Influenza Targets

Description

The present invention relates to a pharmaceutical composition comprising an inhibitor of influenza virus replication. Yet another aspect is a screening method for identification of new targets for the prevention, alleviation or/and treatment of influenza.

In view of the threatening influenza pandemic, there is an acute need to develop and make available lastingly effective drugs. In Germany alone the annual occurrence of influenza causes between 5,000 and 20,000 deaths a year (source: Robert-Koch Institute). The recurring big influenza pandemics are especially feared. The first big pandemic, the so-called "Spanish Flu", cost about 40 million lives in the years 1918-1919 including a high percentage of healthy, middle-aged people. A similar pandemic could be caused by the H5N1 influenza virus, which at the moment replicates mainly in birds, if acquired mutations enable the virus to be transmitted from person to person. The probability of a human pandemic has recently grown more acute with the spreading of bird flu (H5N1) worldwide and the infection of domestic animals. It is only a question of time until a highly pathogenic human influenza-recombinant emerges. More recently, a novel influenza virus variant has emerged, i.e. the influenza A (H1N1) 'swine flu' strain, posing an unpredictable pandemic threat. The methods available at the moment for prophylaxis or therapy of an influenza infection, such as vaccination with viral surface proteins or the use of antiviral drugs (neuraminidase inhibitors or ion channel blockers), have various disadvantages. Already at this early stage resistance is appearing against one of our most effective preparations (Tamiflu), which may make it unsuitable to contain a pandemic. A central problem in the use of vaccines and drugs against influenza is the variability of the pathogen. Up to now the development of effective vaccines has required accurate prediction of the pathogen variant. Drugs directed against viral components can rapidly lose

their effectiveness because of mutations of the pathogen.

An area of research which has received little attention up to now is the identification of critical target structures in the host cell. Viruses are dependent on certain cellular proteins to be able to replicate within the host. The knowledge of such cellular factors that are essential for viral replication but dispensable (at least temporarily) for humans could lead to the development of novel drugs. Rough estimates predict about 500 genes in the human genome which are essential for viral multiplication. Of these, 10% at least are probably dispensable temporarily or even permanently for the human organism. Inhibition of these genes and their products, which in contrast to the viral targets are constant in their structure, would enable the development of a new generation of antiviral drugs in the shortest time. Inhibition of such gene products could overcome the development of viral escape mutants that are not longer sensitive to antiviral drugs.

It is the object of the present invention to provide screenings methods for compounds suitable for the prevention, alleviation or/and treatment of an influenza virus infection.

In the context of the present invention, it was surprisingly found that modulation (activation or inhibition) of particular genes leads to reduction of influenza virus replication. Tables 1, 2, 3 and 4 describe targets for the prevention, alleviation or/and treatment of an influenza virus infection.

Examples of genes which upon downregulation increase the influenza virus replication are described in Tables 1, 2, 3 and 4. Thus, by increasing expression or/and activity of these genes or/and gene products, the influenza virus replication can be reduced.

Examples of genes which upon downregulation decrease the influenza virus replication are also described in Tables 1, 2, 3 and 4. Thus, by decreasing expression or/and activity of these genes or/and gene products, the

influenza virus replication can be reduced.

Subject of the present invention is thus a screening method covering different aspects related to influenza virus infection, in particular influenza virus replication. A first aspect of the present invention is a screening method for identification of a compound suitable for the prevention, alleviation or/and treatment of an influenza virus infection, comprising the steps

(a) providing a cell or/and a non-human organism capable of being infected with an influenza virus and capable of expressing a gene, wherein the gene or/and gene product thereof is capable of modulating an influenza virus replication,

(b) contacting the cell or/and the organism of (a) with an influenza virus and with a compound known to be capable of modulating the expression or/and activity of the gene of (a) or/and the gene product thereof,

(c) determining the amount of influenza virus produced by the cell or/and the organism, and

(d) selecting a compound which reduces the amount of the influenza virus produced by the cell or/and the organism.

The gene of (a) may be selected from from Table 1, Table 2, Table 3 and Table 4. Preferably, the gene of (a) is selected from Table 4.

The method of the present invention may comprise a cellular screening assay. A cellular screening assay includes the determination of the activity or/and expression of a gene of (a) or/and the gene product thereof. The screening assay may be performed in vivo or/and in vitro.

Another aspect of the present invention is a screening method for identification of a compound suitable for prevention, alleviation or/and treatment of an influenza virus infection, comprising the steps

(i) providing a cell or/and a non-human organism capable of

expressing a gene, wherein the gene or/and gene product thereof is capable of modulating an influenza virus replication,

(ii) contacting a compound with the cell or/and the organism of (i),

(iii) determining the amount or/and the activity of gene product of the gene of (i), and

(iv) selecting a compound which modulates the amount or/and the activity of the gene product of (i).

The gene of (i) may be selected from Table 1, Table 2, Table 3 and Table 4. Preferably, the gene of (i) is selected from Table 4.

The compound of (iv) may reduce the amount of the influenza virus produced by the cell or/and the organism.

"Modulation" in the context of the present invention may be "activation" or "inhibition". Modulation of the expression of a gene may be downregulation or upregulation, in particular of transcription or/and translation. It can easily be determined by a skilled person if a gene is upregulated or downregulated. In the context of the present invention, upregulation (activation) of gene expression may be an upregulation by a factor of at least 2, preferably at least 4. Downregulation (inhibition) in the context of the present invention may be a reduction of gene expression by a factor of at least 2, preferably at least 4. Most preferred is essentially complete inhibition of gene expression, e.g. by RNA interference.

Modulation of the activity of the gene may be decrease or increase of the activity. In the context of the present invention, “activity” of the gene or/and gene product includes transcription, translation, posttranslational modification, modulation of the activity of the gene or/and gene product. The activity may be modulated by ligand binding, which ligand may be an activator or inhibitor. “Inhibition of the activity” may be a decrease of activity of a gene or gene product by a factor of at least 2, preferably at least 4. “Inhibition of the activity” includes essentially complete inhibition of activity.

"Activation of the activity" may be an increase of activity of a gene or gene product by a factor of at least 2, preferably at least 4.

The activity may also be modulated by an miRNA molecule, an shRNA molecule, an siRNA molecule, an antisense nucleic acid, a decoy nucleic acid or/and any other nucleic acid, as described herein. Modulation may also be performed by a small molecule, an antibody, an aptamer, or/and a spiegelmer (mirror image aptamer).

An activator of a gene identified by the method of the present invention may be suitable of reducing the amount of the influenza virus produced by a cell or/and an organism. In Tables 1, 2, 3 and 4, genes are described which upon inhibition (e.g. by siRNA) increase virus replication. Therefore, upon activation of these genes, virus replication may be reduced. In the tables, such genes are characterized by positive z-scores or/and by negative values of normalized percent inhibition (NPI).

An inhibitor of a gene identified by the method of the present invention is suitable of reducing the amount of the influenza virus produced by a cell or/and an organism. In Tables 1, 2, 3 and 4, genes are described which upon inhibition (e.g. by siRNA) decrease virus replication. In the tables, such genes are characterized by negative z-scores or/and by positive values of normalized percent inhibition (NPI).

Modulation may be performed by a single nucleic acid species or by a combination of nucleic acids comprising 2, 3 4, 5, 6 or even more different nucleic acid species, which may be selected from sequences of Tables 1, 2, 3, and 4 and fragments thereof. Preferred combinations are described in Table 4. It is also preferred that the combination modulates one gene, for instance selected from Tables 1, 2, 3, and 4. A combination of two nucleic acid species is preferred.

Modulation may be a knock-down performed by RNA interference. The

nucleic acid or the combination of nucleic acid species may be an siRNA, which may comprise a sequence selected from the sequences of Tables 1, 2, 3, and 4 and fragments thereof. It is preferred that the combination knocks down one gene, for instance selected from Tables 1, 2, 3, and 4. A combination of two siRNA species is preferred.

In the context of the present invention, a "target" includes a nucleotide sequence in a gene or/and a genome, a nucleic acid, or/and a polypeptide which is involved in regulation of influenza virus replication in a host cell. The target may be directly or indirectly involved in regulation of influenza virus replication. In particular, a target is suitable for reduction of influenza virus replication, either by activation of the target or by inhibition of the target.

Examples of targets are genes and partial sequence of genes, such as regulatory sequences. The term “target” also includes a gene product such as RNA, in particular mRNA, tRNA, rRNA, miRNA, piRNA. A target may also include a polypeptide or/and a protein encoded by the target gene. Preferred gene products of a target gene are selected from mRNA, miRNA, polypeptide(s) and protein(s) encoded by the target gene. The most preferred gene product is a polypeptide or protein encoded by the target gene. A target protein or a target polypeptide may be posttranslationally modified or not.

"Gene product" of a gene as used herein includes RNA (in particular mRNA, tRNA, rRNA, miRNA and piRNA), a polypeptide or/and a protein encoded by said gene.

The cell employed in step (a) may be any cell capable of being infected with an influenza virus. Cell lines suitable for the production of an influenza virus are known. Preferably the cell is a mammalian cell or an avian cell. Also preferred is a human cell. Also preferred is an epithelial cell, such as a lung epithelial cell. The cell may be a cell line. A suitable lung epithelial cell line is A594. Another suitable cell is the human embryonic kidney cell line 293T. In

one embodiment of the present invention, the method of the present invention employs a cell as described herein.

The non-human organism employed in step (a) may be any organism capable of being infected with an influenza virus.

The influenza virus employed in the method of the present invention may be an influenza A virus. The influenza A virus may be selected from influenza A viruses isolated so far from avian and mammalian organisms. In particular, the influenza A virus may be selected from H1N1, H1N2, H1N3, H1N4, H1N5, H1N6, H1N7, H1N9, H2N1, H2N2, H2N3, H2N4, H2N5, H2N7, H2N8, H2N9, H3N1, H3N2, H3N3, H3N4, H3N5, H3N6, H3N8, H4N1, H4N2, H4N3, H4N4, H4N5, H4N6, H4N8, H4N9, H5N1, H5N2, H5N3, H5N6, H5N7, H5N8, H5N9, H6N1, H6N2, H6N3, H6N4, H6N5, H6N6, H6N7, H6N8, H6N9, H7N1, H7N2, H7N3, H7N4, H7N5, H7N7, H7N8, H7N9, H9N1, H9N2, H9N3, H9N5, H9N6, H9N7, H9N8, H10N1, H10N3, H10N4, H10N6, H10N7, H10N8, H10N9, H11N2, H11N3, H11N6, H11N9, H12N1, H12N4, H12N5, H12N9, H13N2, H13N6, H13N8, H13N9, H14N5, H15N2, H15N8, H15N9 and H16N3. More particularly, the influenza A virus is selected from H1N1, H3N2, H7N7, H5N1. Even more particularly, the influenza A virus is strain Puerto Rico/8/34, the avian influenza virus isolate H5N1, the avian influenza strain A/FPV/Bratislava/79 (H7N7), strain A/WSN/33 (H1N1), strain A/Panama/99 (H3N2), or a swine flu strain H1N1, such as A/HH/04/2009.

The influenza virus may be an influenza B virus. In particular, the influenza B virus may be selected from representatives of the Victoria line and representatives of the Yamagata line.

The at least modulator of influenza virus replication employed in the method of the present invention of the present invention may be selected from the group consisting of nucleic acids, nucleic acid analogues such as ribozymes, peptides, polypeptides, antibodies, aptamers, spiegelmers, small molecules and decoy nucleic acids.

The modulator of influenza virus replication may be a compound having a molecular weight smaller than 1000 Dalton or smaller than 500 Dalton. In the context of the present invention, "small molecule" refers to a compound having a molecular weight smaller than 1000 Dalton or smaller than 500 Dalton.

