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(54) **Titre : UBIQUITINE LIGASES E3 BACTERIENNES PENETRANT DANS LES CELLULES DESTINEES A ETRE UTILISEES EN IMMUNOTHERAPIE**
(54) **Title: CELL-PENETRATING BACTERIAL E3-UBIQUITIN-LIGASES FOR USE IN IMMUNOTHERAPY**

(57) **Abrégé/Abstract:**

The present invention relates to cell-penetrating effector proteins of type III secretion system (T3SS)-containing bacteria of the genus Salmonella or Shigella and variants, fragments and immunomodulatory domains thereof, for use in immunotherapy. The present invention further relates to cell-penetrating effector proteins of type III secretion system (T3SS)-containing bacteria of the genus Salmonella or Shigella and variants, fragments and immunomodulatory domains thereof, for delivering cargo molecules into eukaryotic cells.



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(54) Title: CELL-PENETRATING BACTERIAL E3-UBIQUITIN-LIGASES FOR USE IN IMMUNOTHERAPY

(57) Abstract: The present invention relates to cell-penetrating effector proteins of type III secretion system (T3SS)-containing bacteria of the genus Salmonella or Shigella and variants, fragments and immunomodulatory domains thereof, for use in immunotherapy. The present invention further relates to cell-penetrating effector proteins of type III secretion system (T3SS)-containing bacteria of the genus Salmonella or Shigella and variants, fragments and immunomodulatory domains thereof, for delivering cargo molecules into eukaryotic cells.

5 Cell-penetrating bacterial E3-ubiquitin-ligases for use in immunotherapy

FIELD OF THE INVENTION

The present invention relates to isolated recombinant effector proteins of type III secretion system (T3SS)-containing bacteria of the genus *Salmonella* or *Shigella* or variants, fragments or
10 immunomodulatory domains thereof, for use in immunotherapy.

Immunotherapy is the treatment of disease by inducing, enhancing, or suppressing an immune response. The active agents of immunotherapy are called immunomodulators. The present invention relates to the use of cell-penetrating bacterial E3 ubiquitin ligases from bacteria of the
15 genus *Salmonella* or *Shigella* as immunomodulators. Agents that inhibit or prevent activity of the immune system are called immunosuppressives. Optionally the present invention relates to the use of cell-penetrating bacterial E3 ubiquitin ligases from bacteria of the genus *Salmonella* or *Shigella* as immunosuppressives.

20 In particular the present invention relates to cell-penetrating recombinant effector proteins of type III secretion system (T3SS)-containing bacteria of the genus *Salmonella* or *Shigella* that modulate cytokines and/or cytokine receptors of eukaryotic cells and/or eukaryotic genes which respond to cytokines, after said effector proteins have autonomously penetrated into said eukaryotic cells. Alternatively the present invention relates to of cell-penetrating variants, fragments or
25 immunomodulatory domains of cell-penetrating effector proteins of type III secretion system (T3SS)-containing bacteria of the genus *Salmonella* or *Shigella*, wherein said variants, fragments or immunomodulatory domains modulate cytokines and/or cytokine receptors of eukaryotic cells and/or eukaryotic genes which respond to cytokines, after said variants, fragments or immunomodulatory domains have autonomously penetrated into said eukaryotic cells.

30 In one aspect the present invention relates to the use of isolated recombinant effector proteins of type III secretion system (T3SS)-containing bacteria of the genus *Salmonella* or *Shigella*, wherein said effector proteins are characterized in that they are recombinantly produced or chemically synthesized, comprise an E3 ubiquitin ligase domain, preferably an Novel E3 ubiquitin ligase,
35 optionally comprise at least one Leucin-rich repeat and are cell-penetrating proteins, which translocate into eukaryotic cells without the requirement of a bacterial T3SS, or variants, fragments or immunomodulatory domains of said effector proteins, for the preparation of a pharmaceutical composition for regulating inflammatory reactions of the immune system, treating diseases caused

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by autoimmunity, treating acute inflammation or chronic inflammation, treating of inflammatory disorders, and/or suppressing the immune system.

Moreover the present invention relates to a pharmaceutical composition, comprising an isolated
5 effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it is recombinantly produced or chemically synthesized, comprises an E3 ubiquitin ligase domain, optionally comprises at least one Leucin-rich repeat, and is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

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The present invention further relates to the use of cell-penetrating effector proteins of type III secretion system (T3SS)-containing bacteria of the genus *Salmonella* or *Shigella* or variants, fragments and immunomodulatory domains of said effector proteins, for delivering cargo molecules into eukaryotic cells. Preferably the present invention relates to the use of cell-penetrating effector
15 proteins of type III secretion system (T3SS)-containing bacteria of the genus *Salmonella* or *Shigella* or variants, fragments and immunomodulatory domains of said effector proteins, for the preparation of pharmaceutical compositions for delivering linked cargo molecules into eukaryotic cells. Preferably said effector proteins, or variants, fragments and immunomodulatory domains thereof, that are linked to said cargo molecules modulate cytokines and/or cytokine receptors of
20 eukaryotic cells and/or eukaryotic genes which respond to cytokines, after said variants, fragments or immunomodulatory domains have autonomously penetrated into said eukaryotic cells.

Furthermore the present invention relates to the use of an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the
25 effector protein is characterized in that it is recombinantly produced or chemically synthesized, comprises an E3 ubiquitin ligase domain, preferably a Novel E3 ubiquitin ligase, optionally comprises at least one Leucin-rich repeat, is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS, or the use of a variant or fragment of said effector protein for delivering at least one cargo molecule across the membrane of a
30 eukaryotic cell, wherein the fusion construct of cargo molecule and said effector protein or variant or fragment thereof is a cell-penetrating construct, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

Hence the present invention provides pharmaceutical compositions comprising an isolated effector
35 protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it is recombinantly produced or chemically synthesized, comprises an E3 ubiquitin ligase domain, preferably a Novel E3 ubiquitin ligase, optionally comprises at least one Leucin-rich repeat, is a cell-penetrating protein, which

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translocates into eukaryotic cells without the requirement of a bacterial T3SS, or the use of a variant or fragment of said effector protein for delivering at least one cargo molecule across the membrane of a eukaryotic cell, wherein the fusion construct of cargo molecule and said effector protein or variant or fragment thereof is a cell-penetrating construct, which translocates into
5 eukaryotic cells without the requirement of a bacterial T3SS.

BACKGROUND OF THE INVENTION

Immunosuppression involves reactions that reduce the activation or efficiency of the immune system. Immunosuppressive reactions are either due to intentional medical actions or derivable
10 from a natural background and are, thus, largely pathogenic for the body.

In the first case of intentional medical actions, immunosuppressive activity of compounds is used for the modulation, in particular the controlled and purposeful inhibition or prevention of the activity of the immune system. The corresponding compounds are generally summarized as
15 immunosuppressants or immuno-suppressive drugs. Immunosuppressive drugs are a heterogenic collection including the following groups: glucocorticoids, cytostatics, antibodies, drugs acting on immunophilins, TNF-binding proteins and interferons. Immunosuppressives are used, for example, to prevent the rejection of transplanted organs and tissues (e.g., bone marrow, heart, kidney, liver), treat autoimmune diseases or diseases that are most likely of autoimmune origin (e.g., rheumatoid
20 arthritis, multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, sarcoidosis, focal segmental glomerulosclerosis, Crohn's disease, Behcet's Disease, pemphigus, and ulcerative colitis) or treat non-autoimmune inflammatory diseases (e.g., long term allergic asthma control).

In the second case of natural background, immunosuppression can occur, for example, in
25 malnutrition, aging, many types of cancer (such as leukemia, lymphoma, multiple myeloma), and certain chronic infections such as acquired immunodeficiency syndrome (AIDS). The unwanted effect of this immunosuppression is immunodeficiency that results in increased susceptibility to pathogens such as bacteria and virus. Moreover many microbial pathogens have evolved, in order to successfully infect a host organism, intriguing mechanisms to subvert host defenses
30 (Sansonetti, 2004). These microbes circumvent and undermine innate and specific host defenses.

One of the most fascinating and widespread pathogenicity modules of Gram-negative pathogens is the type III secretion system (T3SS) that targets essential cytoplasmic processes of the host cell by directly injecting so-called effector proteins into the cytoplasm via a molecular injection machine
35 ('molecular syringe') (Cornelis, 2002a; Cornelis, 2002b; Cornelis & Wolf-Watz, 1997). Bacterial effector proteins interfere with signaling mechanisms of the host cells, including those triggering immune responses. Especially pathogenic bacteria of the genus *Yersinia*, *Shigella*, or *Salmonella* harbor a wide range of effector proteins that target signaling mechanisms such as MAPK signaling

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cascades or pathways leading to repression of NF- κ B activation (Matsumoto & Young, 2009). Moreover numerous effector proteins of pathogenic bacteria, which utilize type III or IV secretion systems to deliver effector proteins into host cells, usurp the host ubiquitin pathways (Hicks and Galan, 2010; Angot, 2007).

