Dissecting the mechanism of signaling-triggered nuclear export of newly synthesized influenza virus ribonucleoprotein complexes

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Influenza viruses (IV) exploit a variety of signaling pathways. Previous studies showed that the rapidly accelerated fibrosarcoma/mitogen-activated protein kinase/extracellular signal-regulated kinase (Raf/MEK/ERK) pathway is functionally linked to nuclear export of viral ribonucleoprotein (vRNP) complexes, suggesting that vRNP export is a signaling-induced event. However, the underlying mechanism remained completely enigmatic. Here we have dissected the unknown molecular steps of signaling-driven vRNP export. We identified kinases RSK1/2 as downstream targets of virus-activated ERK signaling. While RSK2 displays an antiviral role, we demonstrate a virus-supportive function of RSK1, migrating to the nucleus to phosphorylate nucleoprotein (NP), the major constituent of vRNPs. This drives association with viral matrix protein 1 (M1) at the chromatin, important for vRNP export. Inhibition or knockdown of MEK, ERK or RSK1 caused impaired vRNP export and reduced progeny virus titers. This work not only expedites the development of anti-influenza strategies, but in addition demonstrates converse actions of different RSK isoforms.

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The pathway regulates important cellular functions involved in proliferation, differentiation, cell metabolism, and immune response (22). Downstream targets of the pathway can either be directly phosphorylated by the MAPK ERK or by ERK-activated protein kinases like the p90 ribosomal S6 kinases (RSKs), which are exclusively activated by ERK1/2 (23).

In previous studies we have shown that viral activation of the Raf/MEK/ERK pathway, induced by hemagglutinin (HA) accumulation in the cellular membrane, supports vRNP nuclear export (15, 24, 25). These findings indicated that vRNP export is a Raf/MEK/ERK signaling-induced event, ensuring timely regulation of the export late in the infection cycle when the pathway is activated. Accordingly, influenza virus infection triggers activation of the pathway in an unusual biphasic manner, with a very early phase directly after infection and a later phase that requires productive infection. By using a variety of inhibitors of the kinase.

Fig. 1.  ERK1/2 knockdown and MEK-inhibition result in chromatin retention of progeny vRNPs. (A) Cellular localization of WSN vRNA 7 h.p.i. after an ERK1/2 knockdown. See also SI Appendix, Fig. S1D. (B) Dense chromatin analysis of PB2-vRNA 7 h.p.i. after an ERK1/2 knockdown. Same laser and detector settings were used. See also SI Appendix, Fig. S1E. (C) Cellular localization of WSN vRNPs (NP) and M1 7 h.p.i. after an ERK1/2 knockdown. See also SI Appendix, Fig. S1J. (D) Dense chromatin analysis of vRNPs (NP) and M1 7 h.p.i. after an ERK1/2 knockdown. Same laser and detector settings were used. See also SI Appendix, Fig. S1K. (E) Cellular localization of WSN vRNA 7 h.p.i. after CI-1040 (10, 15 μM) and LMB (5 nM) treatment 3 h.p.i. DMSO (0.1%) and MeOH (0.1%) served as negative controls. See also SI Appendix, Fig. S1L. (F) Dense chromatin analysis of PB2-vRNA 7 h.p.i. after CI-1040 (10, 15 μM) and LMB (5 nM) treatment. DMSO (0.1%) and MeOH (0.1%) served as negative controls. Same laser and detector settings were used. See also SI Appendix, Fig. S1M. (G) Cellular localization of WSN vRNPs (NP) and M1 7 h.p.i. after CI-1040 (10 μM) and LMB (5 nM) treatment 3 h.p.i. DMSO (0.1%) and MeOH (0.1%) served as negative controls. See also SI Appendix, Fig. S1R. (H) Dense chromatin analysis of vRNPs (NP) and M1 7 h.p.i. after CI-1040 (10 μM) and LMB (5 nM) treatment. Same laser and detector settings were used. See also SI Appendix, Fig. S1S. (A–H) Representative images of three independent experiments. Dashed squares indicate zoom-in areas. (Scale bar, 20 μm.)
MEK, which represents the bottleneck of the Raf/MEK/ERK cascade, it was shown that the MEK blockade not only suppressed both activation phases but, in addition, led to strongly decreased progeny virus titer correlating with a nuclear retention of newly synthesized vRNPs of both influenza A (IAV) and B viruses (IBV) (15, 24, 26–28). Accordingly, treatment also impaired viral replication in vivo (26, 27). Importantly, no escape mutants could be detected after multipassage use of the MEK inhibitor U0126 in contrast to treatment with virus-directed drugs such as Amantadine (24). In addition, Oseltamivir-resistant influenza strains are still fully sensitive to MEK inhibitor treatment (27). These findings indicate the inability of the virus to compensate for the missing cellular function, suggesting that MEK inhibition might be suitable as an antiviral strategy.

