New Host Factors Important for Respiratory Syncytial Virus (RSV) Replication Revealed by a Novel Microfluidics Screen for Interactors of Matrix (M) Protein*

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Although human respiratory syncytial virus (RSV) is the most common cause of bronchiolitis and pneumonia in infants and elderly worldwide, there is no licensed RSV vaccine or effective drug treatment available. The RSV Matrix protein plays key roles in virus life cycle, being found in the nucleus early in infection in a transcriptional inhibitory role, and later localizing in viral inclusion bodies before coordinating viral assembly and budding at the plasma membrane. In this study, we used a novel, high throughput microfluidics platform and custom human open reading frame library to identify novel host cell binding partners of RSV matrix. Novel interactors identified included proteins involved in host transcription regulation, the innate immunity response, cytoskeletal remodeling, membrane remodeling, and cellular trafficking. A number of these interactions were confirmed by immunoprecipitation and cellular colocalization approaches. Importantly, the physiological significance of matrix interaction with the actin-binding protein cofilin 1, caveolae protein Caveolin 2, and the zinc finger protein ZNF502 was confirmed. siRNA knockdown of the host protein levels resulted in reduced RSV virus production in infected cells. These results have important implications for future antiviral strategies aimed at targets of RSV matrix in the host cell. Molecular & Cellular Proteomics 14: 10.1074/mcp. M114.044107, 532–543, 2015.

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1 The abbreviations used are: RSV, Respiratory Syncytial Virus; M, Matrix protein; F, Fusion protein; N, Nucleo protein; P, Phospho protein; IB, Inclusion Bodies; RNP, Ribo Nucleo Protein; ARE, Apical Recycling Endosomes; NDV, Newcastle Disease Virus; MOI, Multiplicity of Infection; PING, Protein Interaction Network Generator; ORF, Open Reading Frame; FBS, Fetal Bovine Serum; BSA, Bovine Serum Albumin; PFA, Paraformaldehyde; LSM, Laser Scanning Microscope; ZNF, Zinc Finger proteins.
complexes from IBs to the sites of budding (16). We recently showed that ordered oligomerization of M is central to infectious filamentous virus production (17), potentially through providing the framework for filament morphology (18), in conjunction with M2–1, which serves as a bridging protein between the oligomeric M layer and RNP in the mature virus (19).

Additional to the crucial role of M in RSV filament morphology and infectivity, M has been suggested to recruit cellular factor(s) during virus assembly (20–23). Proteins involved in apical recycling endosomes (ARE)-mediated protein sorting (e.g. Myosin 5 beta), have been shown to be essential for RSV assembly (24) with budding of released virus believed to be Vps4-independent and to require Rab11a FIP2 protein (25). However, only Importin-β1 (5) and CRM1 (8) (see above) are known to be direct interactors of M. A proteomic screen for cellular interactors of RSV M, N, and F proteins identified only limited numbers of proteins, none of which could be validated to bind directly to M (26). Overall, the network of RSV-cell interactions is still mostly unknown, with limited targets identified.

Protein microarrays technology allows the interrogation of protein–protein interactions, which could possibly overcome the obstacles mentioned above (27). Here we use an in vitro protein expression and interaction analysis platform based on a highly parallel and sensitive microfluidics affinity assay (28) to identify new host factors interacting with RSV M. This is the first time microfluidics has been used to screen for host factors interacting with a protein from a negative strand RNA virus. A range of factors were identified for the first time, including proteins involved in host transcription and translation regulation, innate immunity response, plasma membrane remodeling, cytoskeleton regulation, and cellular trafficking, with a number verified by coprecipitation. Of these, we present initial characterization of key caveolae structural component Caveolin (Cav) and the actin-binding protein Cofilin1 (Cof1) as cellular factors that colocalize with M in viral inclu- sions and filaments, and of the zinc finger protein ZNF502, which appears to interact with RSV M in the nucleus. These and the other host factor-RSV M interactions identified here for the first time may be exciting possibilities as targets for anti-RSV approaches in the future.

**EXPERIMENTAL PROCEDURES**

**Microfluidics** — The microfluidic devices were fabricated on silicone molds performed as described previously (29, 30). To prevent nonspecific adsorption and to control suitable binding orientation, all of the accessible surface area within the microfluidic device was chemically modified. Biotinylated-BSA (1 μg/μl, Pierce, Tel Aviv, Israel) was flowed for 20 min through the device, allowing binding of the BSA to the epoxy surface. Streptavidin (0.5 μg/μl) (Neutravidin, Pierce) was then added for 20 min. The “button” valve was then closed and biotinylated-BSA was flowed over once again as above. Following the passivation step, the “button” valve was released and the appropriate anti-penta-His (Qiagen, Valencia, CA) or -V5 (Pierce) biotinylated antibody applied at concentrations of 0.1–0.2 mg/ml enabling binding to exposed Streptavidin, specifically activating the area under the “button.” Hepes (50 mM, Biological Industries, Kibbutz Beit Haemek, Israel) was used for washing unreacted substrates between each of the surface chemistry steps.