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Ubiquitylation results in the covalent attachment of ubiquitin to a lysine residue on a target protein. Following the initial conjugation, subsequent ubiquitin molecules can be ligated to one of seven lysines in the previously attached ubiquitin molecule, resulting in polyubiquitylation of various linkages. Therefore, a substrate can be monoubiquitylated at a single lysine residue, multi-ubiquitylated at multiple lysine residues, or polyubiquitylated at one or more lysine residues. The type of ubiquitylation and the topology of the ubiquitin chains formed direct substrate fate. Ubiquitylation can signal for proteasome-dependent degradation or function as non-proteolytic signals important for DNA repair, signal transduction and vesicular trafficking.

Ubiquitylation involves an enzymatic cascade resulting in the formation of an isopeptide bond between ubiquitin and internal lysine residues of a substrate protein. This process involves an ubiquitin-activating enzyme (E1), which forms a thioester bond between a catalytic cysteine and the carboxy terminal glycine residue of ubiquitin. The ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2). Finally, an ubiquitin ligase (E3) facilitates the covalent conjugation of ubiquitin from an ubiquitin-loaded E2 to one or more lysine residues in the substrate. Therefore, E3 ubiquitin ligases confer specificity to the reaction through substrate binding. E3 ubiquitin ligases are defined by their ability to facilitate the transfer of ubiquitin from a cognate E2 to a specific substrate. There are two major known types of E3 ubiquitin ligases in eukaryotes, which possess distinct structural and mechanistic properties: the RING (really interesting new gene)/U-box domain and the HECT (homologous to E6-associated protein C terminus) domain. Bacterial effector proteins belonging to these ubiquitin ligase families are e.g. NleG2-3 from *Escherichia coli* ssp., LubX from *Legionella* spp. or SopA from *Salmonella* ssp (Hicks and Galan, 2010; Angot, 2007). In addition to that, another family of E3 ubiquitin ligases has been described that possesses a structural domain (termed NEL-domain for Novel E3 Ligase), which is distinct from either the RING or HECT domains (see e.g. Hicks and Galan, 2010). NEL E3 ligases comprise a large family of a bacterial effector proteins encoded by pathogenic bacteria, including *Shigella* ssp., *Salmonella* ssp., *Yersinia* spp., *Pseudomonas* spp., and *Escherichia coli* ssp.. Examples of bacterial NEL E3 Ubiquitin ligases are IpaH1, IpaH1.4, IpaH2, IpaH2.5, IpaH3, IpaH4, IpaH4.5, IpaH5, IpaH6, IpaH7, IpaH7.8, IpaH9.8 (from *Shigella* spp.); Slrp, SspH1, SspH2 (from *Salmonella* spp.); YPA_3361, YPA_3364 (from *Yersinia* spp.); PflO1_4099, PflO1_4565, PP_2212, PP_2394, PSPTO_1492, PSPTO_4093 (from *Pseudomonas* spp.); Ecol5_0100486, Ecol5_01001536, Ecol5_01001967, Ecol5_01003958, Ecol5_01004202, Ecol5_01004539, Ecol5_01004764, Ecol5_01004830, and Ecol5_01004885 (from *Escherichia coli* ssp.) (Hicks and Galan, 2010). Several of these bacterial

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NEL E3 ligases comprise an N-terminal leucine-rich repeat (LRR) domain and are therefore also classified as leucine-rich repeat (LRR) proteins. If said LRR is a LRR of the LPX-subtype, the proteins are called effector proteins of the LPX-subtype or effector proteins of LPX-family (Miao et al, 1999). Examples of effector proteins which comprise a NEL-domain and belong to the LPX-subtype are SspH1, SspH2, SlrP, IpaH4.5, IpaH7.8, and IpaH9.8.

For T3SS-dependent translocation of effector proteins, bacterial attachment to target cells is essential. Only cells that are contacted directly are infected via the T3SS injection machine. Surprisingly, it was recently shown that the *Yersinia* protein YopM, a secreted effector protein, is able to translocate into eukaryotic cells independently of the T3SS. Besides a T3SS-dependent translocation, YopM is able to autonomously penetrate the eukaryotic cell membrane and integrate into the cell cytosol (Rüter et al, 2010; Scharnert et al, 2013). It was suggested that the N-terminal α -helices of YopM mediate autonomous cell-penetration. Moreover it was shown that YopM can thereby deliver heterologous cargos into eukaryotic cells. Furthermore it was shown that YopM down-regulates the transcription of pro-inflammatory cytokines (e. g. TNF α , IL-12, IL-15, and IL-18) after autonomous penetration into host cells (Rüter et al, 2010).

Cell-penetrating peptides (cell-penetrating proteins, cell-permeable protein, CPPs) are proteins that are able to cross the cell membrane on their own. CPPs such as the trans-activator of transcription (Tat) protein encoded by the human immunodeficiency virus type I (HIV-1) are usually relatively short proteins or peptides (5-40 amino acids) with the ability to enter cells by different mechanisms (Frankel & Pabo, 1988) (Green & Loewenstein, 1988). Since the early studies on CPPs, numerous natural and synthetic peptides have been described to penetrate eukaryotic plasma membranes and deliver heterogeneous cargos into the host cell (Langel, 2011). The uptake mechanisms of most CPPs are poorly understood. Various uptake mechanisms of CPPs are discussed in the literature. It appears that different mechanisms can be involved in uptake of the diverse CPPs rather than a general mechanism. Initial binding of CPP to the plasma membrane seems to depend on electrostatic interactions of positively charged amino acids with negatively charged plasma membrane components. Two different models are currently discussed describing potential uptake mechanisms that follow the initial binding of the CPP to the plasma membrane. CPP uptake might be mediated by different endocytic uptake mechanisms including macropinocytosis, Clathrin-dependent and independent endocytosis, and Caveolae-dependent endocytosis, wherein after uptake, CPP need to escape from endosomal compartments during intra-cellular transport. Moreover internalization of the CPPs might occur by direct membrane penetration mechanisms, including inverted micelle and pore formation (Trabulo et al, 2010). CPPs have the ability to cross cellular membranes, either alone or in association with cargo molecules.

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YopM was the first identified bacterial CPP and opened the class of bacteria-derived CPPs within the heterogeneous group of CPPs (Rüter et al, 2010). YopM shares the ability of known CPPs to translocate across eukaryotic plasma membranes and it has the capacity to deliver molecular cargos such as GFP intracellularly. However, YopM does not show sequence homologies to known CPPs, indicating unique features that promote the uptake of YopM.

Crossing the plasma membrane is a prerequisite for intracellular targeted drug and/or compound delivery (for example in gene therapy where the gene/nucleic acid has to be delivered to an intracellular compartment). Cell penetrating peptides (CPPs) are known to transport cargo molecules attached to them into cells primarily by endocytosis. Nevertheless, there is an ongoing need in the art to provide compounds, which are able to cross the plasma membrane of higher cells. Especially in the field of immunotherapy, there is a great need for improved strategies for delivering immunomodulatory agents into cells. Moreover, self-delivering immunomodulatory agents would greatly improve the current immunotherapeutic strategies.

Although the above-mentioned immunosuppressive drugs are valuable medical tools, they are not without side effects and risks. Because the majority of them act non-selectively, the immune system is less able to resist infections and the spread of malignant cells. Furthermore, the production of the majority of immunomodulatory drugs is time-consuming and expensive. Therefore, there is a need for the provision of new, effective, cell-specific, selectively acting, and inexpensive immunomodulators, particularly immunosuppressants, preferably with lower side effects and risks.

The solution to the above-indicated technical problem is achieved by providing the embodiments as characterized herein and summarized below.

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SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a pharmaceutical composition A, comprising an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it

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a) is recombinantly produced or chemically synthesized,

b) comprises an E3 ubiquitin ligase repeat,

c) optionally comprises at least one Leucin-rich repeat, and

d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

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Alternatively, the present invention provides a pharmaceutical composition B, comprising a variant, fragment, or immunomodulatory domain of an effector protein as defined in the in the previous paragraph.

- 5 Optionally the pharmaceutical composition B can be characterized in that said variant, fragment, or immunomodulatory domain is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

- 10 The pharmaceutical composition B can be characterized in that optionally said variant, fragment, or immunomodulatory domain comprises an E3 ubiquitin ligase domain and optionally at least one Leucin-rich repeat.

- 15 Optionally, the pharmaceutical composition B can be characterized in that said variant, fragment, or immunomodulatory domain comprises an E3 ubiquitin ligase domain and optionally at least one Leucin-rich repeat.

