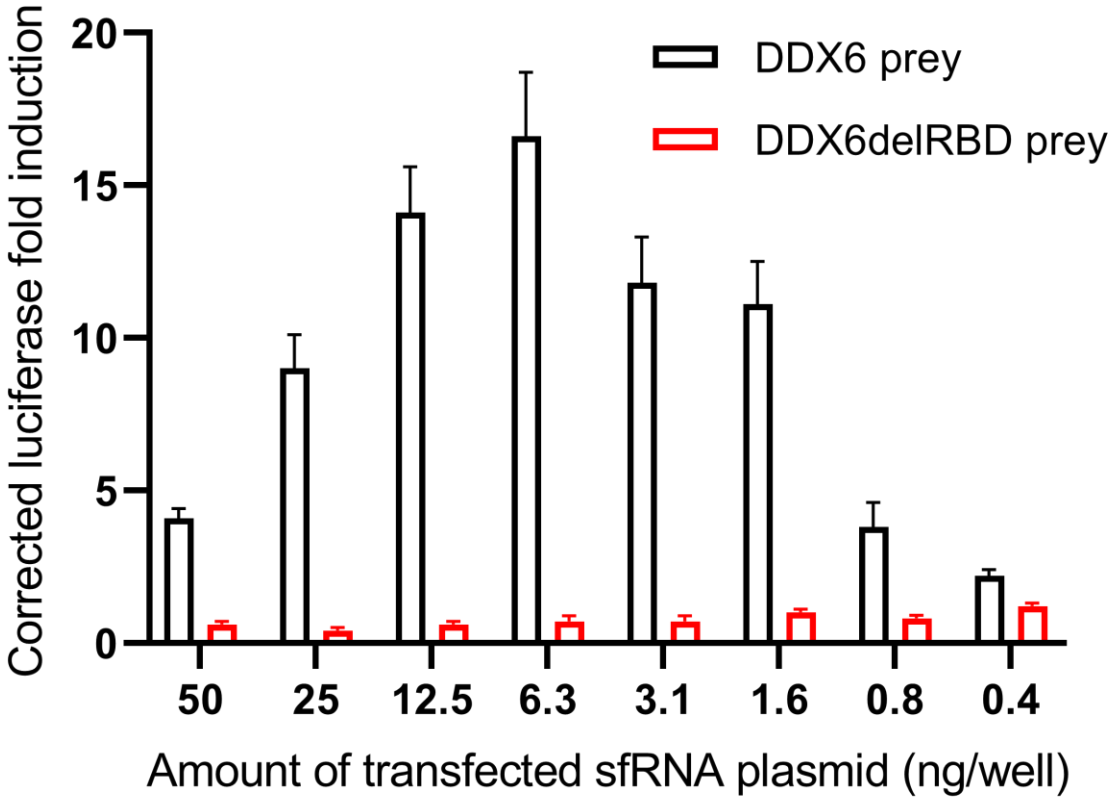



**Figure 2 The RNA-binding domain of DDX6 is required for DENV sfRNA-binding.**
HEK293T cells were transfected with 4 plasmids, encoding the MS2dimer-Tyk2-HA, DDX prey, the pXP2d2-rPAP1-luciferase reporter, and decreasing amounts of the eGFP-DENV sfRNA plasmid. For quantification, the average bioluminescence signals as assessed in triplicates for DDX6 (*black bars*) or a DDX6 deletion mutant lacking its C-terminal RNA-binding domain (delRBP, *red bars*) was divided by the average bioluminescence signal obtained when the DDX6 prey was replaced by the negative control prey, or by the average bioluminescence signal obtained when eGFP-asDENV sfRNA was transfected as negative control bait instead of eGFP-DENV sfRNA. As most conservative estimate, the lowest value of both corrected luciferase ratios is shown ± SD. Data shown are from a representative experiment out of three independent biological