**Protein Network Interaction Generator (PING)** — Proteins were expressed on the device or in a test tube (small evaluation screens) using an expression mix of rabbit reticulocyte quick coupled transcription and translation reaction (Promega, Madison, WI). In this study, coding regions for 500 proteins from the human proteome were assembled using PCR (30) and selected clones from the public genome-scale lentiviral sequence-confirmed expression library of human ORFs (open reading frames) in a Gateway vector system (31) as templates. PCR products were generated by using specific primers encoding c-Myc and Penta-His tags at the N and C-terminal, respectively, for all of the ORFs. The linear synthetic genes were spotted onto the glass substrate and the microfluidic device attached to the array. Protein expression on the device was performed by flushing the device with 12.5 μl of the expression mix, following opening of the “neck valve,” to initiate in vitro transcription/translation of all of the proteins in parallel. The “sandwich” valves were then closed to isolate each unit cell from its environment, and the device incubated on a hot plate for 2.5 h at 32 °C. Expressed proteins then diffused through the DNA channel to the reaction chamber and immobilized to the surface via binding of the C-terminal Penta-His tag to the appropriate surface-attached antibody under the “button” area. Proteins were labeled fluorescently using 0.01 mg/ml Cy3-labeled anti-c-Myc antibody (Sigma, St. Louis, MO).

In small evaluation screens, PCR-generated human ORF linear expression templates were added directly to rabbit reticulocyte lysate off-chip in the presence of a FluoroTect™ Green Lys (Promega) in vitro Translation Labeling System for labeling and detection of proteins synthesized in a final volume of 25 μl rabbit reticulocyte lysate and 1 μg of the relevant DNA in an Eppendorf microfuge tube. After incubation at 32 °C for 2.5 h with agitation (600 rpm), proteins were flushed into the device to allow binding to the surface. RSV M protein was produced after cloning of the coding sequence into the pCAG vector using the same PCR approach as above, but with C-terminal primer encoding the V5 tag. For all experiments, RSV M was generated by in vitro transcription/translation from the linear PCR fragment in a final volume of 25 μl in an Eppendorf microfuge tube, which was then incubated as above, before flushing into the device as above.

**Analysis of Microfluidics** — Interaction ratios are defined as the fluorescence ratio for RSV M and the surface bound human protein based on the extent of labeling by 0.01 mg/ml of Alexa 647-labeled anti-V5 (Abd-Serotec, Oxford, UK) and Cy3-labeled anti-c-Myc (Sigma) antibodies, respectively. Measurements were performed using a microarray scanner (LS Reloaded, Tecan) with fluorescent excitation at 635 nm (for RSV M) and 575 nm (for human proteins). In small screens, human proteins were expressed in presence of fluorescent Lys (Fluo- roTect™ Green Lys, Promega) and protein expression levels determined with a microarray scanner (LS Reloaded, Tecan) with fluorescent excitation at 488 nm, where the signals were normalized to the number of lysines in each protein (30).

**Cell Culture and RSV Virus Preparation** — HEp-2 (ATCC CCL-23™), 293T, and Vero cells (both cell lines were provided by W. Barclay, Imperial College London) were maintained in DMEM medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% l-glutamine, and 1% penicillin-streptomycin. The transformed human bronchial epithelial cell line BEAS-2B (ATCC) was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 1% L-glutamine, and 1% penicillin-streptomycin. Transfections were performed with Lipo- fectamine 2000 (Invitrogen) used according to the manufacturer’s specifications. Plaque-purified human RSV (A2 strain from ATCC) or
recombinant (r) A2 RSV strain was cultured in Vero cells. In the case of cell-associated virus, the culture supernatant was removed when extensive cytopathic effects were observed, and the cells resuspended in serum free SPGA media (218 mM sucrose, 7.1 mM KH2PO4, 4.9 mM sodium glutamate, and 1% BSA), followed by centrifugation (13000 × g, 15 min, 4 °C) and storage at −80 °C. RSV titers were determined by immune-plaque assay in triplicate on HEp-2 cells.

**Calcium Phosphate Transfection**—Overnight cultures of 293T cells, seeded at 1 × 10^6 cells/60 mm plate in 5 ml DMEM/10% FBS, were transfected with 4 μg of each plasmid. Plasmid DNA was added to sterile H2O to 215 μl total volume. In a separate Eppendorf microfuge tube 250 μl of 2×HeBS buffer (42 mM Hepes, 270 mM NaCl, 1 mM KCl, and 10 mM Dextrose, pH 7.1) was mixed with 5 μl 100×PO4 (1.4 mM Na2HPO4). Diluted DNA was mixed with the HeBS/PO4 solution, 30 μl of 3.2 μM CaCl2 was added gently, and the mix was immediately added to cells. Media was changed after 6 h and cells were harvested 36 h post-transfection for immune-preparation assay.