The nucleic acid employed in the present invention may be an antisense nucleic acid or a DNA encoding the antisense nucleic acid.

The nucleic acid or/and nucleic acid fragment employed in the present invention may have a length of at least 15, preferably at least 17, more preferably at least 19, most preferably at least 21 nucleotides. The nucleic acid or/and the nucleic acid fragment may have a length of at the maximum 29, preferably at the maximum 27, more preferably at the maximum 25, especially more preferably at the maximum 23, most preferably at the maximum 22 nucleotides.

The nucleic acid employed in the present invention may be a microRNA (miRNA), a precursor, a fragment, or a derivative thereof. The miRNA may have the length of the nucleic acid as described herein. The miRNA may in particular have a length of about 22 nucleotides, more preferably 22 nucleotides.

A further aspect of the present invention is a pharmaceutical composition comprising at least one inhibitor of influenza virus replication optionally together with a pharmaceutically acceptable carrier, adjuvant, diluent or/and additive, for the prevention, alleviation or/and treatment of an influenza virus infection.

In the pharmaceutical composition of the present invention, the at least one inhibitor may be selected from the group consisting of nucleic acids, nucleic acid analogues such as ribozymes, peptides, polypeptides, and antibodies,

and compounds having a molecular weight below 1000 Dalton.

The influenza virus infection may be an influenza A virus infection or an influenza B virus infection, as described herein.

The at least one inhibitor in the pharmaceutical composition of the present invention may be capable of modulating gene expression or/and gene product activity. Modulation of the expression or/and gene product activity may be activation, as described herein. Modulation of the expression or/and gene product activity may be inhibition, as described herein.

The inhibitor may be a modulator as described herein.

The pharmaceutical composition may comprise a nucleic acid being RNA or DNA. Preferably, the nucleic acid in the pharmaceutical composition is selected from

(a) RNA, analogues and derivatives thereof,
(b) DNA, analogues and derivatives thereof, and
(c) combinations of (a) and (b).

In the pharmaceutical composition of the present invention, the at least one inhibitor may comprise

(a) a nucleic acid comprising a nucleotide sequence selected from sequences of Table 1, Table 2, Table 3 and Table 4,
(b) a fragment of the sequence of (a) having a length of at least 70%, at least 80 %, at least 90 %, at least 95 %, at least 98 %, or at least 99% of the sequence of (a),
(c) a nucleic acid comprising a sequence which is at least 70 %, at least 80 %, at least 90 %, at least 95 %, at least 98 %, or at least 99% identical to the sequence of (a) or/and (b), or/and
(d) a sequence complementary to the sequence of (a), (b) or/and (c).

In the pharmaceutical composition, the nucleic acid of (a) preferably

comprises a nucleotide sequence selected from the sequences of Table 4 and fragments thereof.

Suitable inhibitors of influenza virus replication in the pharmaceutical composition of the present invention are RNA molecules capable of RNA interference. The nucleic acid in the pharmaceutical composition of the present invention may comprise

(i) an RNA molecule capable of RNA interference, such as siRNA or/and shRNA,
(ii) a miRNA,
(iii) a precursor of the RNA molecule (i) or/and (ii),
(iv) a fragment of the RNA molecule (i), (ii) or/and (iii),
(v) a derivative of the RNA molecule of (i), (ii) (iii) or/and (iv), or/and
(vi) a DNA molecule encoding the RNA molecule of (i), (ii) (iii) or/and (iv).

A preferred nucleic acid is

(i) a miRNA,
(ii) a precursor of the RNA molecule (i), or/and
(iii) a DNA molecule encoding the RNA molecule (i) or/and the precursor (ii).

Yet another preferred nucleic acid is

(i) an RNA molecule capable of RNA interference, such as siRNA or/and shRNA,
(ii) a precursor of the RNA molecule (i), or/and
(iii) a DNA molecule encoding the RNA molecule (i) or/and the precursor (ii).

RNA molecules capable of RNA interference are described in WO 02/44321 the disclosure of which is included herein by reference. MicroRNAs are described in Bartel D (Cell 136:215-233, 2009), the disclosure of which is included herein by reference.

The RNA molecule of the present invention may be a double-stranded RNA molecule, preferably a double-stranded siRNA molecule with or without a single-stranded overhang alone at one end or at both ends. The siRNA molecule may comprise at least one nucleotide analogue or/and deoxyribonucleotide.

The RNA molecule of the present invention may be an shRNA molecule. The shRNA molecule may comprise at least one nucleotide analogue or/and deoxyribonucleotide.

In the pharmaceutical composition of the present invention the nucleic acid may be an antisense nucleic acid or a DNA encoding the antisense nucleic acid.

In the pharmaceutical composition of the present invention, the nucleic acid may have a length of at least 15, preferably at least 17, more preferably at least 19, most preferably at least 21 nucleotides. In the pharmaceutical composition of the present invention the nucleic acid may have a length of at the maximum 29, preferably at the maximum 27, more preferably at the maximum 25, especially more preferably at the maximum 23, most preferably at the maximum 21 nucleotides.

The pharmaceutical composition of the present invention may comprise an antibody. Preferably the antibody is directed against a polypeptide comprising

(a) an amino acid sequence encoded by a nucleic acid or/and gene selected from sequences of Table 1, Table 2, Table 3, and Table 4 and complementary sequences thereof,

(b) a fragment of the sequence of (a) having a length of at least 70%, at least 80 %, at least 90 %, at least 95 %, at least 98 %, or at least 99% of the sequence of (a), or/and

(c) an amino acid sequence comprising a sequence which is at least 70

%, at least 80 %, at least 90 %, at least 95 %, at least 98 %, or at least 99% identical to the sequence of (a) or/and (b).

Preferably, the pharmaceutical composition comprises a polypeptide of (a) comprising an amino acid sequence encoded by a nucleic acid or/and gene selected from Table 4.

The antibody of the present invention may be a monoclonal or polyclonal antibody, a chimeric antibody, a chimeric single chain antibody, a Fab fragment or a fragment produced by a Fab expression library.

Techniques of preparing antibodies of the present invention are known by a skilled person. Monoclonal antibodies may be prepared by the human B-cell hybridoma technique or by the EBV-hybridoma technique (Köhler et al., 1975, Nature 256:495-497, Kozbor et al., 1985, J. Immunol. Methods 81,31-42, Cote et al., PNAS, 80:2026-2030, Cole et al., 1984, Mol. Cell Biol. 62:109-120). Chimeric antibodies (mouse/human) may be prepared by carrying out the methods of Morrison et al. (1984, PNAS, 81:6851-6855), Neuberger et al. (1984, 312:604-608) and Takeda et al. (1985, Nature 314:452-454). Single chain antibodies may be prepared by techniques known by a person skilled in the art.

Recombinant immunoglobulin libraries (Orlandi et al, 1989, PNAS 86:3833-3837, Winter et al., 1991, Nature 349:293-299) may be screened to obtain an antibody of the present invention. A random combinatory immunoglobulin library (Burton, 1991, PNAS, 88:11120-11123) may be used to generate an antibody with a related specifity having a different idiotypic composition.

Another strategy for antibody production is the in vivo stimulation of the lymphocyte population.

Furthermore, antibody fragments (containing $F(ab')_2$ fragments) of the present invention can be prepared by protease digestion of an antibody, e.g.

by pepsin. Reducing the disulfide bonding of such $F(ab')_2$ fragments results in the Fab fragments. In another approach, the Fab fragment may be directly obtained from an Fab expression library (Huse et al., 1989, Science 254:1275-1281).

Polyclonal antibodies of the present invention may be prepared employing an amino acid sequence encoded by a nucleic acid or/and gene selected from Table 1, Table 2, Table 3 and Table 4 or immunogenic fragments thereof as antigen by standard immunization protocols of a host, e.g. a horse, a goat, a rabbit, a human, etc., which standard immunization protocols are known by a person skilled in the art.

The antibody may be an antibody specific for a gene product of a target gene, in particular an antibody specific for a polypeptide or protein encoded by a target gene.

Aptamers and spiegelmers share binding properties with antibodies. Aptamers and spiegelmers are designed for specifically binding a target molecule.

The nucleic acid or the present invention may be selected from (a) aptamers, (b) DNA molecules encoding an aptamer, and (c) spiegelmers.

The skilled person knows aptamers. In the present invention, an "aptamer" may be a nucleic acid that can bind to a target molecule. Aptamers can be identified in combinational nucleic acid libraries (e.g. comprising $>10^{15}$ different nucleic acid sequences) by binding to the immobilized target molecule and subsequent identification of the nucleic acid sequence. This selection procedure may be repeated one or more times in order to improve the specificity. The person skilled in the art knows suitable methods for producing an aptamer specifically binding a predetermined molecule. The aptamer may have a length of a nucleic acid as described herein. The aptamer may have a length of up to 300, up to 200, up to 100, or up to 50

nucleotides. The aptamer may have a length of at least 10, at least 15, or at least 20 nucleotides. The aptamer may be encoded by a DNA molecule. The aptamer may comprise at least one nucleotide analogue or/and at least one nucleotide derivatives, as described herein.

The skilled person knows spiegelmers. In the present invention, a "spiegelmer" may be a nucleic acid that can bind to a target molecule. The person skilled in the art knows suitable methods for production of a spiegelmer specifically binding a predetermined molecule. The spiegelmer comprises nucleotides capable of forming bindings which are nuclease resistant. Preferably the spiegelmer comprises L nucleotides. More preferably, the spiegelmer is an L-oligonucleotide. The spiegelmer may have a length of a nucleic acid as described herein. The spiegelmer may have a length of up to 300, up to 200, up to 100, or up to 50 nucleotides. The spiegelmer may have a length of at least 10, at least 15, or at least 20 nucleotides. The spiegelmer may comprise at least one nucleotide analogue or/and at least one nucleotide derivatives, as described herein.

The skilled person knows decoy nucleic acids. In the present invention, a "decoy" or "decoy nucleic acid" may be a nucleic acid capable of specifically binding a nucleic acid binding protein, such as a DNA binding protein. The decoy nucleic acid may be a DNA molecule, preferably a double stranded DNA molecule. The decoy nucleic acid comprises a sequence termed "recognition sequence" which can be recognized by a nucleic acid binding protein. The recognition sequence preferably has a length of at least 3, at least 5, or at least 10 nucleotides. The recognition sequence preferably has a length of up to 15, up to 20, or up to 25 nucleotides. Examples of nucleic acid binding proteins are transcription factors, which preferably bind double stranded DNA molecules. Transfection of a cell, an embryonated egg, or/and a non-human animal, as described herein, with a decoy nucleic acid may result in reduction of the activity of the nucleic acid binding protein to which the decoy nucleic acid binds. The decoy nucleic acid as described herein may have a length of nucleic acid molecules as described herein. The decoy

nucleic acid molecule may have a length of up to 300, up to 200, up to 100, up to 50, up to 40, or up to 30 nucleotides. The decoy nucleic may have a length of at least 3, at least 5, at least 10, at least 15, or at least 20 nucleotides. The decoy nucleic acid may be encoded by a DNA molecule. The decoy nucleic acid may comprise at least one nucleotide analogue or/and at least one nucleotide derivatives, as described herein.

The pharmaceutical composition as described herein is preferably for use in human or veterinary medicine.

The pharmaceutical composition of any of the preceding claims further comprises an agent suitable of transportation of the at least inhibitor of influenza virus infection into a cell, in particular into a lung epithelial cell.