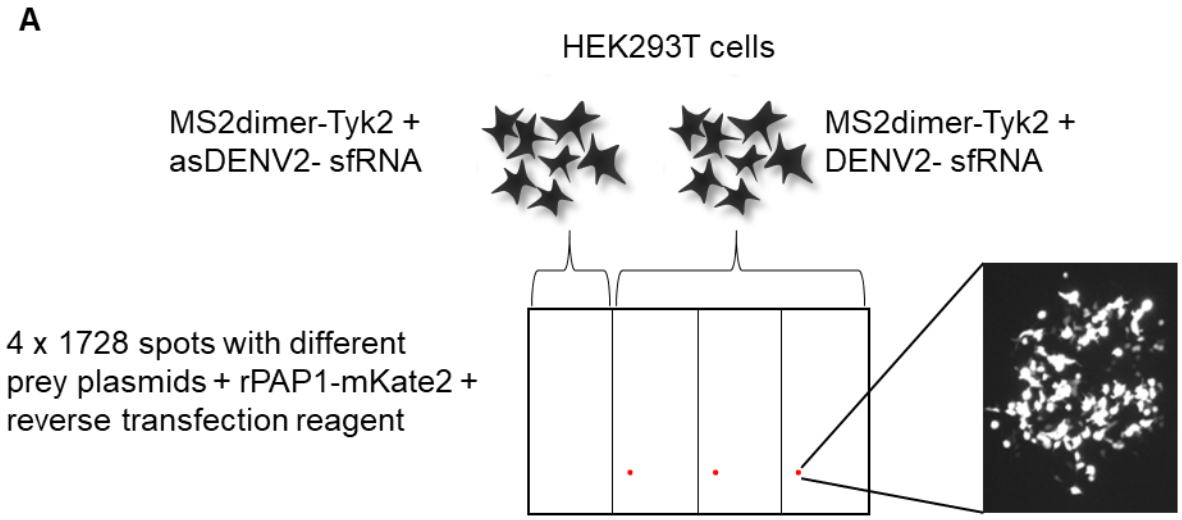
repeats, all done with triplicate technical replicates. Expression control of the MS2dimer-Tyk2-HA, Flag-tagged prey plasmids (DDX6, DDXdelRBD and the negative control prey), eGFP as well as β-actin as loading control are shown in Figure S2.

A strong signal, up to 17 times higher than the background, was obtained with the DDX6 prey and DENV sfRNA over a broad range of transfected eGFP-sfRNA DNA amounts. Furthermore, the interaction was clearly lost when the C-terminal RNA binding domain of DDX6 was deleted, indicating that the obtained signal is specific and depends on the cognate RNA-protein interaction between bait and prey.

**Identification of interacting protein partners for the DENV sfRNA using a cell microarray based screening platform**

To check whether our RNA-KISS method could also be used to perform a close to proteome-wide screen for RNA-binding proteins, we performed a cell microarray based screen wherein the DENV sfRNA was interrogated against a 18K prey library. 15K preys were generated using the ORFeome8.1 library,[27] and ORFeome Collaboration entry clones,[28] kindly provided by the CCSB (Center for Cancer Systems Biology, Dana-Farber Cancer Institute, Boston, US). Single-colony, fully sequenced, full size human open reading frames (ORFs) were C-terminally fused to the Flag-tagged gp130 moiety.[23] Additionally, roughly 3K transmembrane region containing proteins of this collection were also N-terminally fused to the gp130 part resulting in a 18K prey collection. Instead of a luciferase reporter, for screening purposes the far-red fluorescent protein mKate2 was used as read-out.[23] Each 1692 different prey DNA plasmids and 36 controls, all mixed with the STAT-responsive mKate2 reporter and reverse transfection reagent, were spotted in fourfold on 4-well plates.[23] HEK293T cells were transfected with the plasmids encoding the MS2dimer-Tyk2 and the DENV sfRNA and seeded on 3 out of the 4 prey-spotted wells. To serve as background

control in 1 well HEK293T cells were seeded that were transfected with plasmids encoding the MSdimer-Tyk2 and the asDENV sfRNA. In total 11 4-well plates were utilized to cover the complete 18K prey library. The obtained fluorescent signals were normalized to account for plate and within-plate effects, and were used to calculate the rank product statistic for each prey in the screen, and subsequently the p-value and Q-value (to compensate for the multiple testing problem). This analysis was done using the MAPPI-DAT tool as described in Gupta *et al*.[31] Besides the fluorescence intensity for each spot it was determined how many particles display a signal above background. This particle count reflects the amount of cells (or clusters of cells) giving a positive signal.[31] For the averaged particle count, the average of the particle counts seen for a particular spot in all 3 wells was calculated. In Figure 3 all preys are displayed with the corresponding log-transformed Q-value against the averaged particle count.