**Immune-plaque Assay**—Overnight cultures of HEp-2 (seeded at 4 × 10^5 cells/well in 96-well plate) were infected with virus stocks diluted in a separate 96-well plate using doubling dilutions in serum-free DMEM media, starting at 1/10 for supernatant virus and 1/50 for cell-associated virus. Cells were washed once with serum-free DMEM, and 50 μl of each of the virus dilution added to triplicate wells and left for 2 h at 37 °C. One hundred microliters of 2% FBS DMEM DMEM was then added and the plates incubated for further 48 h at 37 °C. In the case of immunostaining, medium was discarded and the cells washed in 100 μl phosphate buffered saline (PBS) and fixed for 20 min with 100 μl absolute methanol containing 2% hydrogen peroxide. After washing with 200 μl PBS/1% BSA, cells were incubated with biotinylated anti-RSV antibody (1:500, AbD Serotec) for 2 h, washed three times in PBS/1% BSA, and then stained with ExtrAvidin peroxidase (1:500, Sigma) for 2 h in the dark. Cells were washed three times and plaques visualized using Sigma Fast Diamobenzidine (DAB) Peroxidase Substrate (Sigma).

**Viral Plaque Assay (PFA Fixation/Crystal Violet)**—Overnight cultures of Vero cells were seeded at a density of 2 × 10^6 cells/well in 24-well plates to form an even monolayer. Viral stocks to be assayed were diluted 1:10 (50 μl into 450 μl DMEM 2% FCS) from 10^-1 to 10^-5 and 200 μl used to infect duplicate wells. Infected cells were placed at 37 °C for 2 h then washed with DMEM/2% FCS then overlaid with 500 μl of 0.8% Aquacalidil/DMEM 2% FCS and incubated at 37 °C for 4–6 days. Cells were fixed with 4% Paraformaldehyde (PFA) for 12 h then stained with 0.4% Crystal Violet/20% Methanol in PBS. Plaques were then imaged on an Olympus SX61 Microscope.

**Visualization of Virus-like Filaments**—Overnight cultures of HEp-2 cells (seeded at 4 × 10^5 cells/well in 6-well plates on 16-mm glass coverslips) were transfected to express plasmids (0.4 μg each) encoding the RSV A2 WT M protein along with pcDNA3.1 codon optimized plasmids encoding the RSV A2 N, P, and F, using Lipofectamine 2000 (Invitrogen). Cells were fixed 24 h post-transfection, immunostained, and imaged as described below.

**RSV Infection for Infectivity Assay and Viral Filament Visualization**—Overnight cultures of HEp-2 or BEAS-2B cells, seeded at 4 × 10^5 cells/well in 6-well plates with or without 16 mm glass coverslips, were infected with recombinant RSV WT (rA2) or WT RSV at a multiplicity of infection (MOI) of three. Cells were fixed 24 h postinfection, immunostained and imaged as described below, to visualize filaments. For infectivity assay, cell-associated and released virus fractions were harvested and virus titers of cell-associated and supernatant fractions were determined using the immune-plaque assay or PFA fixation/Crystal Violet assay (above).

**Immunostaining and Imaging**—Cells were fixed with 4% paraformaldehyde in PBS for 10 min, blocked with 3% BSA in 0.2% Triton X/PBS for 10 min, and communostained with monoclonal anti-M (1:200, gift from Mariethe Ehnlund, Karolinska Institute, Sweden), polyclonal anti-RSV (1:200, Abcam, Cambridge, UK), monoclonal anti-N (1:500, Abcam), polyclonal anti-G (1:100, Abcam), polyclonal anti-Cav1 (1:500, Abcam), polyclonal anti-Cav2 (1:50, Abcam), or polyclonal anti-Cof1 (1:500, Abcam) antibodies followed by species specific secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568 (1:1000, Invitrogen). Images were obtained on Zeiss 5 PASCAL confocal laser scanning microscope (LSM) using 63×/1.4 Plan-Apochromat oil lens (averaging 4×). Images were acquired using Zeiss LSM Image Browser software (4.2.0.121, Zeiss, Cambridge, UK).

**Coprecipitation Experiments with One-STREP-FLAG (OSF) Tagged M**—293T cells were seeded (1.5 × 10^6 cells/60 mm dish) and cotransfected with 4 μg of each of the relevant expression plasmids using the calcium phosphate method (above). Cells were harvested 36 h post-transfection and lysed in 300 μl lysis buffer (50 mM Tris, pH 7.4, and 150 mM NaCl) supplemented with protease inhibitor mixture (Sigma) and 1% Triton X-100 for 10 min. Lysates were clarified by centrifugation (11,600 × g, 6 min, 4 °C), and streptactin-tagged proteins affinity purified by incubation with StreptTactin Sepharose for 2 h (40 μl slurry, Qiagen). The beads were washed three times in wash buffer (20 mM Tris, pH 7.4, and 150 mM NaCl) supplemented with 0.1% Triton X-100, and bound proteins detected by Western blot.