- The pharmaceutical composition A or B as described above can be further characterized in that said type III secretion system (T3SS)-containing bacterium is classified as *Salmonella bongori*, *Salmonella enterica*, *Salmonella subterranean*, *Salmonella typhi*, *Salmonella typhimurium*,
20 *Salmonella enterica* serovar *typhimurium*, *Salmonella enteritidis*, *Salmonella pullorum*, *Salmonella dublin*, *Salmonella arizonae*, *Salmonella choleraesuis*, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella sonnei*, or *Shigella boydii*.

- 25 Optionally the pharmaceutical composition A or B can be characterized in that the E3 ubiquitin ligase domain is classified as Novel E3 Ligase.

- 30 Optionally the pharmaceutical composition A or B can be characterized in that the E3 ubiquitin ligase domain is closer to the C-terminus of said effector protein than to the N-terminus of said effector protein.

Optionally the pharmaceutical composition A or B can be characterized in that the Leucin-rich repeat(s) is/are a Leucin-rich repeat of the LPX-subtype.

- 35 Optionally the pharmaceutical composition A can be characterized in that the Leucin-rich repeat(s) is/are closer to the N-terminus of said effector protein than to the C-terminus of said effector protein.

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Optionally the pharmaceutical composition A or B can be characterized in that said effector protein is a bacterial effector protein of the LPX-Subtype.

- 5 Optionally the pharmaceutical composition A or B can be characterized in that said effector protein is SlrP, SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, and IpaH9.8.

Optionally the pharmaceutical composition A or B can be characterized in that said effector protein has an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2,
10 SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9.

Optionally the pharmaceutical composition A or B can be characterized in that said effector protein is encoded by a polynucleotide selected from the group consisting of SEQ ID NO: 10, SEQ ID NO:
15 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, or SEQ ID NO: 18.

Optionally the pharmaceutical composition A or B can be characterized in that said effector protein, variant, fragment or immunomodulatory domain comprises an amino acid sequence selected from
20 the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27.

Optionally the pharmaceutical composition A or B can be characterized in that said effector protein, variant, fragment or immunomodulatory domain comprises at least one Leucine-rich repeat,
25 preferably at least one Leucine-rich repeat that is comprised in an amino acid sequence selected from the group consisting of SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, or SEQ ID NO: 36.

Optionally the pharmaceutical composition A or B can be characterized in that said effector protein, variant, fragment or immunomodulatory domain comprises at least one amino acid sequence
30 selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO:
35 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65 or SEQ ID NO: 550, and/or at least one amino acid

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sequence that corresponds to a C-terminally or N-terminally truncated fragment of an amino acid sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, or SEQ ID NO: 550.

Optionally the pharmaceutical composition A or B can be characterized in that the composition comprises no auxiliary agent which can cause the penetration of said effector protein into a eukaryotic cell.

Optionally the pharmaceutical composition A or B can be characterized in that the composition comprises no auxiliary agent selected from the group consisting of T3SS-containing bacterium, bacterium of the genus *Salmonella* or *Shigella*, and cell-penetrating molecule, wherein "cell-penetrating molecule" includes CPPs that are different from an effector protein as defined above and cell-penetrating nanoparticles.

Optionally the pharmaceutical composition A or B can be characterized in that said effector protein or variant, fragment or immunomodulatory domain thereof is linked to a cell-specific targeting agent.

Optionally the pharmaceutical composition A or B can be characterized in that said effector protein or variant, fragment or immunomodulatory domain thereof is linked to a cargo molecule, wherein optionally said cargo molecule displays a therapeutical and/or diagnostic activity and/or wherein optionally said cargo molecule comprises at least one compound selected from the group consisting of nucleic acids, polypeptides, organic molecules, small organic molecules, metals, nanoparticles, viruses, modified viruses, viral vectors, antibodies and/or plasmids.

Optionally the pharmaceutical composition A or B can be characterized in that said effector protein or variant, fragment or immunomodulatory domain thereof ubiquitinates itself and/or eukaryotic proteins after said effector protein or variant, fragment or immunomodulatory domain has autonomously penetrated into a eukaryotic cell.

Optionally the pharmaceutical composition A or B can be characterized in that said effector protein or variant, fragment or immunomodulatory domain thereof modulates cellular pathway(s) of the

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innate immune system of eukaryotic cells after it has autonomously penetrated into said eukaryotic cells.

5 Optionally the pharmaceutical composition A or B can be characterized in that said effector protein or variant, fragment or immunomodulatory domain thereof modulates cytokines and/or cytokine receptors of eukaryotic cells and/or eukaryotic genes which respond to cytokines of eukaryotic cells after said effector protein or variant, fragment or immunomodulatory domain has autonomously penetrated into said eukaryotic cells.

10 Optionally the pharmaceutical composition A or B can be characterized in that said effector protein or variant, fragment or immunomodulatory domain thereof downregulates the expression of cytokines and/or cytokine receptors after said effector protein or variant, fragment or immunomodulatory domain has autonomously penetrated into said eukaryotic cells.

15 Preferably the pharmaceutical composition A or B can be used in immunomodulatory therapy of animals or humans.

Optionally the pharmaceutical composition A or B can be used in immunosuppressive therapy of animals or humans.

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Preferably the pharmaceutical composition A or B can be used in the regulation of inflammatory reactions of the immune system, treatment of diseases caused by autoimmunity, acute inflammation or chronic inflammation, treatment of inflammatory disorders, and/or for suppressing the immune system.

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Furthermore, the pharmaceutical composition A or B may be for use in delivering cargo molecules across the cell membrane of a eukaryotic cell.

30 In one embodiment the present invention provides a method of treatment of a disease or of diseases caused by autoimmunity, treatment of acute inflammation, chronic inflammation, inflammatory disorders, pathogenic inflammatory reactions of the immune system, and/or a method of suppressing the immune system in a subject, comprising administering a therapeutically effective amount of a pharmaceutical composition A or B as defined above to said subject.

35 In another embodiment the present invention provides a kit comprising the pharmaceutical composition A or B as defined above.

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In a further aspect the present invention relates to the use of an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it

- a) is recombinantly produced or chemically synthesized
- 5 b) comprises an E3 ubiquitin ligase domain, preferably an Novel E3 ubiquitin ligase
- c) optionally comprises at least one Leucin-rich domain;
- d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS;

or to the use of a variant, fragment or immunomodulatory domain of said effector protein, for the
10 preparation of a pharmaceutical composition for regulating inflammatory reactions of the immune system, treating diseases caused by autoimmunity, treating acute inflammation or chronic inflammation, treating of inflammatory disorders, and/or suppressing the immune system.

In another aspect the present invention relates to the use of an isolated effector protein of a type III
15 secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it

- a) is recombinantly produced or chemically synthesized
- b) comprises an E3 ubiquitin ligase domain, preferably an Novel E3 ubiquitin ligase
- c) optionally comprises at least one Leucin-rich domain;
- 20 d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS;

or to the use of a variant, fragment of said effector protein for the preparation of a pharmaceutical composition for delivering at least one cargo molecule across the membrane of a eukaryotic cell, wherein the fusion construct of cargo molecule and said effector protein or variant or fragment
25 thereof is a cell-penetrating construct, which translocates into eukaryotic cells without the requirement of a bacterial T3SS. Additionally, the effector protein can be e) linked to the at least one cargo molecule.

Preferably said delivered cargo molecule comprises at least one compound selected from the
30 group consisting of nucleic acids, polypeptides, organic molecules, small organic molecules, metals, dye, nano-particles, viruses, modified viruses, viral vectors, antibodies and/or plasmids.

Finally, the present invention provides isolated effector proteins of type III secretion system (T3SS)-containing bacteria of the genus *Salmonella* or *Shigella* or variants, fragments or
35 immunomodulatory domains thereof linked to a cargo molecule and/or cell-specific targeting agent. These effector proteins can be used in immunotherapy.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors identified several effector proteins of T3SS-containing bacteria of the genus *Salmonella* and *Shigella* as cell-penetrating proteins. Doing so, the present inventors identified a new subclass of bacteria-derived CPPs, all being effector proteins that comprise a C-terminal Novel E3 ubiquitin ligase domain. The present inventors first identified SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, IpaH9.8 and SlrP as bacterial cell-penetrating proteins. Moreover the inventors succeeded in predicting protein transduction domains (PTDs) within said effector proteins and thereby identified several potential cell-penetration mediating domains within these proteins. The structural similarities of SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, IpaH9.8 and SlrP indicate a general concept of T3SS-independent translocation. Interestingly the inventor demonstrated that cell-penetration of recombinant SspH2 affects membrane integrity of HeLa cells with no effects on cell viability, suggesting a potential direct uptake mechanism, e.g. by the formation of a transient membrane pore.

On the one, the present invention discloses SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, IpaH9.8 and SlrP as novel cell-penetrating effector proteins and provides the use these effector proteins as novel CPPs to deliver cargo molecules into eukaryotic cells.