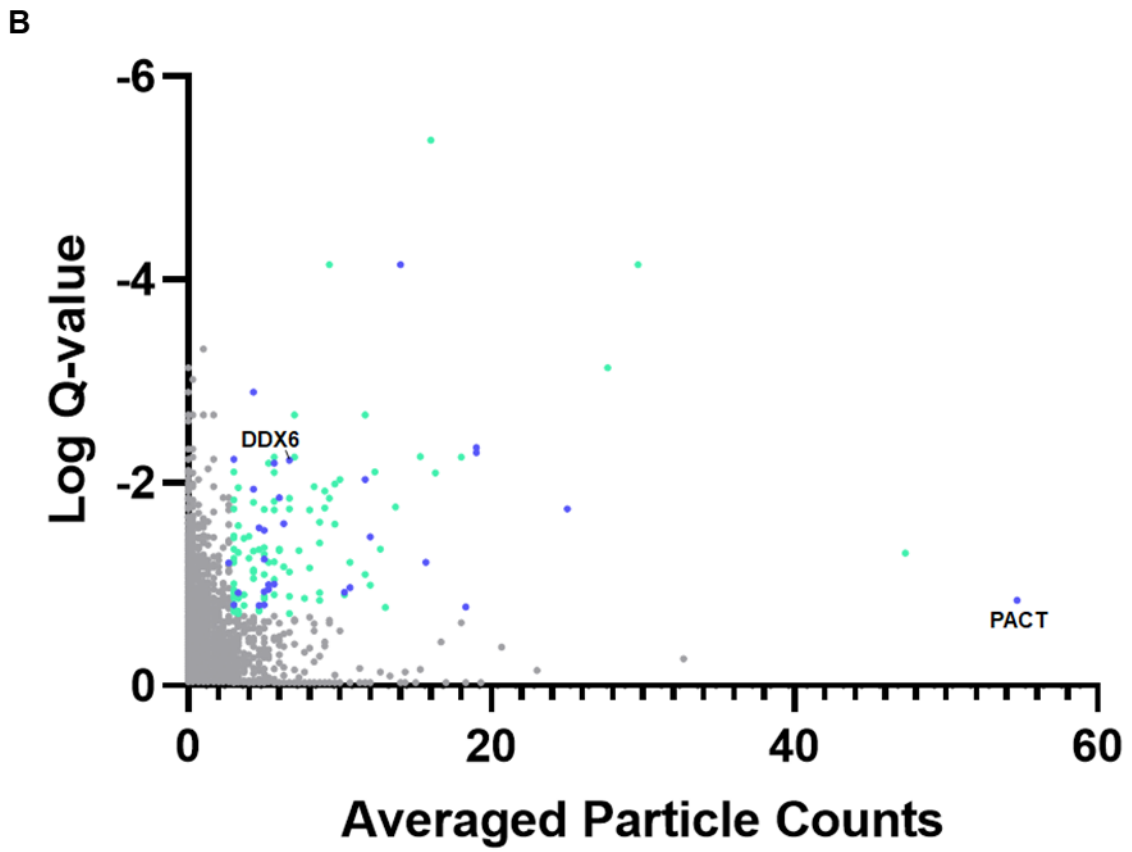



**Figure 3. Lay-out and result of the RNA-KISS cell microarray screen**
(A) Schematic overview of the cell microarray screen. 1728 reverse transfection mixtures, consisting of 1692 different prey plasmids and 36 controls together with a STAT responsive mKate2 reporter and a reverse lipid-based transfection reagent, are spotted in 4-fold on a 4-well plate. One well is seeded with HEK293T cells transfected with pMet7-MS2dimer-Tyk2(AA589-1187)-HA-tag, and the pEGFP.C1-asDENV sfRNA negative control bait plasmid using polyethyleneimine, to serve as background controls. The three other wells are seeded with HEK293T cells transfected with the pMet7-MS2dimer-Tyk2(AA589-1187)-HA-tag, and the original pEGFP.C1-DENV sfRNA bait plasmid. To screen the 18K prey library 11 different 4-well plates are transfected. The interaction of the DENV sfRNA with a spotted prey will result in a localized fluorescent signal (indicated as red spot). An example image of a positive spot is given. (B) Analysis of the RNA-KISS cell microarray screen. The obtained fluorescent signals were normalized and ranked using the MAPPI-DAT tool,[31] resulting in an individual Q-value for each prey. Additionally, for each prey the average of the particle count of the 3 wells, transfected with MS2dimer-Tyk2 and the DENV sfRNA, is calculated. All preys are displayed with the corresponding log-transformed Q-value against the averaged particle count. Green dots represent positively scoring hits (with Q-value below 0.2 and an averaged particle count of at least 3). Blue dots represent positively scoring hits that have known RNA-binding activity (see Figure 4C). DDX6 and PACT are indicated.