**Small Interfering RNA (siRNA) Depletion**—For knockdown of Caveolin1, Caveolin2, and Coiflin1, overnight cultures of HEp-2 cells were seeded at 4 × 10^5 cells/well in six-well plates or 2 × 10^5 cells/well in 12-well plates on 16 mm glass coverslips and siRNA depletion experiments were performed 24 h later with siRNA (20 nm Qiagen FlexiTube siRNA using Lipofectamine RNAiMax, Invitrogen), the medium changed 6 h later, and the cells infected at 48 h after siRNA treatment with WT A2 RSV at an MOI of three. Cells and supernatant were harvested 24 h later for Western blot and virus titer (cell associated and released virus) analysis and/or immunostaining.

In the case of ZNF502 knockdown, sub-confluent monolayers of 293T cells were seeded into 12-well plates overnight. Cells were transfected with 20 nm ZNF502 SMARTPool siRNA or negative control siRNA (Dharmacon, Mulgrave, Australia) using 2 μl DharmaFECT1 transfection reagent as recommended by the manufacturer. Forty-eight hours post transfection, cells were infected with (r) A2 RSV strain at an MOI of one, and cell associated and supernatant released virus was harvested for analysis by plaque assay 24 h postinfection.

**SOS-PAGE and Western Blotting**—Protein samples were analyzed on 12% polyacrylamide gels and subjected to electrophoresis in Tris-glycine buffer. All samples were boiled for 3 min prior to electrophoresis. Gels were then transferred to PVDF membrane (Roche Diagnostics, Sussex, UK). The blots were blocked with 5% nonfat milk in Tris-buffered saline (pH 7.4) followed by incubation in monoclonal anti-Myc (9E10 hybridoma), monoclonal anti-FLAG (1:1000, Sigma), polyclonal anti-Cofilin1 (1:1000, Abcam), polyclonal anti-Caveolin1 (1:1000, Abcam), polyclonal anti-Caveolin2 (1:1000, Abcam), polyclonal anti-ZNF502 (1:1000, Abcam), or monoclonal anti-β actin (1:500, Abcam), and horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit antibody (1:10000, Invitrogen). Western blots were developed using freshly prepared chemiluminescent substrate (100 mM Tris-HCl, pH 8.8, 1.25 mM luminol, 0.2 mM p-convamic acid, and 0.05% H2O2) and exposed to Fuji autoradiography films.

**RESULTS**

**High Throughput Screen for M-host Interactions using Microfluidics**—We performed a high-throughput microfluidics-based screen (see Experimental Procedures section for de-
tails) using RSV M as a prey to identify interactors from a custom library of 500 specific human genes. Encoded proteins were expressed on chip from cDNAs and immobilized on the chip surface (see schematic in Fig. 1A). RSV M protein was expressed separately using a coupled in vitro transcription/translation system, and subsequently flowed over the chip using the microfluidics plumbing. Protein immobilization through the C terminus and labeling through the N terminus ensures that only full-length proteins are tested. The custom library included genes from pathways reported to be involved in the replication of RSV and related viruses. Each bar represents an average of eight replicates, with an additional 268 spots lacking cDNAs serving as negative controls. The X and Y axes represent the coordinates in the two-dimensional protein arrays. Only signals with three standard deviations above the negative background level were scored as positive for protein expression. Interaction was defined as the ratio between the fluorescence signals for M (detected with Alexa Fluor 647) and the human protein (detected with Cy3), and normalized relative to the highest ratio. Signals with two standard deviations above negative background were scored as hits.

The screen was performed four times, with the results of a typical screen shown in Fig. 1B, where the data points represent the average signal for the eight replicates for each protein. Ninety-three of the expressed proteins gave a ratio of two standard deviations above background once or more (see supplemental Table S1). The majority of these were proteins involved in cellular transcription and mRNA translation and...
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Table I