The carrier in the pharmaceutical composition may comprise a delivery system. The person skilled in the art knows delivery systems suitable for the pharmaceutical composition of the present invention. The pharmaceutical composition may be delivered in the form of a naked nucleic acid, in combination with viral vectors, non viral vectors including liposomes, nanoparticles or/and polymers. The pharmaceutical composition or/and the nucleic acid may be delivered by electroporation.

Naked nucleic acids include RNA, modified RNA, DNA, modified DNA, RNA-DNA-hybrids, aptamer fusions, plasmid DNA, minicircles, transposons.

Viral vectors include poxviruses, adenoviruses, adeno-associated viruses, vesicular stomatitis viruses, alphaviruses, measles viruses, polioviruses, hepatitis B viruses, retroviruses, and lentiviruses.

Liposomes include stable nucleic acid-lipid particles (SNALP), cationic liposomes, cationic cardiolipin analogue-based liposomes, neutral liposomes, liposome-polycation-DNA, cationic immunoliposomes, immunoliposomes, liposomes containing lipophilic derivatives of cholesterol,

lauric acid and lithocholic acid. Examples of compounds suitable for liposome formation are 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE); 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS); cholesterol (CHOL); 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).

Nanoparticles include $CaCO_3$ nanoparticles, chitosan-coated nanoparticle, folated lipid nanoparticle, nanosized nucleic acid carriers.

Polymers include polyethylenimines (PEI), polyester amines (PEA), polyethyleneglycol(PEG)-oligoconjugates, PEG liposomes, polymeric nanospheres.

The pharmaceutical composition may be delivered in combination with atelocollagen, carbon nanotubes, cyclodextrin-containing polycations, fusion proteins (e.g. protamine-antibody conjugates).

An RNA or/and a DNA molecule as described herein may comprise at least one nucleotide analogue. As used herein, "nucleotide analogue" may refer to building blocks suitable for a modification in the backbone, at least one ribose, at least one base, the 3' end or/and the 5' end in the nucleic acid. Backbone modifications include phosphorothioate linkage (PTs); peptide nucleic acids (PNAs); morpholino nucleic acids; phosphoroamidate-linked DNAs (PAs), which contain backbone nitrogen. Ribose modifications include Locked nucleic acids (LNA) e.g. with methylene bridge joining the 2' oxygen of ribose with the 4' carbon; 2'-deoxy-2'-fluorouridine; 2'-fluoro (2'-F); 2'-O-alkyl-RNAs (2-O-RNAs), e.g. 2'-O-methyl (2'-O-Me), 2'-O-methoxyethyl (2'-O-MOE). A modified base may be 2'-fluoropyrimidine. 5' modifications include 5'-TAMRA-hexyl linker, 5'-Phosphate, 5'-Amino, 5'-Amino-C6 linker, 5'-Biotin, 5'-Fluorescein, 5'-Tetrachloro-fluorescein, 5'-Pyrene, 5'-Thiol, 5'-Amino, (12 Carbon) linker, 5'-Dabcyl, 5'-Cholesterol, 5'-DY547 (Cy3™ alternate). 3' end modifications include 3'-inverted deoxythymidine, 3'-puromycin, 3'-dideoxy-cytidine, 3'-cholesterol, 3'-amino modifier (6 atom), 3'-DY547 (Cy3™ alternate).

In particular, nucleotide analogues as described herein are suitable building blocks in siRNA, antisense RNA, and aptamers.

As used herein, "nucleic acid analogue" refers to nucleic acids comprising at least one nucleotide analogue as described herein. Further, a nucleic acid molecule as described herein may comprise at least one deoxyribonucleotide and at least one ribonucleotide.

An RNA molecule of the present invention may comprise at least one deoxyribonucleotide or/and at least one nucleotide analogue. A DNA molecule of the present invention may comprise at least one ribonucleotide or/and at least one nucleotide analogue.

Derivatives as described herein refers to chemically modified compounds. Derivatives of nucleic acid molecules as described herein refers to nucleic acid molecules which are chemically modified. A modification may be introduced into the nucleic acid molecule, or/and into at least one nucleic acid building block employed in the production of the nucleic acid.

In the present invention the term "fragment" refers to fragments of nucleic acids, polypeptides and proteins. "Fragment" also refers to partial sequences of nucleic acids, polypeptides and proteins.

Fragments of polypeptides or/and peptides as employed in the present invention, in particular fragments of an amino acid sequence encoded by a nucleic acid or/and gene selected from Table 1, Table 2, Table 3 and Table 4 may have a length of at least 5 amino acid residues, at least 10, or at least 20 amino acid residues. The length of said fragments may be 200 amino acid residues at the maximum, 100 amino acid residues at the maximum, 60 amino acid residues at the maximum, or 40 amino acid residues at the maximum.

A fragment of an amino acid sequence as described herein may have a length of at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99% of the sequence.

A fragment of a nucleotide sequence as described herein may have a length of at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99% of the sequence.

A fragment of a nucleic acid molecule given in Tables 1, 2, 3, and 4 may have a length of up to 1000, up to 2000, or up to 3000 nucleotides. A nucleic acid fragment may have a length of an siRNA molecule, an miRNA molecule, an aptamer, a spiegelmer, or/and a decoy as described herein. A nucleic acid fragment may also have a length of up to 300, up to 200, up to 100, or up to 50 nucleotides. A nucleic acid fragment may also have a length of at least 3, at least 5, at least 10, at least 15, or at least 20 nucleotides.

In the present invention, specific embodiments refer to any individual gene, nucleic acid sequence or/and gene product described in the present application. In a specific embodiment, an individual gene is selected from the genes described in Table 1, Table 2, Table 3, and Table 4. In another specific embodiment, an individual gene product is selected from the gene products produced by the genes described in Table 1, Table 2, Table 3, and Table 4. In yet another specific embodiment, an individual nucleic acid sequence is selected from the nucleic acid molecules described in Table 1, 2, 3 and 4. Further specific embodiment refer to any combination of genes, gene products and nucleic acid molecules described in the Tables 1, 2, 3, and 4.

In the present invention, a reference to Table 4 is a reference to a target, gene or/and nucleotide sequence selected from ACTN1, ATP6AP2, ATP6V1B2, BNIP3L, BRUNOL6, CUEDC2, CYC1, FNTB, GCLC, GNRH2, GRIN2C, GRP, HARBI1, HSPD1, ICAM2, KCNJ12, KPNB1, LAMC2,

LOC440733, MKL1, MRPS12, MYEF2, NDUFV3, NECAP2, ODZ4, PIK3R6, PPARA, RAB4A, SCAF1, SCARB1, SERPINA6, SERPINB2, SERPINE2, SEZ6L2, TBL3, TRERF1, TRIM60, and TUBB4.

In the present invention, a reference to Table 4 may also be a reference to a target gene or/and nucleotide sequence selected from ACTN1, BNIP3L, BRUNOL6, CUEDC2, CYC1, GCLC, GNRH2, GRIN2C, GRP, HARBI1, HSPD1, ICAM2, KCNJ12, LAMC2, LOC440733, MKL1, MRPS12, MYEF2, NDUFV3, NECAP2, ODZ4, PIK3R6, PPARA, RAB4A, SCAF1, SCARB1, SERPINA6, SERPINB2, SERPINE2, SEZ6L2, TBL3, TRERF1, TRIM60, and TUBB4.

Yet another aspect of the present invention is the use of an inhibitor of influenza virus replication capable of inhibiting or activating the expression of a gene selected from Table 1, Table 2, Table 3 and Table 4, or/and of inhibiting or activating a gene product thereof, for the manufacture of a medicament or/and vaccine for the prevention, alleviation or/and treatment of an influenza virus infection. Preferably, the gene is selected from Table 4. Preferably, those genes which upon inhibition by e.g. siRNA, as disclosed herein, result in decrease of virus production are activated, wherein those genes which upon inhibition by e.g. siRNA, as disclosed herein, result in increase of virus production are inhibited.

In the context of the present invention, "manufacture of a medicament or/and vaccine" includes the production of influenza virus, wherein the amount of influenza virus is increased by activating or inhibiting a gene selected from Tables 1, 2, 3 and 4, preferably Table 4. Preferably, those genes which upon inhibition by e.g. siRNA, as disclosed herein, result in decrease of virus production are activated, wherein those genes which upon inhibition by e.g. siRNA, as disclosed herein, result in increase of virus production are inhibited in the production of the medicament or/and vaccine.

Yet another aspect of the present invention is method for production of an

influenza virus, wherein the amount of influenza virus is increased by activating or inhibiting a gene selected from Tables 1, 2, 3 and 4, preferably Table 4. Preferably, those genes which upon inhibition by e.g. siRNA, as disclosed herein, result in decrease of virus production are activated, wherein those genes which upon inhibition by e.g. siRNA, as disclosed herein, result in increase of virus production are inhibited in the production of the influenza virus. In the method for production of an influenza virus, at least one gene selected from Tables 1, 2, 3 and 4, preferably Table 4, may be overexpressed if activation leads to an increased virus production.

Suitable methods for the production of influenza viruses, for instance in embryonated eggs or/and cell culture, are known in the state of the art.

Yet another aspect of the present invention is a method of prevention, alleviation or/and treatment of an influenza virus infection, comprising administering to a subject in need thereof a therapeutically effective amount of an inhibitor of influenza virus replication, as described herein. In the method of prevention, alleviation or/and treatment of an influenza virus infection, delivery systems and delivery methods as described herein may be used.

Yet another aspect of the present invention is the use of a nucleic acid comprising a gene sequence or/and a nucleotide sequence selected from Table 1, Table 2, Table 3, and Table 4 and fragments thereof in a method for screening for compounds or/and targets suitable for the prevention, alleviation or/and treatment of an influenza virus infection. Preferably a combination of at least two nucleic acids is used. It is also preferred that the nucleic acid or the combination is selected from Table 4. The combination may inhibit expression or/and activity of a gene, preferably selected from Tables 1, 2, 3 and 4, more preferably selected from Table 4.

The invention is further illustrated by the following figures, tables and examples.

Figure and Table legends:

Figure 1 | Genome-wide RNAi screen reveals host factors required for the influenza infectious cycle. a, Outline of the screening procedures. b, Negative Log(p-values) of enriched terms according to the GO of the cellular compartments. Numbers of identified factors per ontology, numbers of genes associated with the GO term, and the enrichment factors are indicated. c, Interaction amongst hits associated with RNA splicing, as assessed using the STRING interaction database. Green circles, primary hit; white circle, non-hit. Members of ribosomal and spliceosomal multi-protein complexes are enclosed in larger circles. Thick grey border indicates hits identified in Reactome analysis (see Fig. 10).

Figure 2 | Host cell factors affect replication of a broad range of influenza virus variants. a, Four siRNAs per gene were individually transfected in A549 cells followed by infection with influenza A/WSN/33 or A/Hamburg/04/2009 viruses (both at MOI 0.001) in four independent experiments. Infectious viral particles (IVP) were quantified at 48 h p.i. using the replication assay and analysed by calculating the normalised percent of inhibition. **b**, Venn diagram of hits validated in **a**. **c**, siRNAs (as indicated) were transfected in A549 cells and then infected (48 h later) with the avian H5N1 strain (A/Vietnam/1203/2004, MOI 0.1). Plaque forming units (PFU) were quantified at 20 h p.i. using the replication assay. Data show mean + standard error of the mean (S.E.M) of duplicate samples.