As mentioned above, SspH1, SspH2, IpaH1.4, IpaH 2.5, IpaH3, IpaH4.5, IpaH7.8, IpaH9.8 and SlrP all comprise a C-terminal *Novel E3 ligase* (NEL) domain and, apart from IpaH2.5 and IpaH1.4, they belong to effector proteins of the LPX-subtype. Effector proteins of the LPX-subtype share the presence of a Leucine-rich repeat (LRR) motif and an N-terminal α -helical structure (Miao et al, 1999).

Several bacterial effector proteins, which comprise an E3 ubiquitin ligase domain are known to interfere with the host immune response after being injected into host cells by the T3SS (Angot, 2007; Hicks and Galan, 2010). Surprisingly the present inventors found out that SspH1, an effector protein and E3-ubiquitin ligase of *Salmonella enterica serovar thyphimurium*, is able to modulate the eukaryotic immune response when contacted with eukaryotic cells in an isolated, recombinantly expressed form. As depicted in the examples the inventors showed that recombinant SspH1 is able to autonomously penetrate into eukaryotic cells without the requirement of the *Salmonella* T3SS. Following translocation recombinant SspH1 localises to the cytoplasm as well as to the nucleus of the eukaryotic cell and surprisingly acts as functional E3 ubiquitin ligase that is able to self-ubiquitinate and form polyubiquitin chains within the eukaryotic host cell. Furthermore, the inventors found out that recombinant SspH1 binds and ubiquitinates the host kinase PKN1 and downregulates the expression of IL-8 in eukaryotic cells after it has autonomously penetrated into said eukaryotic cells.

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Accordingly the present inventors not only identified SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, IpaH9.8 and SlrP as novel cell-penetrating proteins, they also found out that these effector proteins are promising self-delivering immunotherapeutic agents.

5

In sum, the inventors revealed that bacterial E3 ubiquitin ligases are able to modulate the immune response of eukaryotic cells in the absence of the pathogenic bacteria from which they derive. The inventors demonstrated that isolated recombinant NEL effector proteins are able to interfere with pathways of the innate immune system of eukaryotic cells after having autonomously penetrating
10 into said eukaryotic cells.

Hence, on the other, the present invention discloses the use of isolated recombinantly expressed or chemically synthesized bacterial cell-penetrating effector proteins, which comprise an immunomodulatory E3-ubiquitin-ligase domain, as immune therapeutic agents.

15

As summarized above the present invention provides the use of cell-penetrating bacterial E3 ubiquitin ligases from bacteria of the genus Salmonella or Shigella as immunomodulators.

In particular embodiments the present invention provides cell-penetrating recombinant effector
20 proteins of type III secretion system (T3SS)-containing bacteria of the genus Salmonella or Shigella that modulate cytokines and/or cytokine receptors of eukaryotic cells and/or eukaryotic genes which respond to cytokines, after said effector proteins have autonomously penetrated into said eukaryotic cells. Alternatively the present invention provides cell-penetrating variants, fragments or immunomodulatory domains of cell-penetrating effector proteins of type III secretion
25 system (T3SS)-containing bacteria of the genus Salmonella or Shigella, wherein said variants, fragments or immunomodulatory domains modulate cytokines and/or cytokine receptors of eukaryotic cells and/or eukaryotic genes which respond to cytokines, after said variants, fragments or immunomodulatory domains have autonomously penetrated into said eukaryotic cells.

30 In further embodiments the present invention provides the use of isolated recombinant effector proteins of type III secretion system (T3SS)-containing bacteria of the genus Salmonella or Shigella, wherein said effector proteins are characterized in that they are recombinantly produced or chemically synthesized, comprise an E3 ubiquitin ligase domain, preferably an Novel E3 ubiquitin ligase, optionally comprise at least one Leucin-rich repeat and are cell-penetrating
35 proteins, which translocate into eukaryotic cells without the requirement of a bacterial T3SS, or variants, fragments or immunomodulatory domains of said effector proteins, for the preparation of a pharmaceutical composition for regulating inflammatory reactions of the immune system, treating

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diseases caused by autoimmunity, treating acute inflammation or chronic inflammation, treating of inflammatory disorders, and/or suppressing the immune system.

Moreover the present invention provides a pharmaceutical composition, comprising an isolated
 5 effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it is recombinantly produced or chemically synthesized, comprises an E3 ubiquitin ligase domain, optionally comprises at least one Leucin-rich repeat, and is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

10

In other embodiments the present invention further provides the use of cell-penetrating effector proteins of type III secretion system (T3SS)-containing bacteria of the genus *Salmonella* or *Shigella* or variants, fragments and immunomodulatory domains of said effector proteins, for delivering cargo molecules into eukaryotic cells. Preferably the present invention relates to the use
 15 of cell-penetrating effector proteins of type III secretion system (T3SS)-containing bacteria of the genus *Salmonella* or *Shigella* or variants, fragments and immunomodulatory domains of said effector proteins, for the preparation of pharmaceutical compositions for delivering linked cargo molecules into eukaryotic cells. Preferably said effector proteins, or variants, fragments and immunomodulatory domains thereof, that are linked to said cargo molecules modulate cytokines and/or cytokine receptors of eukaryotic cells and/or eukaryotic genes which respond to cytokines,
 20 after said variants, fragments or immunomodulatory domains have autonomously penetrated into said eukaryotic cells.

Further embodiments of the present invention provide the use of an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*,
 25 wherein the effector protein is characterized in that it is recombinantly produced or chemically synthesized, comprises an E3 ubiquitin ligase domain, preferably a Novel E3 ubiquitin ligase, optionally comprises at least one Leucin-rich repeat, is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS, or the use of a variant or fragment of said effector protein for delivering at least one cargo molecule across the
 30 membrane of a eukaryotic cell, wherein the fusion construct of cargo molecule and said effector protein or variant or fragment thereof is a cell-penetrating construct, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

In another embodiment the invention relates to the use of an isolated effector protein of a type III
 35 secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it is recombinantly produced or chemically synthesized, optionally comprises an E3 ubiquitin ligase domain, optionally comprises at least one Leucin-rich

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repeat, is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS, and is linked to at least one cargo molecule, for delivering the at least one cargo molecule across the membrane of a eukaryotic cell, wherein the fusion construct of cargo molecule(s) and said effector protein is a cell-penetrating construct, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

Optionally the present invention provides pharmaceutical compositions comprising an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it is recombinantly produced or chemically synthesized, comprises an E3 ubiquitin ligase domain, preferably a Novel E3 ubiquitin ligase, optionally comprises at least one Leucin-rich repeat, is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS, or the use of a variant or fragment of said effector protein for delivering at least one cargo molecule across the membrane of a eukaryotic cell, wherein the fusion construct of cargo molecule and said effector protein or variant or fragment thereof is a cell-penetrating construct, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

In another aspect, the present invention relates to an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella* or a variant, fragment or immunomodulatory domain of said effector protein, wherein said effector protein, variant, fragment or immunomodulatory domain is linked to a cargo molecule and/or cell-specific targeting agent. In the context of the present application the following proteins are denoted as "compound(s) of the invention" or "polypeptide(s) of the invention":

i) isolated effector protein of a type III secretion system (T3SS)-containing bacterium, preferably of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it

- a) is recombinantly produced or chemically synthesized,
- b) comprises an E3 ubiquitin ligase domain,
- c) optionally comprises at least one Leucin-rich repeat, and
- d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS;

ii) variants, fragments or immunomodulatory domain of an effector protein as defined under point i); and

iii) SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, IpaH9.8 and SlrP as well as variants, fragments or immunomodulatory domain of SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, IpaH9.8 and SlrP.

The term "effector protein" as defined under point i), includes "variants", "fragments" or "immunomodulatory domains" as defined under point ii).

- 5 The terms "SspH1", "SspH2", "IpaH1.4", "IpaH2.5", "IpaH3", "IpaH4.5", "IpaH7.8", "IpaH9.8" and "SlrP", as disclosed herein, include "variants", "fragments" and "immunomodulatory domains" of these proteins, namely SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, IpaH9.8 and SlrP.

10 The terms "effector protein" and "effector protein of the invention" and the like refer herein to effector proteins of bacteria which contain a type III secretion system (T3SS), as well as to fragments of said effector proteins, such as a immunomodulatory domain or protein transduction domain, and to variants of said effector proteins. Preferably these effector proteins are secreted via the T3SS of a pathogenic gram-negative bacterium and promote infection and/or suppress host cell defences. Hence the term "effector protein(s)" preferably refers herein to bacterial T3SS

15 effector proteins. Effector proteins of the present invention preferably comprise an E3 ubiquitin ligase domain and interfere with the immune response of a subject. More preferably effector proteins of the present invention comprise an NEL E3 ubiquitin ligase domain and/or belong to effector proteins of the LPX subtype. Even more preferably effector proteins of the present invention are able to autonomously translocate in an isolated recombinant form into eukaryotic

20 cells without the requirement of a bacterial T3SS. Preferably effector proteins of the present invention modulate cytokines and/or cytokine receptors of eukaryotic cells and/or eukaryotic genes which respond to cytokines of eukaryotic cells after said effector proteins have autonomously penetrated into said eukaryotic cells. "Modulate" as used herein refers to inducing, enhancing, and/or suppressing. The present invention includes, but is not limited to the following effector

25 proteins: SspH1 (Salmonella-secreted protein), SspH2, IpaH1.4 (Invasion plasmid antigen), IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, IpaH9.8 and SlrP.