While it has been already known for quite a while that the Raf/MEK/ERK cascade triggers vRNP export, in the present study we have identified the full chain of events that lead to the signaling-driven nuclear export of vRNPs.

Results

Inhibition of the Raf/MEK/ERK Pathway Results in Retention of Progeny vRNPs at the Chromatin and Reduced Binding to the M1-Protein. The inhibition of the Raf/MEK/ERK pathway by specific MEK inhibitors, such as U0126 (15), Trametinib (28), or CI-1040 (27) led to a reduction of progeny virus titer, concomitant with the retention of newly synthesized vRNPs in the nuclei of infected cells. The aim of the present study was to unravel the molecular chain of events that links virus-induced activation of the kinase pathway to the nuclear export of vRNPs. Inhibitors might have off-target effects; therefore, we first aimed to confirm by genetic means that the antiviral action of MEK inhibitors is indeed due to inhibition of the kinase pathway. The kinases ERK1 and 2 are the only known direct downstream targets for MEK (29). Thus, we knocked down expression of ERK1/2 with specific small interfering RNAs (siRNAs). We tested the knockdown efficiency in A549 cells and chose a concentration of 100 nM siRNA for further experiments (SI Appendix, Fig. S1B). Indeed, progeny virus titer of Wilson-Smith neurotropic (WSN), which we used as a model IAV strain, were significantly decreased after a total infection time of 24 h in the ERK1/2 knockdown cells compared to control (siCtrl) (SI Appendix, Fig. S1C). These reduced virus titer correlated well with a nuclear retention of viral RNA (vRNA) and viral NP, polymerase acidic protein (PA), and M1 proteins (Fig. I4 and C and SI Appendix, Fig. SI D, E, H, and J), that are all constituents of vRNP complexes. Furthermore, we analyzed the proteins associated with low-soluble dense chromatin in an in situ fractionation assay. First, soluble proteins were extracted from the cytoplasm and the nucleoplasm, followed by a chromatin digestion with DNase I and an extraction step using the MEK inhibitor U0126 in contrast to treatment with virus-directed drugs such as Amantadine (24). In addition, Oseltamivir-resistant influenza strains are still fully sensitive to MEK inhibitor treatment (27). These findings indicate the inability of the virus to compensate for the missing cellular function, suggesting that MEK inhibition might be suitable as an antiviral strategy.

As it is already known that the Crm1/exportin 1 inhibitor LMB leads to a nuclear retention of vRNPs at the chromatin (14, 34), we used LMB as a control treatment. The incubation of infected cells with either CI-1040 or LMB, starting 3.0 h p.i., led to nuclear retention of vRNPs (Fig. 1 E and G and SI Appendix, Fig. S1 L, N, P, and R). In addition, higher immunofluorescence signals of vRNA, NP, M1, and PA could also be detected at the chromatin (Fig. 1 F and H and SI Appendix, Fig. S1 M, O, Q, and S), confirming the findings of the ERK1/2 knockdown. In addition, stochastic optical reconstruction microscopy (STORM) was used to determine nuclear NP and M1 protein spatial distribution at high resolution (Fig. 2 A and B and SI Appendix, Fig. S2 G and H). We found areas of NP and M1 colocalization in total nuclei and at the chromatin in CI-1040- and LMB-treated cells, indicating that although NP and M1 are localized to the chromatin at a late step in the replication cycle, assembly of the export complex or its release from the chromatin is impaired under inhibitor treatment. Furthermore, nuclear localization of NP in LMB compared to CI-1040-treated cells appeared to differ (Fig. 2 A and SI Appendix, Fig. S2G). While CI-1040-treated cells NP seems to be distributed evenly in the nucleus, the protein appears to accumulate in proximity to the nuclear membrane in LMB-treated cells. This was a first indication that, although both compounds lead to vRNP retention, their molecular mechanism of antiviral action might be different.