| Gene      | UniProtKB | Protein name                      | Positive screens | Function                                                                 |
|-----------|-----------|-----------------------------------|------------------|--------------------------------------------------------------------------|
| ACP1      | P24666    | Acid Phosphatase 1                | 2                | Protein tyrosine phosphatase                                             |
| AMOT      | Q4VCS5    | Angiomotin                        | 4                | Tight junction maintenance; endothelial cell migration                   |
| AmotL2    | Q9Y2J4    | Angiomotin like protein 2         | 3                | Actin filament regulation; Wnt/β-catenin signaling                       |
| Cav2      | P51636    | Caveolin 2                        | 2                | Caveolae formation; MAPK signaling; MAPK1 and STAT3 activation          |
| Cav3      | P56539    | Caveolin 3                        | 2                | Caveolae formation                                                      |
| CFL1      | P23628    | Cofilin 1                         | 3                | Actin dynamics; F-actin depolymerization; cell morphology; regulation of transcription |
| HIST1H4   | P62805    | Histone core protein 4            | 2                | Core component of nucleosome                                             |
| HSPB2     | Q16082    | Small heat shock protein 27       | 2                | Regulation of DMPK kinase                                               |
| KPN2A     | P52292    | Importin α1 (Rch1)                | 2                | NLS-recognising adaptor protein mediating nuclear import                |
| PIK3CB    | P42338    | Phosphatidylinositol 4, 5 bisphosphate 3 kinase catalytic subunit γ | 3 | Cell growth; immune response; cell motility and morphology |
| PIK3CG    | P48736    | Phosphatidylinositol 4, 5 bisphosphate 3 kinase catalytic subunit β | 3 | Cell growth; immune response; cell motility and morphology |
| PPIA      | P62937    | Cyclophilin A                     | 2                | Accelerates protein folding                                             |
| PTMA      | P06454    | Prothymosin α                     | 2                | Mediates immune function                                                |
| Rab11a    | P62491    | Ras related protein Rab11a        | 3                | Intracellular membrane trafficking, epithelial cell polarization        |
| Rab11b    | Q15907    | Ras related protein Rab11b        | 2                | Intracellular membrane trafficking                                       |
| EXOC6     | Q8TA99    | Exocyst Complex Component 6       | 2                | Vesicular trafficking from the Golgi to plasma membrane                 |
| SMAD3     | P84022    | SMAD family member 3              | 2                | Transcriptional regulator activated by TGF-β                           |
| SUMO3     | P55854    | Small Ubiquitin-like modifier 3   | 3                | Adaptor molecule involved in nuclear transport, DNA replication, signal transduction |
| Tom22     | Q9NS69    | Translocase of outer mitochondrial membrane 22 homolog | 2 | Mediates transport into the mitochondria of cytoplasmically synthesized proteins |
| TUBA6     | Q9BQ87    | Tubulin α                         | 2                | Major component of microtubules                                         |
| VDAC1     | P21796    | Voltage dependent anion channel 1 | 2                | Mitochondrial outer mitochondrial membrane channel protein; implicated in apoptosis |
| ZCCHC7    | Q8NSM26   | CCHC domain-containing zinc finger protein 7 | 2 | Component of the nuclear TRAMP-like complex |
| ZNF501    | Q96CX3    | Zinc finger protein 501           | 2                | DNA/zinc ion binding; involved in transcriptional regulation             |
| ZNF502    | Q8TBZ5    | Zinc finger protein 502           | 2                | DNA/zinc ion binding; involved in transcriptional regulation             |

Abbreviations: Wingless-type (Wnt), mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 3 (STAT3), dystrophy myotonic protein kinase (DMPK), nuclear localization signal (NLS), rat sarcoma (RAS), transforming growth factor (TGF), translocation associated membrane protein (TRAMP).

Splicing regulation, signaling and trafficking pathways, and cytoskeleton related proteins. Table I lists 24 novel binding partners for M that scored positive in two or more screens.

**RSV M Host Interactions Verified**—A selection of the hits was evaluated in more detail using a smaller microfluidic device (Fig. 2A) (see Experimental Procedures for details), whereby human proteins were expressed in the presence of fluorescent Lys (FluoroTect™ GreenLys) with C-terminal Penta-His to ensure that the N-terminal Myc tag is not impacting on proper folding of the protein. Based on the close homology and similar functional role of Cav1 to Cav2/3, Cav1 (not in the original screen) was also tested in these experiments, together with the proteasomal regulator Psme3 (PA28) and green fluorescent protein (GFP) as negative controls. All of the hits (as well as Cav1), showed substantial and specific binding to M under the tested conditions (Fig. 2A).

We then validated a number of the interactions in coimmunoprecipitation experiments in 293T cells using expression constructs for Myc-tagged cellular factor and OSF-tagged RSV M (Fig. 2B); in this assay RSV M was captured onto affinity beads while the cellular protein was in the lysate, in contrast to the binding setup in the microfluidics systems. M2–1, previously shown to interact with M in a cell-free system (10), was used as positive control; additionally, because RSV M is known to form higher order oligomers, we also used this as a positive control (17, 18). As is evident from Fig. 2B, Myc-M2–1 coprecipitated specifically with OSF–M, as did (from left to right in the top panel) Myc–M, -Cav1, -Cav3, -Cof1, -ZNF502, and -Tom22, whereas Myc-Rab11b and AmotL2 did not. Importantly, Cav1, Cav3, Cof1, ZNF502, and Tom22 were all able to interact with RSV M in intact cells. Overall, five out of seven interactions (71%) were confirmed using the coimmunoprecipitation assay.