The findings of the inventors strongly suggest that of effector protein of a type III secretion system (T3SS)-containing bacterium of the genus Salmonella or Shigella (beside the above listed), which

30 are characterized in that they comprise an E3 ubiquitin ligase domain and optionally comprise at least one Leucin-rich repeat are able to autonomously translocate into eukaryotic cells and modulate cytokines and/or cytokine receptors of eukaryotic cells and/or eukaryotic genes which respond to cytokines, after they have autonomously penetrated into eukaryotic cells. Hence said other effector proteins are also part of the invention, e.g. IpaH1, IpaH2, IpaH4, IpaH5, IpaH6,

35 IpaH7, or SopA.

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Moreover it is most likely that also effector proteins of other type III secretion system (T3SS)-containing bacteria than those of the genus *Salmonella* or *Shigella*, which are characterized in that they comprise an E3 ubiquitin ligase domain and optionally comprise at least one Leucin-rich repeat are able to autonomously translocate into eukaryotic cells and modulate cytokines and/or
 5 cytokine receptors of eukaryotic cells and/or eukaryotic genes which respond to cytokines, after they have autonomously penetrated into eukaryotic cells.

Said other type III secretion system (T3SS)-containing bacteria include but are not limited to *Yersinia spp.*, *Escherichia spp.*, *Pseudomonas spp.* and *Chlamydia spp.*. Hence effector proteins
 10 of the invention also include, e.g. YPA_3361, YPA_3364; PflO1_4099, PflO1_4565, PP_2212, PP_2394, PSPTO_1492, PSPTO_4093; Ecol5_01000486, Ecol5_01001536, Ecol5_01001967, Ecol5_01003958, Ecol5_01004202, Ecol5_01004539, Ecol5_01004764, Ecol5_01004830, and Ecol5_01004885 (Hicks and Galan, 2010).

15 The T3SS effector protein YopM as well as YopM variant and YopM fragment are not part of the present invention.

In particular embodiments the effector proteins of the invention have an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4,
 20 SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9 and are preferably encoded by a polynucleotide with an nucleotide sequence selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, or SEQ ID NO: 18.

25 Preferably effector proteins of the present invention are effector proteins of type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*. More preferably effector proteins of the present invention are effector proteins of *Salmonella bongori*, *Salmonella enterica*, *Salmonella subterranean*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella enterica* serovar *typhimurium*, *Salmonella enteritidis*, *Salmonella pullorum*, *Salmonella dublin*, *Salmonella arizonae*,
 30 *Salmonella choleraesuis*, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella sonnei*, or *Shigella boydii*.

Compounds of the invention preferably comprise an E3 ubiquitin ligase domain, optionally a NEL E3 ubiquitin ligase (NEL).

35 The NEL (Novel E3 Ligase) domain is a newly identified structure of ubiquitin E3 ligases that is distinct from HECT and RING domains commonly found in eukaryotic and prokaryotic E3 ligases. In contrast, the NEL domain has only been found in bacterial effector proteins of different

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pathogenic bacteria. These proteins functionally mimic eukaryotic E3 ligases but are structurally different from the eukaryotic HECT and RING domains. These proteins are furthermore characterised by a N-terminal LRR domain that is thought to determine the substrate specificity for the E3 ligase and has been suggested to be involved in autoinhibition of the ligase activity by covering the reserved catalytic Cys residue (Hicks and Galan 2010).. Further characteristics of NELs are known to the skilled person.

Preferably compounds of the invention comprise an E3 ubiquitin ligase domain as marked in Example 7 and Figures 14 to 22. Alternatively compounds of the invention comprise an E3 ubiquitin ligase domain having an amino acid sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27.

In some embodiments compound(s) of the invention comprising a E3 ubiquitin ligase domain as described herein above may additionally comprise at least one leucine-rich repeat. The term "at least one leucine-rich repeat" relates to a leucine rich repeat as present in a polypeptide of a compound of the invention as described herein, as can be determined according to methods known in the art. Such leucine-rich repeats are described in Evdokimov et al. (*J. Mol. Biol.* 312: 807-821 (2001)). The leucine-rich repeat may be in any orientation or order with respect to a second or further leucine-rich repeat and/or with respect to an E3 ligase domain of a compound of the invention and/or with respect to other structural elements in the polypeptides of the invention. The leucine-rich repeat may be N-terminally located or C-terminally located or may be localized at any other suitable position within the polypeptide or molecule. Preferably the leucine-rich repeat is N-terminally located.

Preferably, such compound(s) of the invention has/have the capability of modulate cellular pathway of the innate immune system of eukaryotic cells in a subject. Optionally said compound(s) of the invention has/have the capability of autopenetrating the cell membrane and integrating into the cell cytosol without the requirement of additional factors. Both can be tested in accordance with an assay as described herein.

The leucine-rich repeat (LRR) motif is commonly found in eukaryotic proteins and is thought to be involved in protein-protein interaction. The LPX repeat is a subtype of the LRR motif that has exclusively been found in T3SS- secreted effector proteins of *Salmonella*, *Shigella*, *Yersinia*, *Edwardsiella*, *Rhizobium*, and *Bradyrhizobium* species. Most of the LRRs of SspH1 correspond exactly to the LPX structure (LTSLPxLPxxLxxLxaxxNx). The LPX domain has been suggested to determine the specificity for binding of the LPX proteins to its ligand which may account for the multiple copies of these genes that have been identified in *Salmonella*, *Shigella* and *Yersinia* spec

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(Miao et al. (1999); Haraga et al. (2003). Further characteristics of LRRs and LPX repeats are known to the skilled person.

Compounds of the invention preferably comprise at least one Leucin-rich repeat (LRR), i.e. one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen LRRs. Optionally compounds of the invention belong to the LPX-family, a subtype of the LRR superfamily of protein binding domains (Symmons et al. 1997) of bacterial effector proteins that share the presence of an LRR motif as well as an N-terminal alpha-helical structure. Preferably compounds of the invention comprise at least one Leucin-rich repeat, that either corresponds to a "LRR stretch" as marked in Example 7 and Figures 14 to 22 (marked in light grey; non-edged) or a segment of a "LRR stretch" as marked in Example 7 and Figures 14 to 22 (marked in light grey; non-edged). An "LRR stretch" as marked in Example 7 and Figures 14 to 22 corresponds to a predicted LRR domain. Alternatively compounds of the invention comprise at least one Leucin-rich repeat that is comprised in an amino acid sequence selected from the group consisting of SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, or SEQ ID NO: 36.

Preferably compounds of the invention comprise at least one Leucin-rich repeat that corresponds to at least one of the reference sequences LxxLPxxLPxxLxxLxaxxNx, LTSLPxLPxxLxxLxaxxNx or Lx₆Lx₂l/LPx₃P (Dean P.FEMS Microbiol Rev. 2011 Nov;35(6):1100-25. doi: 10.1111/j.1574-6976.2011.00271.x. Epub 2011 May 18. Review).

The effector protein SspH1 of the present invention, or SspH1 variants, SspH1 fragments or immunomodulatory domains of SspH1 preferably comprise at least one Leucin-rich repeats that correspond to at least one of the reference sequences LxxLPxxLPxxLxxLxaxxNx, LTSLPxLPxxLxxLxaxxNx or Lx₆Lx₂l/LPx₃P.

Compounds of the invention preferably comprise at least one of the Leucin-rich repeats indicated in Example 14. Preferably compounds of the invention comprise at least one Leucin-rich repeat selected from one of the groups of LRRs denoted in Example 14 as group 1, group 2, group 3, group 4, group 5, group 6, group 7, group 8, and group 9. Preferably said Leucin-rich are orientated within a compound of the invention in a way that a leucin-rich repeat with a lower identification number is located n-terminally to a leucin-rich repeat with a higher identification number, e.g. N-terminus -> LRR1 -> LRR2 -> LRR3 -> C-terminus.

Preferably the E3 ubiquitin ligase domain of a compound of the invention is closer to the C-terminus of the compound of the invention than to its N-terminus. Furthermore it is preferred that Leucin-rich repeat(s) of a compound of the invention is/are closer to the N-terminus of the

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compound of the invention than to its C-terminus. More preferably Leucin-rich repeat(s) of a compound of the invention are mainly located within the N-terminal half of the compound of the invention. Furthermore the E3 ubiquitin ligase domain of compounds of the invention is preferably located C-terminal in compared to Leucin-rich repeat(s) of the compound.