According to previous reports, the influenza vRNP nuclear export complex assembles at the chromatin where it enters the nuclear export machinery (14, 35). To validate the retention of the viral proteins NP and M1 at the chromatin (shown in Figs. 1H and 2B and SI Appendix, Figs. S1S and S2H), both essential components in the formation of the vRNP export complex, we first aimed to estimate their protein amounts in different cellular/nuclear compartments in presence or absence of the MEK inhibitor. We chose a time window of 6.5 h to 8.0 h p.i. because this is the prime time period when nuclear export takes place with our model virus (11). Infected treated or untreated cells were separated into cytoplasmic (cyt), nucleoplasmic (nuc), and two fractions regardless of whether MEK was inhibited or not, we confirmed by detection of copurified polymerase basic protein 1 (PB1) and PA as markers for the trimeric polymerase complex, we found striking differences with regard to associated M1 protein (Fig. 2 E and F and SI Appendix, Fig. S2E). vRNP-associated M1 could only be detected in the chromatin fraction extracted with 150 mM NaCl. Furthermore, M1 association was virtually abolished if cells were treated with CI-1040, a pattern that was robustly detected over an observation period from 6.0 h to 7.5 h p.i. (Fig. 2 E and F and SI Appendix, Fig. S2 E and F). This clearly
indicates that MEK inhibition alters the interaction of M1 with vRNPs and thus results in a subsequent block in the assembly of the export complex at a particular chromatin fraction.

**Raf/MEK/ERK Pathway-Dependent Phosphorylation of a Specific Motif within the Nucleoprotein.** The data so far indicate that the Raf/MEK/ERK pathway promotes vRNP export by facilitating M1 association to the vRNP export complex at the chromatin. Since on one hand the Raf/MEK/ERK kinase cascade transmits signals within the cell via timely regulated sequential phosphorylation (36), and on the other hand the viral NP is long known to be differentially phosphorylated throughout the replication cycle (37), we hypothesized that posttranslational phosphorylation of the NP may be the decisive signal for M1 association. To identify phosphorylation sites within the viral NP that are controlled via MEK/ERK, HEK293T cells were infected with the recombinant Strep-PB2-WSN virus. At 3.0 h p.i., the infected cells were treated with dimethylsulfoxide (DMSO) or CI-1040. At 7.0 h p.i., vRNPs were Strep-purified from the total protein lysates. Phosphomodification patterns in the vRNP proteins purified from the cells treated with DMSO and CI-1040 were analyzed by mass spectrometry. We found peptides corresponding to two phosphorylation sites at S269 and S392, which were absent after CI-1040 treatment, indicating that these sites are sensitive to inhibitor treatment.

The validity of the mass spectrometry analysis is reflected by the fact that we additionally found already described phosphorylation sites (S402, S403, S457) (38, 39) (SI Appendix, Fig. S3B), which, however, were not sensitive to CI-1040 treatment. The crystallographic structure of a vRNP bound to RNA and monomeric NP revealed that S269 and S392 are located in close proximity to each other. Furthermore, S269 localizes within the nuclear export signal 2 (NES2) and S392 is located near the NES2 and NES3 of the nucleoprotein (40). A positively charged RNA-binding groove and a loop formed by the viral RNA surrounds the two residues (Fig. 3 A–C and SI Appendix, Fig. S3C).