For further experiments, we decided to focus on the actin-binding protein Cof1, and Cav1, which as a heterodimer with Cav2, is a key component of the caveolae membrane struc-
features that are believed to be important for RSV filament formation. Cav3, which has amino acid sequence similar to Cav1, was not included because it is mostly expressed in muscle cells (32), which are not relevant for RSV infection. In addition, because our hits included a number of transcription factors, including several zinc finger proteins that may be targets of RSV M transcriptional inhibition (6) (Table II), we selected zinc finger protein ZNF502 for further analysis. ZNF502 was also identified as a binding partner of M using mass spectrometric approach (data not shown).

**RSV Colocalizes with Cav1 and Cofilin1 Proteins in Virus-like Structures**—In order to validate whether the identified proteins colocalize with M within RSV-like structures, we used our previously described VLP transfection based assay (Fig. 3). To analyze the subcellular localization of the host proteins in the absence of RSV proteins, HEp-2 cells were transfected to express Myc-tagged CHMP1B, -M2–1, -Cav1, -Cof1, or -ZNF502, where CHMP1B served as a negative control based on the screening results, and M2–1 was the positive control (see above). Cells were fixed and stained with anti-Myc antibodies 24 h post-transfection (Fig. 3A), with Myc-CHMP1B found to localize to endosomal membranes as previously observed (33) and Myc-M2–1 showing a cytoplasmic distribution. Myc-Cav1 localized into caveolae structures at the plasma membrane, Myc-Cof1 was cytoplasmic, possibly associated with the cytoskeleton, and Myc-ZNF502 was nuclear. To analyze colocalization in the presence of RSV proteins, HEp-2 cells were cotransfected with codon-optimized vectors expressing RSV F, N, P, and M protein, together with the plasmids encoding the Myc-tagged proteins as in Fig. 3A. Cells were fixed and stained with anti-RSV in addition to anti-Myc antibodies 24 h post transfection (Fig. 3B). As expected, Myc-CHMP1B showed no alteration in localization in the presence of RSV proteins, whereas Myc-M2–1 showed striking colocalization with RSV proteins in inclusions, in stark contrast to its localization in the absence of RSV proteins. In the presence of RSV proteins, Myc-Cav1 was observed to localize at the base of virus-like filaments, whereas Myc-Cof1 relocated to the nucleus in the presence of ectopically expressed ZNF502—In contrast to the above, Myc-ZNF502 appeared to remain predominantly nuclear even in the presence of RSV proteins (Fig. 3B). To assess whether ZNF502 might affect M subcellular localization, we performed the same experiment but instead staining for M specifically (Fig. 3C). In the absence of Myc-ZNF502, M was predominantly associated with the virus-like filaments, but in its presence, M localization appeared to change. M demonstrated reduced association with filaments, and instead increased localization in both cytoplasm and nucleus.

**Cav1 and Cof1 Localize in Viral Structures in RSV Infected Cells**—To extend the results to infected cells, we performed immunofluorescence on BEAS-2B cells infected with WT A2 virus and stained for RSV and endogenous Cav1, Cav2, or Cof1 (Fig. 4A). Results were comparable to those above; in mock infected cells (upper panel), endogenous Cav1 and Cav2 localized into caveolae structures whereas Cof1 was cytoplasmic. In infected cells, RSV proteins colocalized with Cav1 and Cav2 in viral filaments, whereas Cof1 relocated
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### Table II

| Gene      | Protein name                                      | Number and type of zinc fingers | Function                                      |
|-----------|--------------------------------------------------|---------------------------------|------------------------------------------------|
| Trim27    | Tripartite Motif Containing 27                   | 3, RING and B box               | Transcription repression, E3 ligase of PIK3C2B, inhibition of CD4 activation |
| YY1       | Yin and Yang 1                                   | 4, GLI Kruppel                  | Transcription repression                       |
| ZCCHC7    | Zinc finger CCHC domain containing 7             | 4, CCHC                         | RNA exosome                                    |
| ZNF501    | Zinc finger protein 501                          | 9, C2H2                         | Presumably transcription regulation            |
| ZNF502    | Zinc finger protein 502                          | 14, C2H2                        | Presumably transcription regulation            |
| ZMYM6     | Zinc finger MYM type 6                           | 8, MYM                          | Cell morphology and cytoskeleton               |
| ZNF410    | Zinc finger protein 410                          | 5, C2H2                         | Transcription factor                           |
| NR2C2     | Nuclear receptor subfamily 2, group C            | 2, NR-C4                        | Transcription repression                       |

|          |                                                   |                                 | Antibody: Phosphatidylinositol 4,5 bisphosphate 3 kinase catalytic subunit β (PIK3C2B). |

Introduced into viral IBs (lower panel). Western analysis indicated that Cav1 and Cav2 levels did not change up to 48 h postinfection compared with mock-infected cells, whereas Cof1 levels were reduced in the soluble fraction in infected cells (Fig. 4B), most probably because of relocation into IBs, which are not soluble in the buffer used.