Figure 3 | Dissection of infection processes affected by host cell factors. a, Transfected A549 cells were infected with influenza A/WSN/33 virus (MOI 5) for 3 h (upper panel) and 5 h (lower panel). Samples were stained for nuclei (blue) and NP (green). **b,** At 48 h p.t., A549 cells were infected with influenza A/WSN/33 virus (MOI 1). At 2 h p.i., vRNA and viral mRNA were quantified by qRT-PCR. RNA levels were normalised to the non-targeting (Allstars) siRNA control. **c,** Transfected A549 cells were

infected with influenza A/WSN/33 virus (MOI 10) for 45 min. Samples were stained for influenza virus (green) and CD63 (red). Images are representative of three independent experiments in **a** and **c**.

Figure 4 | In-depth analysis of the impact of p27 and CLK1 on influenza A virus infection. a, Quantification of virus replication in primary NHBE cells after siRNA-mediated target knockdown using the replication assay. Cells were infected with influenza A/WSN/33 virus (MOI 0.1) 48 h p.t. **b**, A549 cells were pretreated with TG003 (50 μM) or DMSO for 24 h and subsequently infected with influenza A/WSN/33 virus (MOI 0.01). IVPs were quantified at 40 h p.i.. **c, d**, Ratio of spliced M2 to unspliced M1 after inhibition of CLK1 by TG003 at the RNA **(c)** or protein level **(d)**. A549 cells were pretreated for 2 h or 24 h with TG003 (50 μM) or DMSO, then infected with influenza A/WSN/33 virus (MOI 4) for 5 h. **e,** C57BL/6 wild-type or homozygous $p27^{-/-}$ mice (n=4) were intranasally infected with influenza A/Puerto Rico/8/34 virus (10× LD50) and at 48 h p.i. IVPs within the lungs were quantified. Student's *t-test* was used to determine p value, * $p = 0.041$. Data in **a**,**b** are mean + standard deviation (SD) of three independent experiments. Blots in **c**,**d** are representative of three independent experiments.

Figure 5 | Screening Controls. Depicted are representative images of the non-targeting (Allstars) and inhibitory (siNP) control samples, stained with an anti-NP antibody and analysed by automatic microscopy. **c,** Graph depicts light units exerted by the corresponding supernatants transferred onto 293T reporter cells.

Figure 6 | Relative frequency distribution of screening data. Shown are data gained from the luciferase reporter assay (left panel), percentage of infected cells (middle panel), and the number of infected cells (right panel) across all screening samples and controls. All data are normalised to the plate median.

Figure 7 | Histogram of Pearson's correlation coefficients calculated for all siRNA screening plates. Distribution of pairwise correlations for the normalised values of number of infected cells derived from all siRNA screening plates. Blue lines indicates all plates, red line indicates sets of replicates. Only values originating from sample wells were used for calculating the correlation coefficients. Control well values were excluded from this analysis.

Figure 8 | Workflow of RNAi screen data analysis. Data analysis procedures (left panel) and associated applied thresholds (right panel) are shown. Raw screening data from from all three read-out parameters was subjected to an analysis pipeline incorporating statistical thresholds at each stage. The data analysis workflow was done separately for all three read-outs and the final hit lists of each one were combined to provide a definitive primary hit list of 287 factors.

Figure 9 | Gene enrichment analysis. Negative Log10(p-values) of enriched terms according to the gene ontology of the molecular function, biological process, and cellular compartments. Values at bars indicate the number of identified factors per ontology, the number of genes associated with the term and the enrichment factor.

Figure 10 | Reactome analysis. The 287 'high-confidence' hits identified in the primary screen, were analyzed using the online web-resource Reactome http://www.reactome.org), a database of biological pathways in human cells. Each pathway is referred to as an event. The hits were uploaded as gene-identifiers using the 'sky-painter' tool, calculating a one-tailed Fisher's exact test for the probability of observing at least N genes from an event. 104 identifiers could be matched to 399 out of 4374 events. Several categories showed a significant overrepresentation such as Gene Expression (p=3.4e-07, 29/384), Transcription (p=1.1e-03, 14/198), Membrane Trafficking (2.5e-03, 6/50) or Influenza- (1.9e-04, 15/187) and HIV- infection (2.5e-01, 14/406). Single events are coloured according to the number of matching

identifiers from blue (1 matching identifier) to red (12 matching identifiers). Prominent categories showing overrepresentation of hits were coloured and important events were marked using an arrow. Several events were further analysed using the STRING database. (Figure 11)

Figure 11 | Interaction networks of the identified hits. Interactions amongst hits associated with vacuolar ATPases, nuclear transport, coat complex formation and translation, as assessed using the STRING interaction database (http://string.embl.de). Green circles, primary hit; dark green circles, primary hit also identified by a *Drosophila*-based influenza screen (13). All hits included in one large circle: members of one multi-protein complex, e.g. 40S ribosomal subunit. Hits with a thick grey border are also included in the Reactome pathway analysis (Figure 10).

Figure 12 | Host cell viability determination by WST-1 assay. A549 cells were transfected with indicated siRNAs followed by adding the WST-1 reagent 48h later to analyse eventually toxic effects due to siRNA transfections. Background subtracted mean values from two replicates are illustrated as a heat map. An siRNA targeting PLK1 was used as positive control. Missing siRNAs (less than four per gene) are indicated by grey boxes.

Figure 13 | Host cell factors affect replication of a H1N1 influenza virus variants. A subset of siRNAs was again transfected in A549 cells that were infected (48 h later) with the A/WSN/33 (**a**) or A/Hamburg/04/2009 (**b**) virus strains. IVPs in the virus containing supernatants were determined using the replication assay. Infection rate is expressed as a percentage of the non-targeting (Allstars) transfected control. Data show mean + S.E.M of duplicate samples. Cells transfected with the non-targeting control (Allstars) exhibited ca. 1.8×10^6 IVP/ml in the supernatant of A/WSN/33 infected and $6.6. \times 10^3$ IVP/ml upon A/Hamburg/04/2009 (A/H/04/09) virus infection. The inhibitory NP siRNA reduced the amount of infectious particles to $2.6. \times 10^4$ IVP/ml (A/WSN/33) and $4.5. \times 10^2$ IVP/ml (A/Hamburg/04/2009), respectively.

Figure 14 | Relative frequency distributions of NP expression. Relative frequency distributions of mean values of nuclear NP 3 h p.i.. Shown are values gained from two separate wells of the Allstars (Allstars W1 and W2) and NP (siNP W1 and W2) control as well as two independent siRNAs for the indicated target genes. Results are representative profiles of three independent experiments.

Figure 15 | Relative frequency distributions of nuclear export of NP. Relative frequency distributions of the ratios of cytosolic to nuclear NP 5 h p.i. Shown are values gained from two separate wells of the Allstars (Allstars W1 and W2) and NP (siNP W1 and W2) control as well as two independent siRNAs for the indicated target genes. Results are representative profiles of three independent experiments.

Figure 16 | P-values of differences between relative frequency distributions. Negative Log10(p-values) of the samples shown in Figures 14 and 15 as assessed by the one-sided Kolmogorov-Smirnov test.

Figure 17 | Quantification of co-localised virus particles. SON knockdown and control cells were infected with influenza A virus (A/WSN/33) for 45 min at 37 °C after incubation on ice. Cells were fixed and stained for influenza A virus and CD63 as described. Confocal pictures were taken and co-localisation was determined as described in Methods. Total numbers of viral particles and co-localised particles were quantified using ImageJ "Analyse particle" function. In total 34 cells were quantified for each condition. Diagram shows mean numbers of particles for two independent experiments. Control, black bars; Son knockdown, hatched bars; ** < 0.005; standard error of the mean (S.E.) is depicted.

Figure 18 | Influence of the chemical CLK1 inhibitor TG003 on cell viability. A549 cells were incubated with TG003 (50 µM, dissolved in DMSO), with DMSO or left untreated. Cell viability was evaluated at the

indicated time points using the WST-1 assay, according to the manufacturer's instructions. Shown are the mean values from three replicates. Error bars indicate the standard deviation.

Figure 19 | Influence of the chemical CLK1 inhibitor TG003 on VSV replication. A549 cells were pretreated with TG003 (50 µM, dissolved in DMSO) or DMSO (as a control), for 24 h and subsequently infected with VSV (MOI 0.01). After infection, the inhibitor or DMSO was added again at identical concentrations. The supernatants of treated or untreated cells were harvested at 24 h p.i. and infectious virus particles quantified by detecting plaques on MDCK cells.

Table 1 | Primary screening data and hit. Primary hit list and screening data. Shown are the Z-scores obtained from the CellHTS and the Genedata Screener® software analysis, and the RSA analysis for the classification of a particular siRNA as a hit. The mean cell number as an indicator for cell viability is shown. siRNAs leading to a mean cell number below 750 were defined as toxic. Gene expression fold changes upon infection, plus corresponding p-values and expression intensities as assessed by microarray analysis are also given.

Table 2 | Hit validation data. Shown are the siRNA IDs as provided by the supplier, the WST assay data, and the normalised percent inhibition data together with the number of validated siRNAs per gene for both tested viruses.

Tables 3 and 4 | Targets identified in the siRNA screen of the Example. Disclosed are oligonucleotide sequences employed in the siRNA screen of example 1. Up to four oligonucleotide sequences ("siRNA1 Target", "siRNA2 Target", "siRNA3 Target", "siRNA4 Target",) specific for a target gene were

employed.

Example

Human host cell factors crucial for influenza virus replication identified by genome-wide RNAi screen

Summary

Influenza A virus, being responsible for seasonal epidemics and reoccurring pandemics, represents a global threat to public health (1). High mutation rates facilitate the generation of viral escape mutants rendering vaccines and drugs directed against virus-encoded targets potentially ineffective (2). In contrast, targeting host cell determinants temporarily dispensable for the host but crucial for virus replication could prevent viral escape.

In this example, the discovery of 287 human host cell genes influencing influenza A virus replication in a genome-wide RNAi screen is described. Using an independent assay we confirmed 168 hits (59%) inhibiting either the endemic H1N1 (119 hits) or the current pandemic swine-origin (121 hits) influenza A virus strains, with an overlap of 60%. Importantly, a subset of these common hits was also essential for replication of a highly pathogenic avian H5N1 strain. In-depth analyses of several factors provided insights into their infection stage relevance. Notably, SON DNA binding protein (SON) (3) was found to be important for normal trafficking of influenza virions to late endosomes early in infection. We also show that a small molecule inhibitor of CDC-like kinase 1 (CLK1) (4) reduces influenza virus replication by more than two orders of magnitude, an effect connected with impaired splicing of the viral M2 mRNA. Furthermore, influenza virus-infected $p27^{-/-}$ (cyclin-dependent kinase inhibitor 1B; Cdkn1b) mice accumulated significantly lower viral titers in the lung providing in vivo evidence for the importance of this gene. Thus, our results highlight the potency of genome-wide RNAi screening for the dissection of virus-host interactions and the identification of drug targets for a broad range of

influenza viruses.

Introduction

During the course of infection, the influenza virus encounters numerous bottle necks, constituted by host cell functions essential or inhibitory for viral propagation (5). Comprehensive knowledge of such critical host cell determinants could provide valuable insight into the molecular mechanisms of viral replication and facilitate the development of a novel generation of drugs that target host cell factors and are thus less prone to select for resistant viral mutants. To identify host cell factors involved in the viral infection cycle in human cells, we conducted a genome-wide RNAi screen using a two-step approach (Fig. 1a): First, A549 human lung epithelial cells, transfected with siRNAs 48 h prior to infection with influenza A H1N1 virus (A/WSN/33), were stained with a virus-specific antibody at 24 h post infection (p.i.) to monitor cell infection rates. Second, virus supernatants from these transfected A549 cells were transferred onto 293T human embryonic kidney reporter cells, containing an inducible influenza virus-specific luciferase construct (FlaA) (6). Assay reliability was confirmed with an siRNA directed against influenza virus nucleoprotein (NP) mRNA (7). Knockdown of NP effectively blocked viral replication, as assessed by immunofluorescence staining and the luciferase reporter assay (Fig. 5). Statistical analyses further confirmed the robustness of our assay controls (NP and the non-targeting Allstars siRNA) and reproducibility of results (Figs. 6 and 7). Using this bipartite assay, we screened a genome-wide siRNA library consisting of ca. 62,000 siRNAs targeting ca. 17,000 annotated genes and ca. 6,000 predicted genes.