5

Compound(s) of the invention are preferably cell-penetrating proteins (CPPs). That means that the compound(s) of the invention are able to cross eukaryotic cell membranes on its/their own. In other words compound(s) of the invention cross eukaryotic cell membranes autonomously. Preferably compound(s) of the invention translocate into eukaryotic cells without the requirement of a bacterial
 10 T3SS or any other auxiliary agent which can cause the penetration of a protein, e.g a compound of the invention, into a eukaryotic cell. "Auxiliary agent" refers to any molecule that is able to deliver a cargo molecule into a eukaryotic cell. Within the present invention auxiliary agents include but are not limited to non-bacterial cell-penetrating peptides, YopM, YopM fragment and YopM variants, and cell-penetrating nanoparticles. Within the present invention auxiliary agent also refers to
 15 functional bacterial secretion systems, preferably to functional T3SS. Hence compounds of the invention are preferably able to penetrate into eukaryotic cells in an isolated form and in the absence of T3SS-containing bacteria. Preferably compounds of the invention translocate into the eukaryotic cytosol, optionally also into the eukaryotic nucleus. Moreover cell-penetrating compounds of the invention are preferably able to facilitate cellular uptake of a molecular cargo.

20

"Isolated" means that the compound(s) of the invention is/are separated out of its/their natural environment, preferably separated from T3SS-containing bacteria which naturally comprise the compound of the invention or the effector protein the compound of the invention is derived from. Compound(s) of the invention may be produced by recombinant expression or chemical protein
 25 synthesis. Hence "isolated" refer to compound(s) of the invention which are separated from the genetically modified host organism used for the production of the compound(s) of the invention. However, as described below, the compound(s) of the invention may be administered as living therapeutics. In this case "isolated" does preferably not mean that compound(s) of the invention are separated from the genetically modified host organism.

30

The ability of compounds of the invention to autonomously penetrate into eukaryotic cells is preferably mediated by at least one protein transduction domain (PTD). The term "Protein transduction domain(s)" as used herein preferably refers to the domain(s) of a cell-penetrating protein that mediate(s) translocation of said protein across the cell membrane. (It cannot be
 35 excluded that the terms "protein transduction domain" and "cell-penetrating protein" are sometimes used herein interchangeable.) How potential PTDs can be predicted is described in the Examples of the invention. Whether predicted PTDs correspond to functional cell-penetrating entities can be

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tested experimentally, for instance as explained in the examples of these invention. Alternatively functional PTDs of compounds of the invention can be identified by deleting and/or mutating predicted PTDs and subsequently investigating whether the deletion- and/or mutation-construct penetrates autonomously into eukaryotic cells. "Mutating" means any amino acid substitution(s) or
 5 amino acid modification(s) that destroy the cell-penetrating ability of a PTD.

The terms "autopenetrating", "autonomously penetrating" and "penetrate/pass/cross the membrane of eukaryotic cells without the assistance of exogenous factors, such as a T3SS" are used herein interchangeably. The terms mean that the compounds of the invention are able to
 10 cross/pass a membrane which separates two different compartments. It is preferred that the mentioned two compartments refer to the exterior and interior of a cell. The "cell membrane" is therefore preferably a plasma membrane that separates the interior of a cell from the exterior. It will be understood that the compounds of the invention preferably cross the plasma membrane from the exterior of the cell towards the interior of the cell.

15 The compounds of the invention may enter eukaryotic cells, without the need to interact with a receptor, i.e. the compounds of the invention may enter eukaryotic cells irrespective of a receptor.

The capability to pass the cell membrane and to enter the cytosol of a cell without the assistance of
 20 exogenous factors can be tested and determined by methods known to a person skilled in the art. The autopenetration of compound(s) of the invention into the cell membrane and its integration into the cell cytosol can be tested by a method of cell fractionation as described e.g. by Kenny B, Finlay BB. Infect Immun. 1997 Jul;65(7):2528-36 and/or by the methods described in the appended examples. For example, such a method comprises the incubation of cells to be tested, e.g. HeLa
 25 cells, with compound(s) of the invention, e.g. a recombinant compound(s) of the invention, for a time period of 10 to 60 min, preferably of 20 to 40 min, more preferably of 25 to 35 min and most preferably of 30 min. The compound(s) of the invention may be present in any suitable medium known to the skilled person. For example, the protein is provided in an infection medium comprising, e.g. DMEM, FCS, L-glutamine, HEPES and methyl- α -D-mannose. Preferably, the
 30 infection medium comprises 500 ml DMEM, 10% (v/v) FCS, 1 mM L-glutamine, 10 mM HEPES and 1% (w/v) methyl- α -D-mannose. For the assay cell culture dishes comprising the cells to be tested, e.g. as a confluent grown surface layer may be incubated with the compound(s) of the invention present in an infection medium as described herein above in any suitable concentration, e.g. a concentration of 1 to 100 μ g per ml, preferably of 5 to 50 μ g per ml, more preferably of 10 to
 35 30 μ g per ml and most preferably of 15 to 25 μ g per ml. Subsequently, the cells may be washed with any suitable buffer known to the skilled person, e.g. with D-PBS/Mg²⁺. Preferably, the washing is carried out in ice-cold buffer and repeated twice. This is optionally followed by an acid-wash with

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0.2 M glycine, pH 2.0. Subsequently, the cells are permeabilised by any suitable means known to the skilled person. Preferably, the cells are suspended in a suitable sonication buffer and the suspension may then be permeabilised by sonication. Subsequently, the resulting suspension may be separated into cell fractions, for example by centrifugation, e.g. at 108.000 x g for 15 min at 4°C. After the fractionation step, the supernatant comprising suspended cytoplasmic proteins may be recovered. A resulting pellet may optionally be washed with any suitable buffer known to the person skilled in the art, e.g. with a sonication buffer. The sonication buffer comprises exemplarily TrisHCl, NaCl, EDTA, EGTA, glycerol, NaVO₄ and NaF. Preferably, the sonication buffer comprises 50 mM TrisHCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30% glycerol, 0.4 mM NaVO₄ and 1 mM NaF. Subsequently, the pellet may be resuspended in any suitable buffer known to the person skilled in the art, e.g. in a Triton buffer, preferably in 1 ml of a Triton buffer comprising 1% (v/v) Triton in a sonication buffer as described herein above. The suspension may then be incubated in a shaker for a suitable period of time known to the skilled person, e.g. for 30 min at 4°C at 15 U/min. Subsequently, the suspension may again be centrifuged, e.g. at 108.000 x g for 15 min at 4°C. A resulting supernatant may be recovered as 'membrane fraction'. Subsequently, the resulting fractions may be precipitated by suitable means known to the skilled person, e.g. with trichloro acetic acid (TCA). For the detection of autopenetration and integration of compound(s) of the invention, a cytoplasmic and membrane fraction obtained by the method as described herein above may be analysed with any method known to the person skilled in the art, for example by way of immunostaining. Exemplarily, the fractions may be analysed by Western-blotting as known to the person skilled in the art and derivable, e.g., from Lottspeich and Zorbas, (Bioanalytik, 1998). The detection may be performed e.g. with an antiserum against the compound(s) of the invention.

A compound of the invention is regarded to be capable of autopenetrating the cell membrane and integrating into the cell cytosol without the requirement of additional factors if a tested molecule can be detected in the cytoplasmic fraction, either associated with vesicles as outlined above or already released in the cytosol, the latter being preferred. More preferably, a compound of the invention is regarded to be capable of autopenetrating the cell membrane and integrating into the cell cytosol without the requirement of additional factors if at least 10%, 20%, 30%, 40%, 50%, 60%, or 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% of the tested compound is detected in the cytoplasmic fraction, in comparison to the total amount of the compound of the invention added. Methods for quantification of the amount of protein uptake are known to the skilled artisan. It is envisaged that the compound of the invention, which is detected in the cytoplasmic fraction, is associated with vesicles as indicated above, and/or already released into the cytosol, the latter being preferred.

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Alternatively, the autopenetration of compound(s) of the invention into the cell membrane and its integration into the cell cytosol can be tested by a translocation coefficient assay as known to the person skilled in the art, for example as described in Langel, Ü. (ed) (*Cell-penetrating peptides: Processes and Applications*, CRC Press, Boca Raton, FL 2002,) and references therein. Briefly, a compound of the invention is linked to a suitable label, for example a dye like for example Cy3 or Cy5 or to gold particles, GFP, RFP etc.. Subsequently, a defined amount of the labelled protein is incubated with target cells, for example such as described herein. Afterwards, the cells are lysed and fractionated, for example such as described herein in the context of the method of cell fractionation. A translocation coefficient $K_T = [\text{protein of interest}_{\text{intracellular}}] / [\text{protein of interest}_{\text{extracellular}}]$ may be determined by measuring the amount of the label in the intracellular cell fractions [protein of interest_{intracellular}] and comparing it with the originally used amount for the incubation [protein of interest_{extracellular}], e.g. by determining the fluorescence of Cy3. Alternatively, ELISA methods may be used or further corresponding methods including e.g. radioactivity counting, biotinylation/cell-ELISA, fluorescence-labeling/spectrophotometer/FACS, resonance energy transfer, HPLC detection, immunodetection, fluorescence correlation microscopy (FCM), cell activity by capillary electrophoresis (CACE), or MALDI-TOF MS, as known to the skilled person, for example such as described in Langel, Ü. (ed) (*Handbook of cell-penetrating peptides*, CRC Press, Boca Raton, FL, 2007), und Langel, Ü. (ed) (*Cell-penetrating peptides: Processes and Applications*, CRC Press, Boca Raton, FL 2002).