Using a threshold of Q-value below 0.2 and averaged particle count above 3 resulted in 120 candidates. Of these 120 candidates thus identified, 8 proteins have been described before to have a (in)direct link with the DENV as shown in Figure 4A. Next to DDX6 we also identified U2AF1 and PTRF, which were previously detected in a DENV2 3'UTR pulldown screen [22]. Furthermore, CNOT2, LZTR1 and PACT were found to enhance or restrict dengue replication in functional screens. [24] and more indirectly, RBPMS and PSCA were shown to affect West-Nile virus - another mosquito-borne flavivirus- infection [33]. Analyzing gene ontologies of our hits showed significant enrichment for proteins (i) found in P-bodies and (ii) involved in the viral defense response (Figure 4B,C,D). Moreover, we could retrieve straightforward evidence of RNA-binding for 28 out of 120 identified genes based on (i) the presence of a classical RNA-binding domains (17) and/or (ii) detection in a comprehensive RNA-interactome screen[2] (13) and/or (iii) RNA-binding gene ontology (19) (Figure 4E).

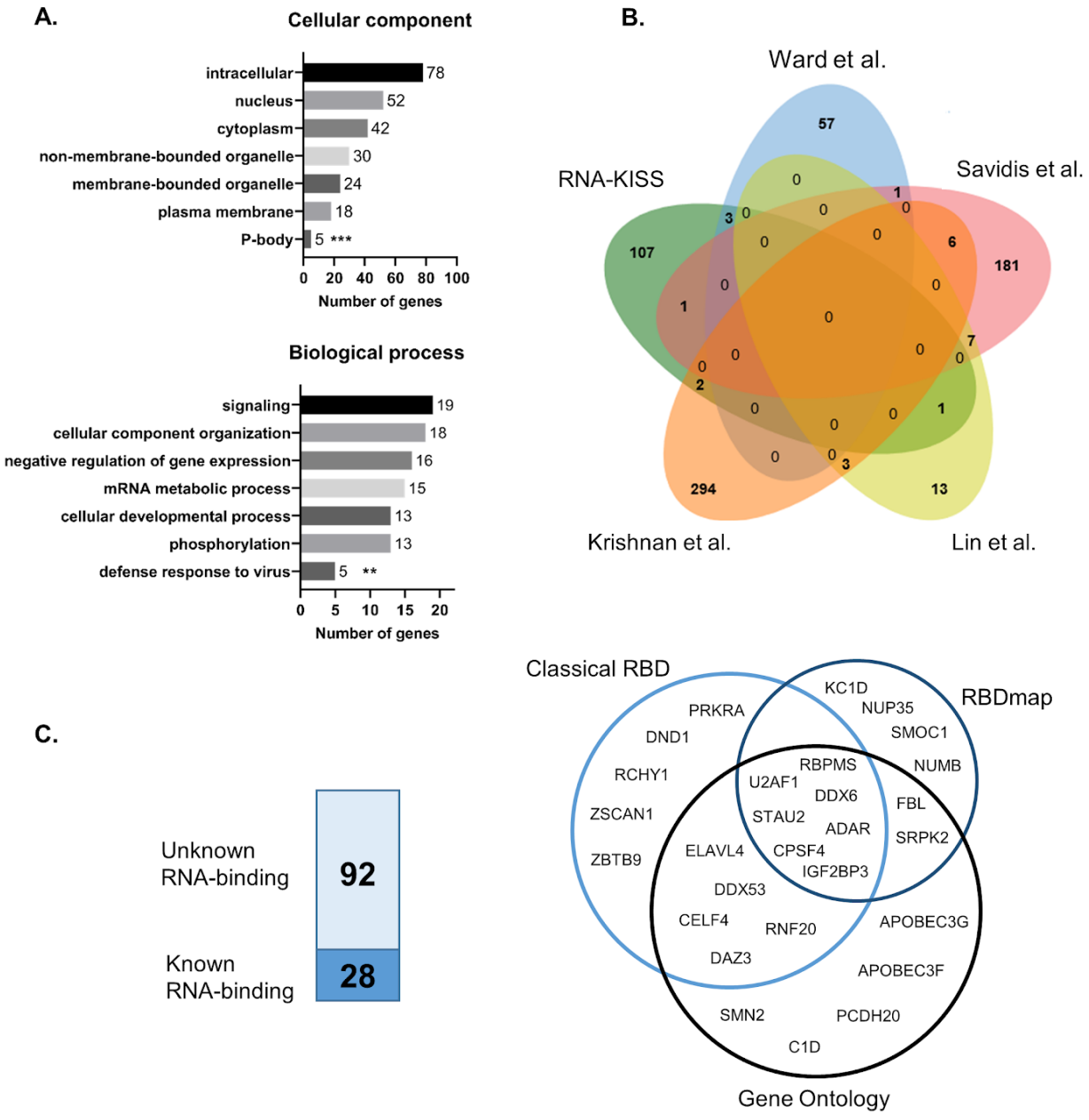