For identification of primary hits, three parameters were included: luciferase expression, the percentage of infected cells, as determined by immunofluorescence microscopy, and the total number of infected cells. After excluding non-expressed genes and toxic siRNAs, primary screening data from all three parameters were separately subjected to an analyses pipeline with statistical checkpoints at each step, finally leading to hit

selection based on Z-scores below -2 (Fig. 8 and Methods). Results from each of the three parameters were combined, and from a total of 22,843 human genes (annotated and predicted) 287 were designated primary hits (Table 1).

Among these high-confidence candidates we found several genes known to play a pivotal role in influenza virus replication, e.g. the nuclear export factors NXF1 (8) and XPO1 (9), as well as the vacuolar ATPase ATP6V0D1 (10,11). Gene ontology (GO) term enrichment analysis revealed our dataset was markedly enriched in gene categories associated with the proton-transporting two-sector ATPase complex, the spliceosome, the small ribosomal subunit, the eukaryotic translation initiation factor 3 (EIF3), the COPI coated vesicle transport and the nuclear pore complex (Fig. 1b and Fig. 9), which comprise functional categories already associated with viral replication. Further bioinformatic analysis using Reactome (12) corroborated the GO results (Fig. 10). In-depth analysis of selected enriched functional categories using the STRING database revealed numerous interactions between factors associated with the same GO term (Fig. 11). Interestingly, we found multiple factors connected with pre-mRNA splicing (Fig. 1c), which escaped detection in a previous RNAi screen using *Drosophila* cells (13). However, the small ribosomal subunit and EIF3 were enriched in the *Drosophila*-based influenza screen (13) but not in other viral RNAi screens (14, 15, 16, 17), indicating these factors could be influenza-specific (18).

Next, we independently ascertained the significance of all 287 primary hits for replication of the influenza A/WSN/33 (H1N1) and the current pandemic swine-origin influenza A/Hamburg/04/2009 (H1N1) viruses. The number of viruses released from siRNA transfected A549 cells was determined by titrating supernatants on Madin-Darby canine kidney (MDCK) cells. For each primary hit four different siRNAs were used individually to knockdown gene function. We found that 119 (A/WSN/33) and 121 (A/Hamburg/04/2009) of the 287 primary hits decreased virus number by more than fivefold in comparison to control samples, with a least two siRNAs (Fig 2a), without impairing cell viability (Fig. 12). In total, 168 primary hits

were validated, comprising an overall validation rate of ~59%. Remarkably, of the factors inhibiting viral replication, 72 were common to both influenza virus strains, indicative of their broad inhibitory potential (Fig. 2b and Table 2).

Validation was extended to the highly pathogenic avian-origin influenza A virus of the H5N1 subtype (A/Vietnam/1203/2004) using a subset of the common siRNAs. The knockdown efficiencies shown in the following Table (percentages of knockdown ± standard deviation as obtained in three independent experiments):

siRNA	Knockdown [%]	SD [%]
ATP6V0D1_1	95%	2%
ATP6V0D1_2	98%	1%
COPG_1	89%	8%
COPG_2	63%	25%
EIF4A3_1	96%	2%
EIF4A3_2	95%	3%
NUP205_1	85%	12%
NUP205_2	83%	7%
NUP98_1	86%	10%
NUP98_2	83%	6%
NXF1_1	53%	39%
NXF1_2	79%	17%
SON_1	77%	19%
SON_2	81%	16%

Strikingly, H5N1 virus replication decreased by more than two orders of magnitude using these siRNAs (Fig. 2c). Likewise, knockdown of identical targets inhibited replication of influenza A/WSN/33 (H1N1) virus and the pandemic A/Hamburg/04/2009 (H1N1) strain (Fig. 13). The observation that a subset of common factors blocked replication of both swine and avian-origin virus variants corroborates that these proteins constitute crucial sub-type independent host-cell checkpoints.

The life-stage relevance of 18 targets, representing a variety of

functional categories and affecting both H1N1 influenza viruses, was assessed by immunofluorescence staining for NP as a marker of viral ribonucleoprotein (vRNP) localisation (19). Typically, vRNP is confined to the nucleus early in infection, but enters the cytoplasm for packaging into progeny virions late in infection (19). Here, upon knockdown of all targets, NP gave a mainly nuclear signal at 3 h p.i. (Fig. 3a, upper panel), shifting towards cytoplasmic staining 2 h later (Fig. 3a, lower panel). In addition to the expected blockage of NP synthesis upon inhibition of NXF1 (8,20), knockdown of several identified hits such as COPG, SON, and ATP6V0C appeared to reduce NP expression levels (Fig. 3a, upper panel) and to delay export of NP from the nucleus (Fig. 3a, lower panel). Relative frequency distribution analysis of NP expression and cytosolic to nuclear NP ratios within single cells corroborated our microscopic observations (Figs. 14-16). In total, knock down of 11 genes significantly reduced NP expression and interfered with nuclear export of NP.

To analyse the impact of targets, shown to affect NP synthesis and localisation, on the synthesis of viral RNA, we infected siRNA-transfected cells with influenza virus and determined the levels of viral genomic RNA (vRNA) and viral mRNA at 2 h p.i. by qRT-PCR (7). Most of the analysed targets had no effect on virus cell entry, as indicated by robust vRNA detection (Fig. 3b). However, for many targets, including identified ATPases and SON, a protein known to repress Hepatitis B virion production (3), plus several factors involved in RNA biogenesis, e.g. NXF1, viral mRNA, synthesis was substantially reduced (Fig. 3b). This demonstrates virus propagation is affected at a stage between virus entry and mRNA synthesis. Knockdown of SON also reduced vRNA levels (Fig. 3b), indicating it functions in an infection step preceding viral mRNA synthesis. Accordingly, considerably less virus particles co-localised with CD63-labelled late endosomes upon SON knockdown (Fig. 3c; Fig. 17), suggesting this factor is important for trafficking of influenza virions early in the infection cycle. Intriguingly, knockdown of the nucleoporin 98kDa (NUP98) increased vRNA

level (Fig. 3b), most likely due to accelerated de novo vRNA synthesis, but at the same time dramatically decreased viral progeny (Fig. 2a; Fig. 13). Consistent with its reported antiviral (8) and proviral functions (13), these seemingly contradictory results suggest NUP98 exerts an inhibitory effect early in the life cycle but is mandatory for completion of viral replication. Taken together, these data reveal that the 11 targets (identified as reducing NP expression levels) interfere with early events in virus replication. In contrast, knockdown of the remaining 7 factors analysed in this set of experiments, such as CLK1 or p27 (CDKN1B), probably exert their function during later infection stages.

To more closely mimic in vivo conditions, we tested the effect of target knockdown on influenza virus replication in primary normal human bronchial epithelial cells (NHBE). Most notably, knockdown of CLK1 and ATP6V0D1 strongly reduced viral growth in these cells (Fig. 4a). We independently assessed the function of CLK1 by treating A549 cells with TG003, a chemical inhibitor of CLK1 (4). Influenza virus propagation was inhibited by more than 93% (Fig. 4b) without exerting detectable toxic effects (Fig. 18). CLK1 regulates alternative splicing in mammalian cells by phosphorylating the splicing factor SF2/ASF (21, 22), therefore we hypothesized that inhibition of CLK1 would affect splicing of viral RNAs. In accordance, TG003 reduced levels of spliced M2 viral RNA, whereas unspliced M1 and NS1/NS2 were unaffected (Fig. 4c, data not shown). Immunoblot analysis corroborated our qRT-PCR results, revealing drastically reduced M2 protein levels following treatment with TG003, whereas M1 protein levels remained relatively constant (Fig. 4d). Since the SF2/ASF complex is important for splicing and the shuttling of spliced viral mRNAs to the cytoplasm (23), it is conceivable that reduction of M2 protein expression was at least partially caused by nuclear retention of its mRNA transcript. Our finding that CLK1 is involved in processing viral M2 mRNA is consistent with the essential role of the SF2/ASF splicing factor in viral M2 ion channel protein production (24). Interestingly, replication of vesicular stomatitis virus (VSV), which, unlike

influenza, does not depend on splicing of its own viral RNA, was only slightly reduced in the presence of TG003 (Fig. 19).

During the primary screen and the hit validation, knockdown of the cell cycle regulator p27 led to a strong inhibition of influenza virus replication. To confirm the impact of p27 on viral replication under in vivo conditions, $p27^{-/-}$ mice were intranasally infected with influenza A/Puerto Rico/8/34 (H1N1) virus. At 2 d p.i., virus load within the lungs of $p27^{-/-}$ mice was significantly reduced (Fig 4e). The observation that a lack of p27 reduces influenza virus replication in vivo but does not affect mouse viability, indicates certain cellular proteins involved in influenza virus replication are dispensable for the host organism.

Thus, this first genome-wide RNAi screen in human cells for factors affecting influenza virus replication has provided new and comprehensive information on host cell determinants of replication, and uncovered potential targets for novel antiviral strategies. We provide in vitro and in vivo evidence for the role of CLK1 and the tumor suppressor p27, using a small molecule inhibitor and a homozygous knockout model, respectively. The majority of the hits analysed in-depth appear to function during early infection processes such as viral protein synthesis and nuclear export of viral RNA. Importantly, most of the validated hits are essential for a broad spectrum of influenza viruses, including the pandemic swine-origin H1N1 influenza virus and even a highly pathogenic avian H5N1 strain. This holds promise for the therapeutic potential of these targets against novel emerging influenza viruses with minimised likelihood of developing drug resistant variants. In conclusion, transient interference with distinct host cell functions during infection is likely to extend our current armament, consisting of vaccines and virus-targeted drugs, in the battle against the recurring threat of seasonal and pandemic influenza virus infections.

In the present screen, a range of cellular functions were identified

which were associated with influenza virus propagation. Amongst the significantly enriched functional categories are the small ribosomal subunit and the translation initiation factor EIF3, splicing associated genes, vesicular (coat complex formation) and nuclear transport, as well as vacuolar ATPases. In contrast, in other viral RNAi-based screens, including an influenza virus screen in *Drosophila* cells, mostly single metabolic functions were enriched in the hit lists (13, 14, 15, 17). This general observation strengthens the impact of performing RNAi screens in homologous host cell models.

The small ribosomal subunits and the translation initiation factor EIF3 components comprised a major cellular function enriched in a recent *Drosophila*-based influenza virus screen (13) but not in other viral RNAi screens (14-17). Yet, only single components of the large ribosomal subunit were included in either the previous or our current influenza screens. Toxicity, as determined by our WST assay (c.f. Fig. 11) and viable cell counts (c.f. Table 1), did not have a major impact on the knockdown cells. Kittler *et al.* found knockdown of many of these genes impacted the cell cycle (arrest) and division, but toxicity was a confounding factor in a minimal number of cases. A *Drosophila* C virus screen identified small as well as large ribosomal subunit genes as enriched and this finding was linked to IRES-mediated translation initiation (18). Translation of influenza mRNAs is initiated in a Cap-dependent and 5'-UTR-mediated manner (Garfinkel et al., Kash et al.) and the initiation factor EIF4E within the EIF4F complex is substituted by the viral polymerase (Burgui et al.). On the other hand, EIF4GI, another member of the EIF4F complex, is targeted by NS1, enhancing preferential translation of late viral mRNAs in particular (Aragon et al.). The eukaryotic 5'-UTR targeting factor GRSF-1, which also enhances translation of influenza mRNAs, was not identified as a hit in our screen (Kash et al.). Besides these known factors, other host cell proteins may play an important role in initiating translation of viral mRNAs (Burgui et al.). The identification of defined translation machinery components in two influenza virus RNAi screens but not other viral screens, suggests these factors could

be influenza virus A specific. We speculate that the small ribosomal subunit as well as EIF3 complete the pre-initiation complex that initiates virus-specific, selective translation and probably contribute to the inhibition of host cell gene translation.