In summary, Cav1, Cav2, and Cof1 subcellular localization was altered in the presence of RSV proteins, and showed marked colocalization with viral components, consistent with a direct interaction with RSV M, whereas ectopically expressed ZNF502 appears to change subcellular localization of Cof1, ZNF502, and Cav2 and RSV M protein. Endogenous Cav1 and Cav2 were found to colocalize with the viral filaments in RSV-infected cells (Fig. 2). Importantly, we observed colocalization of endogenous Cav1 and Cav2 with viral filaments (Fig. 3B and Fig. 4A) raising the question as to whether Cav proteins may be required either for viral filament formation or viral infectivity. To test this, HEp-2 cells were transfected with siRNA specifically targeting Cav1, Cav2, or both with scrambled siRNA serving as control, and cells were infected with WT RSV at an MOI of three 48 h later. Knockdown of Cav1 alone (90% reduction) had no significant impact on cell-associated virus titer. In contrast, depletion of Cav2 (80% reduction) or Cav1 and Cav2 simultaneously (65 and 90%, respectively) resulted in up to 70% reduction in virus titer (Fig. 5D, upper panel). We also checked whether depletion of Cav proteins affect released virus infectivity. Knockdown of Cav2 alone resulted in significant reduction in viral titer (Fig. 5D, lower panel).

Because depletion of Cav2 seemed to affect virus titer both in cell-associated and released virus fraction, we tested whether RSV filaments still formed in Cav-depleted cells. HEp-2 cells were transfected with siRNA specifically targeting Cav1, Cav2, or control scrambled siRNA, and infected with WT RSV at an MOI of three 48 h later. Cells were subsequently fixed 24 h later, and stained with antibodies specific for Cav1 or Cav2 and RSV M protein. Endogenous Cav1 and Cav2 were found to colocalize with the viral filaments in RSV-infected cells, in agreement with staining in BEAS-2B cells (Fig. 4A). Significantly, however, viral filaments still formed in Cav1-depleted and in Cav2-depleted and RSV-infected cells (supplemental Fig. S1).

**Discussion**

This study is the first to use microfluidics technology to perform a high-throughput screen to identify 24 novel host factors that can interact directly with the RSV M protein (see Table I). Further, although previous proteomics-based studies have identified host cell factors important for RSV replication, this is the first to identify and validate host proteins directly interacting with RSV M. Here, we focused on three selected
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We showed that M can interact with all three members of the Caveolin family (Fig. 2 and Table I). Together with cellular proteins and their effect on RSV replication. Significantly, our microfluidics screen identified a total of eight ZNF-containing proteins, mostly transcription factors, as interactors of RSV M (Table II). We focused on ZNF502, hits in particular, characterizing their colocalization with RSV proteins and their effect on RSV replication.

We showed that M can interact with all three members of the Caveolin family (Fig. 2 and Table I). Together with cellular proteins and their effect on RSV replication. Significantly, our microfluidics screen identified a total of eight ZNF-containing proteins, mostly transcription factors, as interactors of RSV M (Table II). We focused on ZNF502, colocalization of Cav1 and Cav2 with M at the viral filaments (Figs. 3B and 4A), this implies that interaction with Caveolin proteins occurs in lipid rafts at the plasma membrane where M oligomerizes and virion budding takes place, and presumably it is the mechanism by which Cav1 can be incorporated into mature virions. Lipid raft integrity has been shown to be essential for proper RSV assembly and release of infectious particles (35–38), with Cav1, an integral component of lipid rafts, shown to be incorporated into mature virions (34, 37). Importantly, our results showed that depletion of Cav2 rather than Cav1 by siRNA has a significant effect on infectivity of both cell-associated and released virus (Fig. 5D). Based on our results showing that RSV filaments were still formed in Cav1 and Cav2 depleted RSV infected cells (supplemental Fig. S1), we speculate that lipid rafts and Caveolin proteins may be required for optimal infectivity of the released RSV viral particles, similar to what has been demonstrated for Newcastle disease virus (NDV) (39). One possibility is that lipid raft integrity, dependent on Cav1/2, is required for viral filament stability. Intriguingly, even though Cav1/2 form heterodimers and are both key components of lipid rafts, only depletion of Cav2 impacted significantly on RSV infectivity. An exciting possibility is that in RSV assembly Cav2 plays a role distinct to that of Cav1 out of the context of the Cav1/2 heterodimer, but this remains to be tested experimentally.