To analyze whether the two serine residues play a functional role in virus replication, WSN mutants with nonphosphorylatable amino acids at the positions 269 and 392 (S269A, S392A, S269A/S392A) were generated. Notably, phospho-mimicking mutants could not be rescued, indicating that permanent negative charges at these positions are not tolerated. The replication efficiency of the S392A-mutants was decreased within multicycle replication experiments by up to two log10. The S269A-mutant showed only a slight increase in the replication efficiency, especially at earlier time points (Fig. 3D). These data indicate a strong virus-supportive

![Fig. 2.](image-url) Treatment of infected cells with CI-1040 results in chromatin retention of progeny vRNPs and decreased vRNP-binding to the M1 protein. (A) STORM analysis of WSN vRNPs (NP) and M1 localization after CI-1040 (10 μM) and LMB (5 nM) treatment. DMSO (0.1%) and MeOH (0.1%) served as negative controls. See also SI Appendix, Fig. S2G. (B) STORM analysis of dense chromatin after WSN infection and CI-1040 (10 μM) or LMB (5 nM) treatment. See also SI Appendix, Fig. S2H. (A and B) Dashed lines mark the nuclear periphery. Squares (Epi) indicate high-resolution areas. (Scale bar, 500 nm.) (C) Fractionation of WSN infected and CI-1040 (10 μM) (+) treated A549 cells. DMSO (0.1%) (-) served as negative control. Results of one out of three independent experiments for each time point are shown. See also SI Appendix, Fig. S2A, C, and D. (D) Total protein amounts of WSN infected and CI-1040 (10 μM) (+) treated A549 cells. DMSO (0.1%) (-) served as negative control. Total infection times are indicated. Results of one out of three independent experiments for each time point are shown. See also SI Appendix, Fig. S2B. (E and F) Experiments were conducted as in C using Strep-PB2-WSN. Fractionation and vRNP purification were performed after the indicated time points. (E) Results of one out of three independent experiments. See also SI Appendix, Fig. S2F. (F) Total Western blot images were cropped to show the ch150 fraction. Indicated time points were analyzed once. Total analysis is shown in SI Appendix, Fig. S2E.
contribution of the phosphoacceptor residue S392 on the viral life cycle.

**The Kinase RSK1 Links Activation of the Raf/MEK/ERK Pathway to the Control of vRNP Export.** The amino acid sequences adjacent to the identified CI-1040 sensitive phosphorylation sites (L-I-L-R-G-S269-V, A-I-R-T-R-S392-G) lack similarity to the consensus sequence of the ERK phosphorylation motive (P-X-S/T-P) (SI Appendix, Fig. S3D) (43). Therefore it appears unlikely that the identified serine residues are directly phosphorylated by ERK. Instead, the consensus target sequences of the ERK-downstream kinase 90 kDa ribosomal S6 kinase (RSK) (R/L-X-R-X-X-S/T; R-R-X-S/T) showed much higher identities (SI Appendix, Fig. S3D) (44). To explore whether RSK is the link between the activation of the Raf/MEK/ERK signaling pathway and the phosphorylation of NP, which subsequently drives nuclear export of newly synthesized vRNPs, RSK activation during the viral life cycle was analyzed. Indeed, in later stages of the infection, not only ERK but also RSK as well as the RSK downstream target glycogen synthase kinase GSK-3β were increased in their phosphorylation (Fig. 4 and SI Appendix, Fig. S4A). This activation could be blocked by incubation with the MEK inhibitor CI-1040, clearly indicating that virus-induced RSK activation is mediated by the Raf/MEK/ERK pathway (SI Appendix, Fig. S4B). To test for a functional involvement of RSK activation, we used a specific inhibitor of RSK, BI-D1870 (45), which led to a concentration-dependent reduction of GSK-3β phosphorylation, confirming its inhibitory effect on RSK activation during the viral life cycle (Fig. 4B and SI Appendix, Fig. S4C). While, similar to MEK inhibitors (Fig. 2D), BI-D1870 did not affect the synthesis and accumulation of viral proteins, we interestingly found an increase of ERK activation after the inhibition of RSK (Fig. 4B and SI Appendix, Fig. S4C). This is indicative of a BI-D1870-mediated inhibition of a negative feedback loop that under normal conditions would prevent the overactivation of the pathway (45, 46). To test whether RSK inhibition would also lead to impaired export of viral RNPs, BI-D1870 was compared side by side with CI-1040 on their impact on localization of the vRNA and viral proteins NP, M1, PA, and NEP (Figs. 4C and 5D and SI Appendix, Figs. S4 D–K and SSD). Similar to CI-1040, treatment with BI-D1870 resulted in a strong impairment of vRNP nuclear export. The effect on nuclear retention correlates with a strong antiviral activity of BI-D1870 (SI Appendix, Fig. S4 L and M), with a 50% effective concentration (EC50) of 2.8 μM (SI Appendix, Fig. S4M) and a selectivity index (SI) of 157.98 (SI Appendix, Fig. S4N). As a control, a second RSK inhibitor, SL0101-1 was used, resulting in similar antiviral effects (SI Appendix, Fig. S4 O–Q). This demonstrates a strong anti-IAV activity of RSK inhibitors in the absence of any toxic side effects in the effective concentrations. Furthermore, if BI-D1870 and CI-1040 were used in a combinatorial treatment, there were no significant additive effects compared to CI-1040 treatment alone, again demonstrating that RSK acts within the same pathway and is directly activated by the Raf/MEK/ERK kinase cascade (Fig. 4D).