**Figure 4: Analysis of the hits identified in the cell microarray screen**

Using a threshold of Q-value below 0.2 and averaged particle count above 3 resulted in 120 human putative DENV sfRNA binding proteins. (A) Frequency distribution of different gene ontologies for the list of hits. The gene list thus obtained was significantly enriched for proteins that are part of P-bodies (GO:0000932, p=0.00074) and involved in the defense to virus infections (GO:0051607; p=0.00507). Functional profiling was done using the g:GOst tool in g:profiler. All identified genes were run as an ordered query ranked according to their Q-value in the RNA-KISS screen. ** P≤0.01, *** P≤0.001. (B) Venn diagram showing the number of overlapping genes identified in orthogonal screens for DENV2 3'UTR binding proteins[22], dependency or restriction factors for dengue virus[24, 34] and the related West Nile virus.[33] (C) 28 out of 120 identified genes

have known RNA-binding activity based on (i) the presence of a classical RNA-binding domains (17) and/or (ii) detection in a comprehensive RNA-interactome screen[2] (13) and/or (iii) RNA-binding gene ontology (19).

One of these RNA binding proteins is PACT, for which we observed - using the luciferase reporter as readout- a robust RNA-KISS signal over a broad range of bait-to-prey ratios, similar as seen with the DDX6 prey (Figure 5). The raw bioluminescence values are displayed in Figure S1, and its expression controls in Figure S2.