Since pre-mRNA splicing is a major cellular function known to be important for gene expression in a variety of viral systems (reviewed by e.g. Engelhardt et al.), we expected this function to be identified in our screen. Yet, the *Drosophila* influenza virus screen does not show the same enrichment of splicing factors. This could be due to the experimental limitations of the *Drosophila* host cell system for influenza A virus infection and replication, therefore other processes might be important in this experimental system. This might also apply to other cellular processes we identified. König *et al.* (17) found many splicing factors in their HIV early stage replication screen. HIV mRNA splicing is a very complex and highly regulated process that ensures co-ordinated expression of the different viral proteins as well as production of unspliced genomic RNA (reviewed by e.g. Stoltzfus et al). Brass *et al.* (16) detected several splicing associated factors amongst the HIV-dependency factors (HDFs) included in their screen. Because the individual flavivirus proteins are derived by co- and post-translational cleavage from a polyprotein translated from an unspliced RNA (e.g. Beasley et al), splicing factors are virtually missing in the Dengue and West Nile virus hit lists (14, 15). Furthermore, vacuolar ATPases are enriched in our screen as well as the West Nile virus screen (14). Both viruses rely upon acidification of the phagosome to enter the cytoplasm (reviewed by e.g. Bouvier et al.). Single vacuolar ATPase subunits were also included in the *Drosophila*-based influenza virus screen (13).

The nuclear transport factors are required for export of the viral RNA into the cytoplasm to be translated and incorporated into new virus particles. The cyclin-dependent kinase inhibitor 1B (p27, also CDKN1B) involved in cell cycle regulation and other cellular processes (Borriello et al.), is associated with this network. Phosphorylation at certain amino acid residues

regulates cellular localisation and thereby function and stability (Ishida et al., Connor et al.). p27 is exported into the cytoplasm by XPO1/RanGTP. p27 is a tumour suppressor in the nucleus, whereas is acts as an oncogene with pro-metastatic capability in the cytoplasm. This functional versatility (reviewed by e.g. Borriello et al.) makes is difficult to trace the step involved in influenza virus replication. To connect it to the cell-cycle arrest associated with knockdown of many ribosomal subunits (see above) is one promising route for future investigation.

Two different COP vesicles operate in the early secretory pathway (reviewed by Lee et al.). COPII vesicles mediate exit from the endoplasmatic reticulum (ER) and transport to the ER-Golgi-intermediate compartment (ERGIC), whereas COPI vesicles are involved in retrograde transport from the Golgi apparatus to the ER or between different Golgi cisternae and in anterograde transport. The influenza glycoproteins HA and NA are synthesised at the ER, transported to the Golgi apparatus and then trafficked to the plasma membrane (Bouvier et al.). Therefore, factors involved in early secretory pathway of the host cell are likely candidates affecting influenza propagation. In the present work, we have shown that knockdown of COPA, COPB1, COPB2, COPD, COPE or COPG reduced number of infectious viruses, demonstrating that these factors are important for the production of infectious influenza A viruses. Specifically, knockdown of COPG dramatically reduced levels of NP at 3 h p.i. (Fig. 3a and Figs.14-16), hinting at a role in early infection processes. These observations are in agreement with a previous RNAi screen that identified COPG as essential for influenza A virus replication in insect cells (13). The underlying mechanism of COPI function in influenza A virus replication is still unknown. Knockdown of COPI constituents could directly affect transport of viral glycoproteins to the plasma membrane. This hypothesis is supported by recent work demonstrating that anterograde transport of proteins in COPB1 knockdown cells is blocked or at least reduced (Styers et al., Rennolds et al.). Interestingly, only components of the COPI machinery have been identified in the present screen. The involvement of COPII vesicles in normal

trafficking of membrane proteins from the ER to the plasma membrane could hint to other functions of COPI during influenza A virus infection including maintenance of the steady-state distribution of Golgi proteins or ER quality control mechanisms (Tu et al., Zerangue et al.). In this scenario, knockdown of COPI proteins would result in incorrect folding or incorrect glycosylation of viral proteins including HA and NA, which either reduce transport of these proteins to the plasma membrane or interfere with the normal function of these proteins. Detailed analysis is on the way to clarify the role of COPI proteins during influenza virus infection.

In summary, these findings highlight the significance of our screen. Many molecular functions of the host cell known, or expected, to play important roles in influenza virus replication were recovered in our analysis. As an extension to previous RNAi-based viral screens (13, 14, 16, 17), which report single functional categories, our findings reveal a range of different molecular networks.

Methods

Summary: siRNA screening

All siRNAs (4 µl/well, 200 nM) were arrayed in 384-well plates. To each well, 8 µl of RPMI medium (Invitrogen, Karlsruhe, Germany) containing 0.35 µl HiperFect (Qiagen) was added and plates were shaken for 1 min. After 10 min incubation at room temperature (RT), a cell suspension (28 µl) of 500 cells was added to give a final siRNA concentration of 20 nM. Cells were incubated at 37 °C and 5% CO_2 for 48 h before infection at MOI 0.12. At 24 hours post infection (p.i.), supernatants were transferred onto freshly seeded 293T reporter cells, incubated for 16 h at 37 °C and 5% CO_2 and then luciferase activities were measured. The A549 cells were fixed, stained for nuclei and NP, and analysed using the Acumen ^{e}X3 Cytometer (TTP Labtech, Royston, U.K.). All multiwell pipetting steps were performed using a Biomek® FX^{P} Laboratory Automation Workstation (Beckman Coulter, Krefeld, Germany). An siRNA library (Qiagen Hu_Genome 1.0 and Human

Druggable Genome siRNA Set V2.0;Qiagen, Hilden, Germany) containing four siRNAs per gene for the druggable genome (25) and two siRNAs per gene for non-druggable and predicted genes was screened three times independently. The following siRNAs with the indicated target sequence were included in all screening plates as controls: siNP (5'-AAGGAUCUUAUUUCUUCGGAG-3'), siPLK1 (5'-CACCATATGAATTGTACAGAA-3') and Allstars (Qiagen, Hilden, Germany).

Cells and viruses

The A549 human lung epithelial cell line (CCL-185, ATCC-LGC, Wesel, Germany) was grown in DMEM media (Invitrogen, Karlsruhe, Germany) supplemented with 4 mM L-glutamine, 4 mM sodium pyruvate, 100 U/ml penicillin/streptomycin and 10% fetal calf serum (FCS, Biochrome, Berlin, Germany) (DMEM complete medium), at 37 °C and 5% CO_2. The human embryonic kidney cell line 293T (CRL-11268, ATCC-LGC) and the Madin Darby Canine Kidney cells (MDCK, CCL-34, ATCC-LGC) were grown in DMEM supplemented with 4 mM L-glutamine, 100 U/ml penicillin/streptomycin and 10% FCS. Primary normal human bronchial epithelial cells (NHBE, CC-2541, Lonza, Cologne, Germany) were grown in Clonetics® BEGM® BulletKit® (CC-3170, Lonza) supplemented with the following growth supplements: BPE, Hydrocortisone, hEGF, Epinephrine, Transferrin, Insulin, Retinoic Acid, Triiodothyronine, GA-1000. Supplements added at 0.5 ml/500 ml medium, except BPE (2 ml/500 ml). Cells were regularly checked for mycoplasma contamination by PCR. The influenza virus strains A/WSN/33 (H1N1) and A/Puerto Rico/8/34 (H1N1) were grown in the allantoic cavities of 11-day-old embryonated chicken eggs. Production of recombinant highly pathogenic influenza A/Vietnam/1203/2004 virus (H5N1) by reverse genetics was done essentially as described previously (26). The pandemic H1N1 A/Hamburg/04/2009 strain was provided by S. Becker (Philipps University, Marburg, Germany) and was propagated in MDCK cells in DMEM supplemented with 1 μg trypsin/ml in the absence of FCS. Virus stocks were titrated by standard plaque assay on MDCK cells using an agar overlay medium (27).

siRNA screening

All siRNAs (4 µl/well; 200 nM) were arrayed in 384-well plates. To each well, 8 µl of RPMI medium (Invitrogen, Karlsruhe, Germany) containing 0.35 µl HiperFect (Qiagen) was added and plates were shaken for 1 min. After 10 min incubation at room temperature (RT), a cell suspension (28 µl) containing 500 cells was added to give a final siRNA concentration of 20 nM. Cells were incubated at 37 °C and 5% CO_2 for 48 h before infection at an MOI of 0.12 (see below). At 24 hours post infection (p.i.), supernatants were transferred onto freshly seeded 293T reporter cells, incubated for 16 h at 37 °C and 5% CO_2 and then luciferase activities were measured (see below). The A549 cells were fixed, stained for nuclei and NP, and analysed using the Acumen ^{e}X3 Cytometer (TTP Labtech, Royston, UK). The number of automatically counted nuclei was further used to estimate cytotoxic effects of specific siRNAs. The siRNA was classified as being toxic, if 750 or fewer nuclei were determined within one well of a 384-well plate. All multiwell pipetting steps were performed using a Biomek® FX^{P} Laboratory Automation Workstation (Beckman Coulter, Krefeld, Germany). An siRNA library (Qiagen Hu_Genome 1.0 and Human Druggable Genome siRNA Set V2.0; Qiagen, Hilden, Germany) containing four siRNAs per gene for the druggable genome (25) and two siRNAs per gene for non-druggable and predicted genes, was screened three times independently. The following siRNAs with the indicated target sequence were included in all screening plates as controls: siNP (5'-AAGGAUCUUAUUUCUUCGGAG-3'), siPLK1 (5'-CACCATATGAATTGTACAGAA-3') and Allstars (Qiagen, Hilden, Germany).

Luciferase reporter assay

To quantify infectious viruses in the supernatants of siRNA transfected A549 cells during the primary RNAi screen, we used a luciferase-based reporter system. 293T cells were transfected in batches with a FluA luc plasmid (6), one day later seeded into 384-well plates at concentrations of 1×10^{4}/well, and subsequently infected with 12.5 µl of virus containing supernatant. At 16 h p.i., Bright-Glo™ firefly luciferase substrate (Promega, Madison, WI, USA)

was added and luciferase activities in cell lysates were measured using the Envision multilabel plate reader (PerkinElmer, Rodgau, Germany). Transfection of 239T cells with the influenza virus-specific luciferase construct (FlaA) induces expression of firefly luciferase transcripts flanked by the untranslated region of the influenza A/WSN/33 virus nucleoprotein (NP) segment. Luciferase expression is therefore only detectable in the presence of the viral polymerase, thus allowing quantification of infectious viruses.

siRNA transfection for validation experiments in 96- and 12-well plates

All siRNAs were purchased from Qiagen. For siRNA transfection of A549 cells in 96-well plates, 20 µl of a 100 nM siRNA dilution in DMEM w/o supplements was mixed with 1 µl HiperFect + 9 µl DMEM medium and incubated for 10 min at RT. Complex formation was stopped by addition of 25 µl DMEM complete medium. Next, 3000 A549 cells in 50 µl DMEM complete medium were seeded into each well and incubated at 37 °C and 5% CO_2 for the indicated time periods. For siRNA transfection of NHBE cells in 96-well plates, BEGM medium (with/without supplements) was used and 15,000 cells/well were seeded. For Western blot experiments, siRNA transfection was carried out in 12-well plates. For each well, 1 µl of a 20 µM siRNA solution was diluted in 99 µl RPMI (Invitrogen) supplemented with 25 mM HEPES (Invitrogen). The mix was incubated at RT for 5 min before addition of 5 µl HiperFect (Qiagen) and further 15 min incubation at RT. Each complex was added to 50,000 A549 cells in 900 µl DMEM complete medium, mixed carefully, and then transferred to 12-well plates. After 6 h incubation at 37 °C and 5% CO_2, the medium was exchanged for fresh DMEM complete medium and the cells were incubated for an additional 48 h using the same growth conditions.