To exclude off-target effects of the RSK inhibitors, the two isoforms RSK1 and RSK2 were knocked down with specific siRNAs and effects on the viral life cycle were analyzed (Fig. 4 E–G and SI Appendix, Fig. S4 R–Y). RSK1 knockdown led to a nuclear retention of newly synthesized vRNPs whereas the RSK2 knockdown had no effect on the nuclear export (Fig. 4E and SI Appendix, Fig. S4 S–W). In multicycle replication analysis, RSK1 knockdown resulted in a strong decrease of viral titers, confirming the data obtained with the inhibitors (Fig. 4F and SI Appendix, Fig. S4X). This was in clear contrast to the RSK2 knockdown, which seemed to have a proviral effect (Fig. 4G and SI Appendix, Fig. S4Z).
Fig. 4. Nuclear retention of progeny vRNPs upon RSK inhibition and RSK1 knockdown. (A) ERK1/2, RSK1, and GSK-3β activation after WSN infection in A549 cells 7 h and 9 h p.i. Results of one out of three independent experiments are shown. See also SI Appendix, Fig. S4A. (B) Increased ERK1/2 and decreased GSK-3β phosphorylation after WSN infection and BI-D1870 treatment in A549 cells 7 h p.i. DMSO (0.1%) served as negative control. Results of one out of four independent experiments are shown. See also SI Appendix, Fig. S4C. (C) Nuclear retention of vRNPs after BI-D1870 (15 μM) treatment in A549 cells 9 h p.i. DMSO (0.1%) served as negative control, Cl-1040 (10 μM) as positive control. Localization of vRNPs (NP) and M1 was analyzed. Representative images of three independent experiments. Dashed squares indicate zoom-in areas (Scale bar, 20 μm.) See also SI Appendix, Fig. S4J and K. (D) Titer reduction of WSN in A549 cells after BI-D1870 (10 μM), Cl-1040 (10 μM), and a combinational treatment (each 10 μM) 24 h p.i. DMSO (0.2%) served as negative control. Data represent means ± SD of four independent experiments, each performed in duplicates. Data passed a one-way ANOVA test followed by Tukey’s multiple comparison test for each time point separately (**P ≤ 0.01; ***P ≤ 0.001). (E) Cellular localization of WSN vRNPs (NP) and M1 9 h p.i. after RSK1 or RSK2 were knocked down in A549 cells. Representative images of three independent experiments. Dashed squares indicate zoom-in areas (Scale bar, 20 μm.) See also SI Appendix, Fig. S4V and W. (F and G) Titers of WSN 24 h p.i. after RSK1 or RSK2 knockdown in A549 cells. Shown are means ± SD of three independent experiments, each performed in triplicates. Data passed a paired two-tailed t test (***P ≤ 0.01; ***P ≤ 0.001). See also SI Appendix, Fig. S4 X and Y. (H and I) Titers of WSN wt or mutants after BI-D1870 (10 μM) treatment in A549 cells 24 h p.i. DMSO (0.1%) served as negative control. Shown are means ± SD of three independent experiments, each performed in triplicates. Statistics: plaque-forming unit (PFU)/mL: Data passed an unpaired two-tailed t test with Welch-correction for each virus individually, Percentage: Data passed a paired two-tailed t test for each virus individually (ns > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).
MEK and RSK Inhibitors Do Not Generally Block Crm1-Mediated Nuclear Export. LMB blocks the general Crm1/exportin1-mediated nuclear export pathway by alkylating and inhibiting Crm1 (48). Since Crm1-mediated export is an essential process for cell homeostasis and LMB acts irreversibly, the compound is toxic and not suitable as a drug. The question now arises, whether MEK and RSK inhibitors would also generally affect Crm1-mediated export, which would disqualify them as possible drug candidates. As a control cargo for the cellular Crm1 export pathway, we used the Ran-binding protein RanBP1, which is known to be exported to the cytoplasm in a Crm1-pathway-dependent manner (49). We compared LMB to the MEK inhibitor CI-1040 and the RSK inhibitor BI-D1870 regarding their effect on the Crm1 pathway. Cells were infected with WSN and treated with the respective inhibitors. Quantification of immunofluorescence stainings 9.0 h p.i. revealed nuclear retention of the viral NP for all tested inhibitors. In contrast, nuclear retention of RanBP1 was only detected in the LMB-treated samples (Fig. 6A and B). Comparable results were found for other MEK and RSK inhibitors, such as ATR-002 and SL0101-1, respectively (SI Appendix, Fig. S5 F and G). This strongly indicates that the inhibition of the Raf/MEK/ERK/RSK pathway has no general effect on the Crm1 export but specifically controls export of the influenza vRNPs.