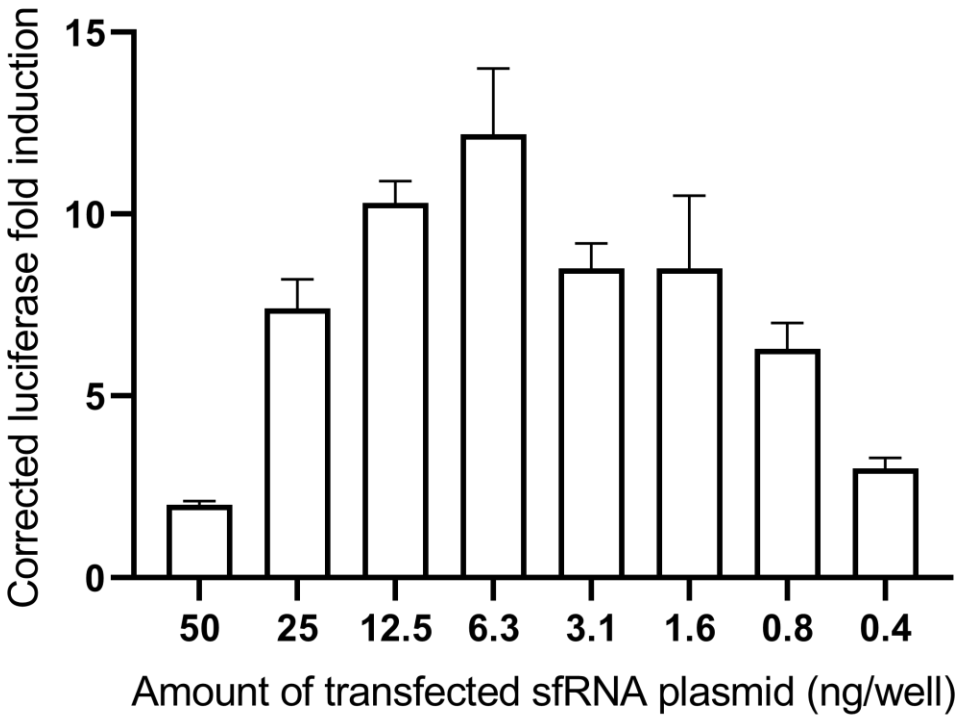



**Figure 5. PACT shows DENV sfRNA-binding in RNA-KISS.**
HEK293T cells were transfected with 4 plasmids, encoding the MS2dimer-Tyk2-HA, PACT prey, the pXP2d2-rPAP1-luciferase reporter, and decreasing amounts of the eGFP-DENV sfRNA plasmid. The average of triplicate bioluminescence signals was divided by the average bioluminescence signal obtained when the PACT prey was replaced by the negative control prey or by the average of the obtained bioluminescence signal when eGFP-asDENV sfRNA was transfected instead of eGFP-DENV sfRNA. The lowest value of both corrected luciferase ratios is shown ± SD. Data shown are from a representative experiment out of three independent biological repeats, all done with triplicate technical replicates. Expression controls of the PACT and negative control prey, MS2dimer-Tyk2-HA and eGFP, as well as β-actin as loading control are shown in Figure S2.

PACT, encoded by the PRKRA gene, is an interferon (IFN)-inducible dsRNA-dependent protein kinase (PKR) activator, and a stress-regulated activator of both PKR and RIG-I (Retinoic Acid-Inducible Gene I)[25,26] that may act as an intracellular pathogen sensor protein. PACT has been identified in a genome-wide knockdown screen as a putative flavivirus host dependency factor,[24] yet without revealing any mechanistic link or molecular detail. Though to be further investigated, it is tempting to hypothesize that direct binding of PACT by sfRNA may modulate its signaling activity and, by this means, helps the DENV to escape innate antiviral immunity. Intriguingly, also another antiviral protein upstream of RIG-I signaling, namely the ubiquitin ligase TRIM25, has been proposed to be targeted by DENV sfRNA to promote viral replication.[14]

## CONCLUSIONS

Our novel RNA-KISS method described here can be used to study binary RNA-protein interactions in living mammalian cells as we illustrated using the well characterized DDX6 - DENV sfRNA interaction. Considering the utmost importance of reliable negative controls for two- and three-hybrid assays in general, we introduced a matched antisense RNA as highly stringent and relevant negative control bait. We demonstrate by employing DENV sfRNA and DDX6 as respective bait and prey that, by optimizing the amount of the sfRNA-expressing construct in our 4-plasmid transfection system, a marked (17-fold) increase in signal over background could be gained. This specificity was further confirmed by the dependency of this RNA-KISS signal on the RNA-binding domain of the DDX6 prey as demonstrated by deletion mutagenesis. Moreover, we validated RNA-KISS as a novel high-throughput approach that is sufficiently robust to perform proteome-wide screens for RNA-protein interactions, as illustrated by screening for novel DENV sfRNA interaction partners in a cell microarray screening platform. Screening 18K human preys for sfRNA-binding resulted in a list of 120 putative host factors consisting of several previously

identified DENV sfRNA binders and known flavivirus host factors such as DDX6, validating our screen, as well as novel putative virus host proteins, such as PACT, that have not been recognized before as sfRNA-binders. Such protein candidates require further investigation to elucidate their molecular interplay with sfRNAs and to assess their potential as targets for interfering with virus infection or replication. RNA-KISS may similarly be used to perform proteome-wide screens with other ncRNAs as bait to identify ncRNA-protein interactions that can shine light on their molecular function and possible role in health and disease. Such insight could lead to the development of more targeted treatments of diseases that originate in the perturbation of cellular RNA-protein interactions.

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**ABBREVIATIONS**

KISS, Kinase Substrate Sensor; DENV, Dengue virus; sfRNA, subgenomic flavivirus RNA; DDX6, DEAD-box helicase 6; RBPs, RNA-binding proteins; ncRNAs, non-coding RNAs; lncRNAs, long non-coding RNAs; EMSA, Electrophoretic Mobility Shift Assay; RAP-MS, RNA-affinity purification methods followed by mass spectrometry analysis; Y3H, yeast three-hybrid; gp130, glycoprotein 130; STAT, signal transducer and activator of transcription; ORFs, open reading frames.