Indirect immunofluorescence labeling

Cells were fixed with 3.7% formaldehyde and permeabilised with 0.3% Triton X-100, 10% FCS in PBS. Samples were sequentially incubated with a primary antibody against the viral nucleoprotein (NP, clone AA5H, AbD Serotec, UK) diluted 1:10000 in PBS with 10% FCS, 0.1% Tween 20 for 1 h

at RT, followed by an incubation with the secondary Cy3 conjugated antibody directed against mouse IgG (1:100 in PBS with 10% FCS, 0.1% Tween 20 and 0.1% Hoechst dye used to stain cellular DNA). Numbers of infected versus non-infected cells were determined using automated microscopy (Olympus, Soft Imaging Solutions, München, Germany) or, for the primary siRNA screen, the Acumen eX3 microplate cytometer (TTP LabTech, Melbourn, UK).

Automated microscopy and image analysis

The numbers of influenza infected and host cells were determined using an automated microscope (Olympus Soft Imaging Solutions). Images were taken with DAPI and Cy3 filter sets (AHF-Analysetechnik, Tübingen, Germany). Scan^R Analysis Software (Olympus Soft Imaging Solutions) was used to automatically identify and quantify influenza nuclear protein (NP) and cell nuclei. For determination of NP localisation, mean and total intensities of NP were analysed. NP located within the same area as the Hoechst staining was defined as nuclear NP. NP located within a 5-pixel-wide ring around the nuclei was defined as cytosolic NP. The distance between the inner edge of the ring and the nuclei was set at 1 pixel. For each experiments identical camera setting were used.

Host cell viability determination by WST-1 assay

Determination of host cell viability upon siRNA tranfection was performed using cell proliferation assay WST-1 (Roche, Mannheim, Germany). WST-1 reagent was added to the cells 48 h after siRNA transfection and incubated at 37 °C for 1.5 h. Absorbance was measured at 460 nm and at the reference wavelength 590 nm. Non-targeting siRNA Allstars and siPLK1 were used as a positive and negative control, respectively.

Virus infection

Cells were washed with PBS and then infected with influenza at the indicated MOIs in infection buffer (PBS supplemented with 0.2% bovine serum albumin) for 60 min at RT. Cells were washed again (in infection buffer) and

incubated for the indicated time periods at 37 °C in DMEM supplemented with 0.2% bovine serum albumin, 4 mM L-glutamine and antibiotics (A549) or BEGM with supplements (NHBE), unless otherwise stated. All infection experiments with A/WSN/33, A/Puerto Rico/8/34 and with A/Hamburg/04/2009 H1N1 viruses were performed under biosafety level (BSL) 2 conditions, whereas BSL 3 conditions were used for experiments with A/Vietnam/1203/2004 (HN51).

Replication assay

To quantify infectious virus particles in infected cell culture supernatants, 5,000 or 12,000 MDCK cells were seeded in 384- or 96-well plates, respectively. One day later the cells were washed twice, infected with a dilution series of cell culture supernatants and incubated at RT for 1 h. Infection buffer (as above) was added (40 µl or 100 µl/well) and plates were incubated at 37 °C, 5% CO_2 for 6 h, followed by fixation with 3.7% formaldehyde, antibody staining and automatic image processing, as described in 'Indirect immunofluorescence labeling'.

Gene enrichment and network analysis

For gene enrichment analysis, we modified the R-script available from the Gaggle web site (http://gaggle.systemsbiology.net/svn/gaggle/PIPE2.0/trunk/PIPEletResourceDir/ GOTableEnrichment/GOEnrichmentScript.R). This script applies the R-package GOstats developed by Falcon, S. and Gentleman, R. (28) and is available at the Bioconductor web site (http://www.bioconductor.org). Briefly, we defined a gene universe consisting of 22843 genes contained and annotated in the genome-wide library and processed the hit list against this universe with respect to molecular function (MF), cellular component (CC) and biological process (BP). Each Gene Ontology term is associated with X number of genes, providing a relative frequency A. In the hit list, the same term is connected to Y genes giving a relative frequency B. B divided by A is the enrichment factor.

The 287 'high-confidence' hits were also uploaded as gene-identifiers using the Sky-Painter tool of the Reactome website (www.reactome.org). Significant events calculated by the application's Fisher's exact test were identified and coloured accordingly. Network analysis was carried out using the STRING database (http://string.embl.de/).

Confocal microscopy

Fusion between influenza viruses and cellular endosomes was detected using confocal microscopy. A549 cells were plated onto cover slips in 12-well plates at a density of 5×10^4 cells/well and directly transfected in suspension with indicated siRNAs, followed by infection with influenza A/WSN/33 virus (MOI 10) 48 h post transfection. During the infection process, cells were kept on ice for 45 min, washed twice with cold infection buffer (see above) and subsequently incubated with pre-warmed infection media (DMEM supplemented with 0.2% bovine serum albumin, 4 mM L-glutamine and antibiotics). After 15, 45 and 90 min cells were fixed with 4% paraformaldehyde and permeabilised for 20 min with 0.2% BSA in PBS and 0.2% Triton X-100. Cells were then incubated for 1 h with antibodies targeting CD63 (Millipore) at a dilution of 1:70 and a polyclonal serum against influenza (1:1000), followed by incubation with a fluorescently labelled secondary antibody (dilution 1:100). Samples were mounted in MOWIOL. Images were taken with a Leica TCS-SP confocal microscope and processed using Adobe Photoshop 11.0.

Immunoblotting

For immunoblotting, cells were washed with PBS and lysed in 1x SDS sample buffer containing 75mM Tris HCl (pH 6.8), 25% glycerol, 0.6% SDS, 7.5% β-mercaptoethanol and 0.001% bromphenol blue. Protein lysates (20 µl) were loaded and separated on a 10% SDS-polyacrylamide gel. Separated proteins were transferred to a PVDF membrane and detected using mouse monoclonal antibodies against viral matrix protein (M1, AbD Serotec, UK), viral ion channel protein (M2,Santa Cruz) or β-actin (Sigma, Germany) at 1:100, 1:1000 or 1:2500 dilution, respectively, followed by

incubation with a secondary sheep anti-mouse IgG Horseradish peroxidase (1:10000). Staining was performed with ECL Western Blotting Detection Reagent (Amersham, Piscataway, NJ, USA). β-actin was used as a loading control. Band intensities were determined using the Aida image analyzer (V.4.03) (2D/ Densitometry) and normalised to β-actin.

Quantitative RT-PCR

For the detection of viral RNA (vRNA) or viral mRNA, quantitative RT-PCR (qRT-PCR) was performed as previously described (7). Briefly, A549 cells infected with influenza A/WSN/33 virus (MOI 1) were lysed with RLT lysis buffer (Qiagen, Hilden, Germany). For reverse transcription of viral mRNA, an oligo(dT)18 primer was used: the negative stranded vRNA of the gene segment PA was converted to cDNA using a PA-specific oligonucleotide (5'-GCTTCTTATCGTTCAGGCTCTTAGG-3'). Resulting cDNAs were quantified by qRT-PCR with oligonucleotides specific for PA (5'-GCTTCTTATCGTTCAGGCTCTTAGG-3' and 5'-CCGAGAAGCATTAAGCAAAACCCAG-3'). GAPDH was amplified using the oligonucleotides, GAPDH for: 5'- GGTATCGTGGAAGGACTCATGAC-3'; GAPDH_rev: 5'- ATGCCAGTGAGCTTCCCGTTCAG-3'. Levels of GAPDH were used for normalisation. All experiments were done in triplicate.

To quantify the levels of spliced and unspliced mRNAs, infection of A549 cells with influenza A/WSN/33 virus was performed at an MOI of 4 for 5 h. RNA was then isolated using the RNeasy Mini Kit (Qiagen) and treated with DNase (Ambion) according to manufacturer's instructions. Reverse transcription of viral mRNA was performed using oligo(dT) primer and the synthesised cDNA was subjected to real-time PCR using primers specific for M1 (5'-GACCAATCCTGTCACCTC-3' and 5'-GATCTCCGTTCCCATTAAGAG-3') and M2 (5'-GAGGTCGAAACGCCTAT-3' and 5'-CTCCAGCTCTATGTTGACAAA-3'), as described previously (29). Levels of M1 and M2 mRNA were normalised to GAPDH.

Validation of RNAi by quantitative PCR

siRNA validation was performed as previously described (30). Briefly, one

day before transfection 3,000 cells per well were seeded onto a 96-well plate. Transfection was performed with a final siRNA concentration of 56 nM with 0.25 µl HiPerFect(Qiagen). Knockdown measurements were performed independently three times. After 48 h, RNA was isolated using the RNeasy 96 BioRobot 8000 system (Qiagen). The relative amount of target mRNA was determined by quantitative PCR using the Quantitect SYBR Green RT–PCR kit following the manufacturer's instructions (Qiagen) and the following primers:

GAPDH forward 5'-GGTATCGTGGAAGGACTCATGAC-3',
GAPDH reverse 5'-ATGCCAGTGAGCTTCCCGTTCAG-3',
ATP6V0D1 forward 5'-TGTCGCAACATCGTGTGGAT-3',
ATP6V0D1 reverse 5'-GAGTGCAATTGAGAGCCTTGG-3',
COPG forward 5'-TCCGCTATGCTGCTGTTCGTA-3',
COPG reverse 5'-GCGGTTTGAATCTGTGACCAG-3',
EIF4A3 forward 5'-TGATCTTGGCTCCCACAAGAG-3',
EIF4A3 reverse 5'-ATTGGTGCCTCCAATGCAG-3',
NUP98 forward 5'-TTCCGGAATCCGATGTCAGA-3',
NUP98 reverse 5'-TGTAAAGCCTTTGGCCGGACT-3',
NUP205 forward 5'-ACCTTCGGAAGGATCTTCCAA -3';
NUP205 reverse 5'-GGAGTCCCAGAATCACCACAA-3';
NXF1 forward 5'-TGAGCAAACGATACGATGGC-3',
NXF1 reverse 5'-TCTGCGATTCAGGACAACGTC-3',
SON forward 5'-CAAGCCTTAGAGCTGGCATTG-3',
SON reverse 5'-GCTTGCGTGATTTGTGTTCAG-3'.

The relative expression levels of target mRNA were normalized against control transfected cells. GAPDH was used as an internal standard.

Chemical inhibitors

The chemical inhibitor TG003 (Sigma-Aldrich, Munich, Germany) directed against the kinase CLK1 was dissolved in DMSO to a concentration of 10 mM.