Broad Anti-Influenza Activity of BI-D1870. To confirm that the effect of RSK inhibition on viral replication is not strain specific, we used a range of different IAV subtypes, including a seasonal H3N2 virus, a swine origin H1N1 pandemic (pdm) virus from 2009, highly pathogenic avian viruses of subtype H5 and H7, as well as an IBV strain to determine the broad anti-influenza virus activity. All tested viruses showed both nuclear retention of newly synthesized vRNPs and a reduction of progeny viral titers of around one log10 in the presence of the RSK inhibitor (Fig. 7). These results confirm a broad dependence of IAV and IBV on the Raf/MEK/ERK/RSK pathway (26–28) and show that RSK is the critical mediator that links the pathway to vRNP export.

In summary, we have identified the complete chain of events that link the nuclear export of newly synthesized vRNPs with viral activation of the Raf/MEK/ERK/RSK pathway (Fig. 7Q). As inhibitors targeting the pathway are not toxic, they may serve as promising candidates for the development of broadly active anti-influenza drugs.

Discussion

Influenza viruses are nuclear-replicating viruses. Hence, the viral genome needs to be exported out of the nucleus at late stages of the replication cycle to be transported to the cellular membrane and packaged in progeny viruses. Since at early and intermediate stages of replication new genomes are needed as templates for the amplification of RNA replication and transcription, vRNP export should not be constitutive but regulated and preferentially promoted in late stages of infection. We showed previously that late-stage viral activation of the Raf/MEK/ERK pathway by accumulation of HA in the host membrane and protein kinase C

![Fig. 5. BI-D1870 treatment results in chromatin retention of progeny vRNPs and decreased binding rates to the M1 protein. (A) Fractionation and Strep-purification of Strep-PB2-vRNP (WSN) after BI-D1870 (10 μM) (+) treatment 7 h p.i. DMSO (−) served as negative control. See also SI Appendix, Fig. S5A. (B) Total protein amounts of the fractionated cell lysates from A. (C) Protein amounts of total cell lysates from A. (D) Cellular localization of WSN vRNA 7 h p.i. after BI-D1870 (10, 15 μM) treatment 3 h p.i. DMSO (0.1%) served as negative control. See also SI Appendix, Fig. SSD. (E) Dense chromatin analysis of PB2-vRNA 7 h p.i. after BI-D1870 (10, 15 μM) treatment. DMSO (0.1%) served as negative control. Same laser and detector settings were used. See also SI Appendix, Fig. S5E.](https://www.pnas.org/content/117/28/16563/tab-figures-supplemental-material)
activation is linked to vRNP nuclear export and may represent such a regulatory principle (15, 25). However, the exact mechanism of how the pathway supports this process was completely enigmatic for a long time. By the use of siRNA knockdown approaches as well as kinase inhibitors we could now not only unravel the complete chain of events how the Raf/MEK/ERK kinase pathway controls the nuclear export of newly produced vRNPs, but at the same time dissect the antiviral mode of action of the MEK inhibitor CI-1040. A derivate of CI-1040, ATR-002 has now successfully passed a phase I clinical trial (clinicaltrials.gov: NCT04385420) and is under further clinical development as an anti-influenza drug.