We also validated the actin-binding protein Cof1 as a host factor interacting with M both in vitro (Table I and Fig. 2A), and in transfected cells (Fig. 2B). We showed that Cof1 localizes into RSV IBs in transfected and in RSV-infected cells (Fig. 3B and Fig. 4A) (see (34)). Cof1 has also been shown previously to be incorporated into mature virions (34), and our work suggests that the incorporation of Cof1 into the virions may be through direct association of M and Cof1. The fact that knocking down Cof1 results in a 50% reduction of infectious virus indicates that Cof1-M interaction plays an essential role in RSV replication. Actin integrity has been shown to be important during RSV infection, presumably through involvement in filament formation (40), where cytoskeletal proteins have been suggested to be involved in the transport of the viral RNP to the sites of budding, which is dependent on M (16). Interestingly, independently of its role as cytoskeleton protein, actin has been shown to be able to act as a transcriptional modulator through binding to the RSV template (41). Our colocalization experiments show that in RSV-infected cells, Cof1 relocates into viral IBs resulting in reduction in Cof1 levels in the cytoplasm (Fig. 4B). We speculate that M may sequester Cof1 in IBs in order to impact on actin polymerization, resulting in an increase of F actin filaments, which could either facilitate trafficking of RSV proteins or viral transcription in IBs. Further work will be required to establish the precise role of RSV M-Cof1 interaction in RSV infection.

**Fig. 3.** A, Subcellular localization of interactors of M in the absence and presence of RSV proteins. HEp-2 cells were transfected to express the indicated Myc-tagged proteins, fixed, and permeabilized 24 h post transfection, and then immunostained using anti-Myc monoclonal followed by Alexa Fluor 568-coupled donkey anti-mouse secondary antibodies, and analyzed by confocal microscopy. B, Colocalization of host factors with RSV viral structures. HEp-2 cells were cotransfected with pcDNA3.1 plasmids encoding RSV P, N, F, and M proteins together with the indicated pCAG encoded Myc-tagged proteins. Cells were fixed, permeabilized 24 h post transfection and immunostained with anti-Myc polyclonal and anti-Myc monoclonal primary antibodies, followed by Alexa Fluor 488-coupled goat anti-mouse and Alexa Fluor 568-coupled donkey anti-rabbit secondary antibodies and analyzed by confocal microscopy. Scale bars represent 10 μm.
showing direct interaction with M both \textit{in vitro} and in transfected cells (Fig. 2). Importantly, partial knockdown of ZNF502 in 293T cells resulted in a 60% reduction of cell associated and 70% reduction of released infectious virus (Fig. 5). Strikingly, ectopic expression of ZNF502 appeared to increase M nuclear accumulation and decrease viral filament formation (Fig. 3). Further work is required to determine the precise contribution of ZNFs such as ZNF502 in RSV infection, and the extent to which this may impact on M transcriptional inhibition, or other activities of M.

Additional to the three host factors above, direct interactors with M included Rab11a, Rab11b, and Sec15, all of which are involved in vesicular trafficking, with Rab11a previously having been shown to be critical for RSV filament formation and infectivity (24). Although Rab11a and Rab11b interacted with M in the microfluidics system, the M-Rab11b interaction could not be verified by coimmunoprecipitation (Fig. 2B). Although this implies that the interactions may not occur in the intact cell, the lack of validation may be attributable to the fact that our microfluidics platform measures interactions at equilibrium. Consequently, the platform is sensitive to weak interactions and to strong interactions with fast off rates. Apart from Rab11a/11b, our hits also included two catalytic subunits (PI3KCB and PI3KCG) of phosphatidylinositol-4,5-bisphos-
phosphate 3-kinase (PI3K), previously shown to be involved in the innate immune response in RSV infection (42, 43). PI3K levels are known to increase in RSV infected cells, which may impact on RSV filament formation (44); whether interaction with M may serve to localize PI3K kinase activity at the site of viral assembly is unclear, but we are currently using specific PI3K inhibitors to test their effect on RSV replication. Further hits included Tom22 (validated in co-IP) and VDAC1, mitochondrial proteins involved in the innate immune response, whose levels and cellular localization change during RSV infection (45). Further work will be required to delineate the exact molecular mechanism of each of these interactions and their effect on RSV replication.

In summary, our novel screening approach has identified a number of new potential host targets for RSV M, shedding light on the cellular mechanisms utilized by RSV for its replication. The host interactors are from a variety of cellular pathways involved in transcription, translation, trafficking, innate immunity signaling and assembly, reflecting the multi-functionality and the ability of viral proteins, and RSV M in particular, to target multiple host proteins. Future work is focused on detailed analysis of the mechanism for the binding interactions, with important potential as targets for antiviral therapy.

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Fig. 5. Depletion of Cof1, Cav2, and ZNF502 negatively affects RSV replication. 4 × 10^5 HEp-2 cells were seeded per well in 6-well plates and transfected with control scrambled or specific siRNA (20 nm) 24 h later, followed by WT A2 RSV infection (MOI of three) 48 h post transfection. 293T cells were used for ZNF502. A and C, Cell lysates were prepared in RIPA buffer 24 h postinfection and subjected to Western analysis using the indicated primary antibodies together with anti-rabbit HRP-conjugated secondary antibody. B and D, Cell-associated or released virus was harvested 24 h postinfection. Virus titer was either determined on HEp-2 cells using an immune-plaque, or by plaque assay on Vero cells as described in the Methods section. Results represent the mean ± S.D. (n = 3), expressed as a % compared with negative control. (see Experimental procedures). * p < 0.05.
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