Animal experiments

Animals were housed and bred under pathogen free conditions, biosafety level 2 according to German Animal Protection Law (Tierschutzgesetz TierSchG). Animal testing was approved by the local authorities (Landesamt für Gesundheit und Soziales Berlin LAGeSo: Reference number G0217/08). C57BL/6/J and $p27^{-/-}$ mice (B6.129S4-*Cdkn1b*^{tm1Mlf}/J) were provided by Charles River (Sulzfeld, Germany) or bred in house, respectively. Mice aged between 7 and 15 weeks were intranasally infected with influenza A/Puerto Rico/8/34 virus (10× LD_{50}; in 50 µl PBS). Two days later, lungs of infected animals were isolated and homogenised, followed by centrifugation at 800 × g for 8 min at 4 °C. The amount of infectious viruses in the supernatant was quantified using the replication assay (see above). Proteins for use in immunoblotting experiments were obtained by adding TRIZOL Reagent (GIBCO BRL) to the remaining cell pellet, according to the manufacturer's instructions.

Data analysis

For identification of primary hits, three parameters were included: luciferase expression, the percentage of infected cells as determined by immunofluorescence microscopy, and the total number of infected cells. The latter parameter was informative because the number of viruses per well correlated with the number of infected cells, with minor influence of cells present. To maximize the robustness of the hit selection and to minimize false positives due to off-target effects, raw screening data from all three parameters were subjected separately to an analysis pipeline incorporating statistical checkpoints at each step (Fig. 8). First, we excluded non-expressed genes by determining constitutive or inducible expression via microarray profiling of non-infected and infected A549 samples (5814 genes were not expressed). Second, we excluded toxic siRNAs which reduced total cell numbers (<750 cells/well) upon transfection were also excluded (1520 siRNAs) using the microscopic assay applied throughout the primary screen. Third, non-toxic siRNAs targeting expressed genes were further analysed. For statistical analysis of luciferase assay data obtained from the genome-

wide screen, the following plate-wise quality control criteria were used: (i) the average signal from the non-targeting control wells (Allstars) was greater than 10,000 counts, and (ii) the difference in signal strength between the non-targeting control (Allstars) and (iii) the inhibitory control (NP) was at least two orders of magnitude. Using Genedata's Screener® software (www.genedata.com), we excluded wells with phenotypes attributable to positional effects. The revised raw data were subjected to statistical analysis using cellHTS (31), an R-implemented software package for the analysis of cell-based high-throughput RNAi screen data. Raw data were normalised using the B-score method to further exclude positional effects (32). Next, a z-score transformation was applied to center and scale the plate-wise data. The z-scores were calculated using the following equation:

$$z = \frac{X - \mu}{\sigma}.$$

where X is a raw score to be standardized, σ is the standard deviation of the population, and μ is the mean of the population. The medians of the centered and scaled values of at least three independent replicates were used for redundant siRNA activity (RSA) analysis (33), which applies a rank-based hypergeometric distribution test to identify hits. Only genes for which two corresponding siRNAs were scored as hits were analysed further. Next, Genedata's Screener® package was used to select all genes with a robust z-score of less than -2.

For the analysis of the hit validation data, for each siRNA the normalised percent inhibition of infectious virus particles was calculated. Briefly, the difference of each sample value subtracted from the median of the non-targeting control (Allstars) values of the particular plate was divided by the difference of the medians of the non-targeting control and the inhibitory control (siNP). An 80% normalised inhibition threshold was applied. Genes were scored as validated hits if at least two siRNAs, which did not impair cell viability, fulfilled this criteria.

The ratios of cytosolic to nuclear NP at 5 h p.i. and levels of total NP at 3 h p.i. in samples tested were non-normally distributed. Therefore, to assess the significance of differences between distributions of the target knockdown samples and non-targeting control reference samples (Allstars), we applied the minimal distance estimation Kolmogorov-Smirnov test using the statistical software environment R (http://www.r-project.org/). The samples sizes are individually defined as the number of main objects per well detected by the automated image analysis package Scan^R.

Significant differences in the amount of infectious viruses gained from the lungs of $p27^{-/-}$ and control mice were tested using a one-tailed t-test assuming different standard deviations for the samples and the controls (Welch-test).

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Claims

1. Pharmaceutical composition comprising at least one inhibitor of influenza virus replication optionally together with a pharmaceutically acceptable carrier, adjuvant, diluent or/and additive, for the prevention, alleviation or/and treatment of an influenza virus infection.

2. The pharmaceutical composition of claim 1, wherein the at least one inhibitor is selected from the group consisting of nucleic acids, nucleic acid analogues such as ribozymes, peptides, polypeptides, and antibodies, and compounds having a molecular weight below 1000 Dalton.

3. The pharmaceutical composition of claim 1 or 2, wherein the influenza virus infection is an influenza A virus infection.

4. The pharmaceutical composition of any of the claims 1 to 3, wherein the at least one inhibitor is capable of modulating gene expression or/and gene product activity.

5. The pharmaceutical composition of claim 4, wherein modulation of the expression or/and gene product activity is activation.

6. The pharmaceutical composition of claim 4, wherein modulation of the expression or/and gene product activity is inhibition.

7. The pharmaceutical composition as claimed in any of the claims 2 to 6, wherein the nucleic acid is RNA or DNA.

8. The pharmaceutical composition as claimed in claim 7, wherein the at least one inhibitor comprises

(a) a nucleic acid comprising a nucleotide sequence selected from sequences of Table 1, Table 2, Table 3 and Table 4,

(b) a fragment of the sequence of (a) having a length of at least 70%, at least 80 %, at least 90 %, at least 95 %, at least 98 %, or at least 99% of the sequence of (a),

(c) a nucleic acid comprising a sequence which is at least 70 %, at least 80 %, at least 90 %, at least 95 %, at least 98 %, or at least 99% identical to the sequence of (a) or/and (b), or/and

(d) a sequence complementary to the sequence of (a), (b) or/and (c).

9. The pharmaceutical composition as claimed in claim 8, wherein the nucleic acid of (a) comprises a nucleotide sequence selected from the sequences of Table 4 and fragments thereof.

10. The pharmaceutical composition as claimed in any of the claims 7 to 9, wherein the nucleic acid comprises

(i) an RNA molecule capable of RNA interference, such as siRNA or/and shRNA,

(ii) a miRNA,

(iii) a precursor of the RNA molecule (i) or/and (ii),

(iv) a fragment of the RNA molecule (i), (ii) or/and (iii),

(v) a derivative of the RNA molecule of (i), (ii) (iii) or/and (iv), or/and

(vi) a DNA molecule encoding the RNA molecule of (i), (ii) (iii) or/and (iv).

11. The pharmaceutical composition as claimed in any of the claims 7 to 10, wherein the RNA molecule is a double-stranded RNA molecule, preferably a double-stranded siRNA molecule with or without a single-stranded overhang alone at one end or at both ends.

12. The pharmaceutical composition as claimed in any of the claims 7 to 11 wherein the RNA molecule comprises at least one nucleotide analogue or/and deoxyribonucleotide.

13. The pharmaceutical composition as claimed in any of the claims 7 to 9, wherein the nucleic acid is an antisense nucleic acid or a DNA encoding the antisense nucleic acid.

14. The pharmaceutical composition as claimed in any of the claims 7 to 13, wherein the nucleic acid has a length of at least 15, preferably at least 17, more preferably at least 19, most preferably at least 21 nucleotides.

15. The pharmaceutical composition as claimed in any of the claims 7 to 14, wherein the nucleic acid has a length of at the maximum 29, preferably at the maximum 27, more preferably at the maximum 25, especially more preferably at the maximum 23, most preferably at the maximum 21 nucleotides.

16. The pharmaceutical composition as claimed in any of the claims 1 to 6 comprising an antibody, wherein the antibody is preferably directed against a polypeptide comprising
    (a) an amino acid sequence encoded by a nucleic acid or/and gene selected from sequences of Table 1, Table 2, Table 3, and Table 4 and complementary sequences thereof,
    (b) a fragment of the sequence of (a) having a length of at least 70%, at least 80 %, at least 90 %, at least 95 %, at least 98 %, or at least 99% of the sequence of (a), or/and
    (c) an amino acid sequence comprising a sequence which is at least 70 %, at least 80 %, at least 90 %, at least 95 %, at least 98 %, or at least 99% identical to the sequence of (a) or/and (b).

17. The pharmaceutical composition as claimed in claim 16, wherein the polypeptide of (a) comprises an amino acid sequence encoded by a nucleic acid or/and gene selected from Table 4.

18. The pharmaceutical composition of any of the preceding claims further comprising an agent suitable of transportation of the at least inhibitor of

influenza virus infection into a cell, in particular into a lung epithelial cell.

19. The pharmaceutical composition of claim 18, wherein the further agent is chitosan, which preferably is formulated in nanoparticles.

20. A screening method for identification of a compound suitable for the prevention, alleviation or/and treatment of an influenza virus infection, comprising the steps

(a) providing a cell or/and a non-human organism capable of being infected with an influenza virus and capable of expressing a gene, wherein the gene or/and gene product thereof is capable of modulating an influenza virus replication,

(b) contacting the cell or/and the organism of (a) with an influenza virus and with a compound known to be capable of modulating the expression or/and activity of the gene of (a) or/and the gene product thereof,

(c) determining the amount of influenza virus produced by the cell or/and the organism, and

(d) selecting a compound which reduces the amount of the influenza virus produced by the cell or/and the organism.

21. The method of claim 20, wherein the gene of (a) is selected from Table 1, Table 2, Table 3 and Table 4.

22. The method of claim 21, wherein the gene of (a) is selected from Table 4.

23. The method of any of the claims 20 to 22 comprising a cellular screening assay.

24. A screening method for identification of a compound suitable for prevention, alleviation or/and treatment of an influenza virus infection, comprising the steps

(i) providing a cell or/and a non-human organism capable of expressing a gene, wherein the gene or/and gene product thereof is capable of modulating an influenza virus replication,

(ii) contacting a compound with the cell or/and the organism of (i),

(iii) determining the amount or/and the activity of gene product of the gene of (i), and

(iv) selecting a compound which modulates the amount or/and the activity of the gene product of (i).

25. The method of claim 24, wherein the gene of (i) is selected from Table 1, Table 2, Table 3 and Table 4.

26. The method of claim 25, wherein the gene of (i) is selected from Table 4.

27. The method of any of the claims 24 to 26, wherein the compound of (iv) reduces the amount of the influenza virus produced by the cell or/and the organism.

28. The method of any of the claims 24 to 27, wherein modulation of the expression of a gene is downregulation or upregulation.

29. The method of any of the claims 24 to 27, wherein modulation of the activity of the gene is decrease or increase of the activity.

30. The screening method of any of the claims 24 to 29, wherein the influenza is selected from influenza A viruses.

31. The screening method of any of the claims 24 to 30, wherein the cell is a mammalian cell, in particular a human cell.

32. Use of an inhibitor of influenza virus replication capable of inhibiting or activating the expression of a gene selected from Table 1, Table 2, Table 3 and Table 4, or/and of inhibiting or activating a gene product thereof,

for the manufacture of a medicament or/and vaccine for the prevention, alleviation or/and treatment of an influenza virus infection.

33. Use of claim 32, wherein the gene is selected from Table 4.

34. Use of a nucleic acid comprising a gene sequence or/and a nucleotide sequence selected from Table 1, Table 2, Table 3, and Table 4 and fragments thereof in a method for screening for compounds or/and targets suitable for the prevention, alleviation or/and treatment of an influenza virus infection.

35. Use of claim 34, wherein a combination of at least two nucleic acids is used.

36. Use of claims 34 to 35, wherein the nucleic acid or the combination is selected from Table 4.

37. Use of claim 36, wherein the combination of nucleic acids inhibits expression or/and activity of a gene.