It is known that the vRNP export complex assembles at the chromatin, where it can interact with the cellular export protein RanBP1. (PKCα) activation is linked to vRNP nuclear export and may represent such a regulatory principle (15, 25). However, the exact mechanism of how the pathway supports this process was completely enigmatic for a long time. By the use of siRNA knockdown approaches as well as kinase inhibitors we could now not only unravel the complete chain of events how the Raf/MEK/ERK kinase pathway controls the nuclear export of newly produced vRNPs, but at the same time dissect the antiviral mode of action of the MEK inhibitor CI-1040. A derivate of CI-1040, ATR-002 has now successfully passed a phase I clinical trial (clinicaltrials.gov: NCT04385420) and is under further clinical development as an anti-influenza drug.

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Crm1 (14). The inhibition of MEK or RSK during the viral life cycle led to a retention of the newly synthesized vRNPs at the chromatin and reduced binding abilities to the viral M1 protein, which is essential for the nuclear export of the viral genome (Figs. 1 E–H, 2, 4, and 5 and SI Appendix, Figs. S1, S2, S4, and S5) (11, 12).

We could identify two new phosphorylation residues within the viral NP, the major constituent of vRNPs (serine 269 and serine 392) (Fig. 3 and SI Appendix, Fig. S3). The sites are located in the loop region (264 to 276; 452 to 463) and the body domain (21 to 149; 273 to 396; 464 to 489) of the protein, respectively, and are located in close proximity to each other. Crystallographic structures of the vRNP helix bound to vRNA revealed that these two serine residues are surrounded by a specifically formed vRNA loop (Fig. 3B and SI Appendix, Fig. S3C) (40, 41).

Signaling pathways are often controlled by negative feedback loops. This is also true for the Raf/MEK/ERK pathway that is negatively controlled by the active downstream kinase RSK (46). If RSK is inhibited, its negative regulating mode of action is missing and the kinases upstream of RSK are over activated (Figs. 4B and 5C and SI Appendix, Fig. S4C). Together with other findings in the present study, this observation allows the conclusion that RSK1 is the predominant downstream effector of ERK that mediates vRNP export. The hyperactivation of ERK in the presence of the RSK inhibitor does not lead to enhanced vRNP export but rather the opposite, indicating that the signal is not further transmitted if RSK is blocked.

Kakugawa and colleagues already previously investigated the role of RSK2 in influenza virus-infected cells (47). They could show via knockdown experiments that RSK2 has an antiviral function as the knockdown resulted in increased IAV and IBV replication. This effect was explained by a role of RSK2 in the innate immune response due to the activation and expression regulation of nuclear factor KB (NF-kB), interferon β (IFNβ), and protein kinase R (PKR). We could fully confirm the antiviral function of RSK2 in our experiments, since RSK2 knockdown lead to higher viral titers (Fig. 4G and SI Appendix, Fig. S4G). In clear contrast, RSK1 knockdown resulted in strongly decreased viral titers and the nuclear retention of newly synthesized vRNPs (Fig. 4 F, I, and M and SI Appendix, Fig. S4X). These findings point to opposite roles of the two RSK subtypes during influenza virus infection. It was shown that messenger RNA (mRNA) and protein expression levels of RSK1 are much higher than RSK2 in cell lines (21 to 149; 273 to 396; 464 to 489) of the protein, respectively, and are located in close proximity to each other. Crystallographic structures of the vRNP helix bound to vRNA revealed that these two serine residues are surrounded by a specifically formed vRNA loop (Fig. 3B and SI Appendix, Fig. S3C) (40, 41).

The hyperactivation of ERK in the presence of the RSK inhibitor does not lead to enhanced vRNP export but rather the opposite, indicating that the signal is not further transmitted if RSK is blocked.

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