20

The test for determining whether a molecule, in particular a compound of the invention, is capable of autopenetrating the cell membrane and integrating into the cell cytosol without the requirement of additional factors is preferably a test as described herein e.g. in the Examples.

25

Compounds of the invention preferably comprise at least one PTD of the PTDs marked in Example 7 and Figures 14 to 22 and designated as "maximal protein-transduction domain" and/or at least one PTD of the PTDs indicated in Example 9 and designated as "predicted PTD sequences". Figure 12 illustrates the PTDs of maximal length as marked in Example 7 and Figures 14 to 22. The in-silico prediction of Example 5 determined several PTDs encompassed within the maximal PTDs. These shorter PTDs corresponds to C-terminally or N-terminally truncated segments of the depicted "maximal protein-transduction domains". Compounds of the invention preferably comprise at least one PTD of these C-terminally or N-terminally truncated segments of the "maximal protein-transduction domains". However, since the PTDs denoted "maximal protein-transduction domains" in Figures 14 to 22 refer to predicted PTDs, it is possible that the effector protein comprise further functional PTDs that C- or N-terminally overlap the PTDs depicted as "maximal protein-transduction domains". Such PTDs, which are one, two, three, four or five amino acids longer than

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the “maximal protein-transduction domains”, either N-terminally or C-terminally, are therefore also encompassed by the present invention.

Example 9 indicates all protein transduction domains determined in Example 5, including the PTDs of maximal length and all shorter PTDs which correspond to C-terminally or N-terminally truncated segments of said PTDs of maximal length. Compounds of the invention preferably comprise at least one PTD denoted herein as “maximal protein-transduction domain” and/or at least one PTD described herein as “C-terminally or N-terminally truncated segments of a “maximal protein-transduction domain” and/or at least at least one PTD of the PTDs indicated in Example 9 and designated as “predicted PTD sequences”.

Compounds of the invention preferably comprise at least one amino acid sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65 or SEQ ID NO: 550, and/or at least one amino acid sequence that corresponds to a C-terminally or N-terminally truncated fragment of an amino acid sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65 or SEQ ID NO: 550.

Other Compounds of the invention preferably comprise at least one amino acid sequence selected from the group consisting SEQ ID NOs: 66 to 529.

“Truncated fragment” as used above refers to the amino acid sequences that are denoted in Example 9 as “Predicted PTD sequences”.

In a second embodiment the present invention provides a pharmaceutical composition, comprising an isolated variant, fragment, or immunomodulatory domain of an effector protein effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein said effector protein is characterized in that it

a) is recombinantly produced or chemically synthesized,

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- b) comprises an E3 ubiquitin ligase domain,
- c) optionally comprises at least one Leucin-rich repeat, and
- d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

5

The term "effector protein", as used herein, includes fragments of the effector proteins of the invention. A "fragment" of an effector protein of the invention is characterized as follows.

10 The term "fragment" relates to a sub-portion of the "effector protein" polypeptides according to the present invention. In particular, the term "fragment" refers to a short amino acid sequence contained in an effector protein according to the invention as described herein. Preferably "fragment" refers to a polypeptide that is characterized in that it

- a) is recombinantly produced or chemically synthesized,
- b) comprises an E3 ubiquitin ligase domain,
- 15 c) optionally comprises at least one Leucin-rich repeat, and
- d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

20 Furthermore, a "fragment" of an effector protein of the invention may correspond to a immunomodulatory domain of a effector protein of the invention. Likewise, a "fragment" of an effector protein of the invention may correspond to a protein transduction domain of an effector protein of the invention.

25 Protein fragments may be "free-standing", i.e. separated out of their natural environment (which is the effector protein, the fragment is derived from), or they may be attached to a polypeptide or comprised within a polypeptide of which the fragment forms a part or region, for example as a single continuous region. "Attached to" includes that the fragment and the polypeptide are expressed/expressable on/from a single nucleic acid as a single continuous region or that both entities are linked/coupled otherwise (for example by way of chemical linkage like

30 biotin/streptavidin etc.). Methods to "attach" two entities, in particular two proteins, are well-known to the skilled person. It is preferred that the polypeptide, to which the fragment of the invention is "attached to" or "comprised within", is heterologous to said fragment or the effector protein, the fragment is derived from respectively, i.e. it is preferably but not exclusively not derived from bacteria of the genus Salmonella or Shigella. Further characteristics of fragments of the invention

35 are described below.

Polynucleotides encoding fragments of the invention are also contemplated.

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The term “effector protein”, as used herein, includes variants of the effector proteins of the invention. A “variant” of an effector protein of the invention is characterized as follows.

In the context of the present invention a “variant” encompasses compounds of the, which
 5 comprise, or alternatively consist of, an amino acid sequence which is at least 40%, 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the polypeptide sequence or an effector protein of the invention, preferably SlrP, SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, or IpaH9.8, more preferably to the SlrP, SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, or IpaH9.8 polypeptide identified as SEQ ID NO: 1, SEQ ID NO:
 10 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9 and/or polypeptide fragments of any effector protein of the invention. Moreover, polynucleotides encoding these variants are also contemplated. Preferably “variant” refers to a polypeptide that is characterized in that it

- a) is recombinantly produced or chemically synthesized,
- 15 b) comprises an E3 ubiquitin ligase domain,
- c) optionally comprises at least one Leucin-rich repeat, and
- d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

20 Preferably “variants” of the invention are generated by deleting, inserting, inversing, repeating, modifying or substituting amino acids of effector proteins of the invention.

Further characteristics of fragments of the invention are described below.

25 The term “effector protein”, as used herein, includes fragments of the effector proteins of the invention, such as the immunomodulatory domain of an effector proteins of the invention. The term “immunomodulatory domain” or “immunomodulatory domain of the invention” refers to a domain of an effector protein of the invention that mediates modulation of pathway(s) of the innate immune system of eukaryotic cells. Preferably immunomodulatory domains of the invention modulates
 30 cytokines and/or cytokine receptors and/or genes which respond to cytokines of eukaryotic cells after said immunomodulatory domains have autonomously penetrated into said eukaryotic cells. More preferably immunomodulatory domains of the invention downregulate cytokines and/or cytokine receptors and/or genes which respond to cytokines of eukaryotic cells after said immunomodulatory domains have autonomously penetrated into said eukaryotic cells.

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Preferably immunomodulatory domains of the invention comprise an E3 ubiquitin ligase domain, preferably a NEL E3 ubiquitin ligase domain. Optionally immunomodulatory domains comprise at

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least one Leucin-rich repeat. Preferably immunomodulatory domains of the invention comprise an E3 ubiquitin ligase domain and at least one Leucin-rich repeat. Preferably an “immunomodulatory domain” polypeptide of the invention comprises an amino acid sequence selected from the group consisting of SEQ ID NO: (NELs und LRRs).

5

Preferably an immunomodulatory domain of the invention is characterized in that it

a) is recombinantly produced or chemically synthesized,

b) comprises an E3 ubiquitin ligase domain,

c) optionally comprises at least one Leucin-rich repeat, and

10

d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

An effector protein, variant, fragment, or immunomodulatory domain according to present invention is preferably a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

15

Hence pharmaceutical compositions of the invention preferably comprise an effector protein, variant, fragment, or immunomodulatory domain of the present invention that is preferably a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

20

An effector protein, variant, fragment, or immunomodulatory domain according to present invention preferably comprises an E3 ubiquitin ligase domain and optionally at least one Leucin-rich domain.

25

Hence pharmaceutical compositions of the invention preferably comprise an effector protein, variant, fragment, or immunomodulatory domain that comprises an E3 ubiquitin ligase domain and optionally at least one Leucin-rich domain.

An effector protein, variant, fragment, or immunomodulatory domain according to present invention preferably comprises an E3 ubiquitin ligase domain that is classified as Novel E3 Ligase.

30

Hence pharmaceutical compositions of the invention preferably comprise an effector protein, variant, fragment, or immunomodulatory domain of the present invention that preferably comprises a Novel E3 ubiquitin ligase domain.

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Pharmaceutical compositions of the invention preferably comprise an effector protein, variant, fragment, or immunomodulatory domain of the invention, which comprises a E3 ubiquitin ligase

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domain that is closer to the C-terminus of effector protein, variant, fragment, or immunomodulatory domain than to its N-terminus.

5 Pharmaceutical compositions of the invention preferably comprise an effector protein, variant, fragment, or immunomodulatory domain of the invention which comprises at least one Leucin-rich repeat of the LPX-subtype. Preferably said Leucin-rich repeat(s) is/are closer to the N-terminus of the effector protein, variant, fragment, or immunomodulatory domain of the invention than to its C-terminus. More preferably said Leucin-rich repeat(s) is/are mainly located within the N-terminal half of the compound of the invention.

10 Effector proteins according to present invention are preferably T3SS effector proteins of the LPX-subtype. Pharmaceutical compositions of the invention preferably comprise effector proteins according to the invention that belong to effector proteins of the LPX-subtype.

15 Effector proteins according to the present invention are preferably is SspH1, SspH2, SlrP, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, and IpaH9.8. Pharmaceutical compositions of the invention preferably comprise SspH1, SspH2, SlrP, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, and IpaH9.8.

20 Preferably an effector protein according to present invention has an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9. Hence pharmaceutical compositions of the invention preferably comprise an effector protein that has an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9.

25 Moreover pharmaceutical compositions of the invention preferably comprise an effector protein that is encoded by a polynucleotide selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, or SEQ ID NO: 18.

30 In the context of the present invention the terms "fragment", "variant" or "immunomodulatory domain" includes that the fragment, variant or immunomodulatory domain is biologically active. The term "biologically active" means that the fragment, variant or immunomodulatory domain has biological activities of the effector protein of the invention from which it is derived. Preferably a fragment, variant or immunomodulatory domain of the invention has a biological activity of SspH1, SspH2, SlrP, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, and IpaH9.8.

35 Preferably "biologically active" means that the fragment, variant or immunomodulatory domain of the invention is capability of autopenetrating the cell membrane and integrating into the cell cytosol

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without the requirement of additional factors and is able to modulates cytokines and/or cytokine receptors and/or genes which respond to cytokines.

5 An effector protein, variant, fragment, or immunomodulatory domain according to present invention preferably comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27.

10 An effector protein, variant, fragment, or immunomodulatory domain according to present invention preferably comprises at least one Leucine-rich repeat, preferably at least one Leucine-rich repeat that is comprised in an amino acid sequence selected from the group consisting of SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, or SEQ ID NO: 36.

15 An effector protein, variant, fragment, or immunomodulatory domain according to present invention preferably comprises at least one amino acid sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, or SEQ ID NO: 550, or are C-terminally or N-terminally truncated fragment thereof.

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25 Alternatively, an effector protein, variant, fragment, or immunomodulatory domain according to present invention preferably comprises at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 66 to 529.

30 Pharmaceutical compositions of the invention preferably comprise an effector protein, variant, fragment, or immunomodulatory domain as defined above.

Optionally effector proteins, variants, fragments, or immunomodulatory domains according to present invention are linked to a cell-specific targeting agent.

35 The compounds of the invention may enter eukaryotic cells without the need to interact with a receptor. Thus, in a further embodiment, the compound(s) of the invention is/are additionally linked to a specific, for example a cell-specific, targeting agent. Such a linkage may be any linkage as described herein above, preferably a peptide linkage.

The term "specific targeting agent" or "cell-specific targeting agent" means a molecule, which allows a (cell)specific interaction with structures on the surface of a cell and thereby facilitates the recognition of different cell types or tissue types in the animal body, preferably in the human body.

5 Molecules which allow such (cell-) specific interaction may, for example, be ligands which specifically interact with receptors or receptor fragments which are located on the surface of a cell (e.g. tumor cells) and which are differentially expressed in specific cell types or tissue types. The term includes any suitable CD antigen as known to the person skilled in the art, for example from (<http://www.pathologyoutlines.com/cdmarkers.html>) more preferably to CD1d, which may be used

10 for the targeting of, e.g., dendritic cells, intestinal epithelial cells, B cell subset, NK T cell subset; CD 11a,b,c,d; CD14 and CD16/18, which may be used for the targeting of, e.g., macrophages; CD23, which may be used for the targeting of e.g., activated mature B cells expressing IgM or IgD (particularly mantle cells), activated monocytes/macrophages, T cell subsets, platelets, eosinophils, Langerhans cells, follicular dendritic cells, or intestinal epithelium; CD54 (also known

15 as ICAM-1), which may be used for the targeting of, e.g., B and T cells and B cell precursors, monocytes, osteoclasts, endothelial cells, and various epithelial cells; CD57, which may be used for the targeting of, e.g., cells of the NK subset, T cell subset, neuroectodermal tissue, retina, brain, prostate, renal proximal tubules; CD64 (also called Fc gamma RI), which may be used for the targeting of antigen presenting cells including macrophages/monocytes, activated granulocytes,

20 dendritic cells or early myeloid cells; CD91 (also known as Low density lipoprotein receptor-related protein 1 (LRP1); also called alpha-2-macroglobulin receptor), which may be used for the targeting of fibroblasts, dendritic cells, macrophages, liver, brain or lung tissue as well as CD-20, CD-45. Furthermore, the term relates to anti-CD antibodies, to molecular danger signals, TLRs, bacterial toxins, e.g. 'trapo' for nerve cells as described in WO 2006/114308 or DEC-205, which is typically

25 present on dendritic cells. In addition, the term relates to a vascular-homing peptide, which may be specific for certain organs or tissues, like e.g. brain, kidney, lung, skin, or heart. More preferably, the term relates to such peptides as mentioned in Arap, W. et al. *Proc. Natl Acad Sci. U.S.A.*, 99:1527-1531 (2002); Rajotte, D. et al., *J. Clin Invest.*, 102:430-437 (1998); Pasqualini, R., and Ruoslahti, E. (2002) *Nat. Rev. Cancer* 2:83; Rajotte, D. and Ruoshlati, E., *J. Biol. Chem.*

30 274:11593-11598 (1999); Essler, M., and Ruoshlati, E., *Proc. Natl Acad. Sci. U.S.A.*, 99:2252-2257 (2002). Tumor homing peptide are also envisaged. The term "tumor homing peptide" means a protein, which comprises an RGD- and/or a NGR motif. Typically, proteins with a RGD motif bind to $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins, which in turn are considered to be specific markers for angiogenic blood vessels (Eliceiri, B.P. and Cheresh, D.A., *Cancer J.* 6:S245-S249 (2000)). Furthermore, proteins

35 with an NGR-Motif may bind to an aminopeptidase N, which in turn is specific for angiogenic, endothelia cells (Pasqualini, R. et al., *Cancer Res.* 60:722-727 (2000)). In a preferred embodiment, a tumor homing peptide comprising an RGD- and/or a NGR motif may be used for the general

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targeting of angiogenic cells indepently of the tumor type involved, as the person skilled in the art would know, e.g. from Arap, W. et al., *Science*, 279:377-380 (1998); Pasqualini, R. et al., *Nat. Biotech.* 15:542-546 (1997)).

In a further preferred embodiment compound of the invention may be fused in frame with the
 5 carboxyl terminus of the heavy chain of the α DEC-205 as described in Boscardin et al. (*J. Exp. Med.*, 203: 599-606 (2006)).

In a further embodiment the term "cell-specific targeting agent" includes agents which bind to (a)
 cell-marker which allow(s) the, preferably specific, targeting of osteoclasts. A particularly preferred
 10 cell-marker for osteoclasts is the calcitonin-receptor, alpha-V-beta3-integrine and/or vitronectine
 (Marta Monjo, Sébastien F. Lamolle, S. Petter Lyngstadaas, H. Jacob Rønold and Jan Eirik Ellingsen 2008 *Biomaterials* 29(28): 3771-3780; Susanne Granholm, Pernilla Lundberg, and Ulf H.
 J. Cell. Biochem. 104(3): 920 – 933; Davies J, Warwick J, Totty N, Philp R, Helfrich M, and Horton
 M 1989 *J. Cell Biol.* 109: 1817–1826; Clove J, Dodds R A, and Gowen M 1992 . *J. Cell Sci.* 103:
 15 267–271). Agents which may bind to this cell-marker are described herein and include for example
 antibodies etc.

In another embodiment the term "cell-specific targeting agent" relates to a virus, preferably an
 attenuated virus, which is linked to a compound of the invention. Such a combination may convey
 20 a cell or tissue tropism depending on the host cell spectrum of the virus used. The term "cell-
 specific targeting agent" also includes retroviridae, adenoviridae etc..

The term "cell-specific targeting agent" also includes an "antibody and functional fragments
 thereof" and refers to a monoclonal or a polyclonal antibody (see Harlow and Lane, "*Antibodies, A*
 25 *Laboratory Manual*", CSH Press, Cold Spring Harbor, USA, 1988) or a derivative of said antibody
 which retains or essentially retains its binding specificity. Preferred derivatives of such antibodies
 are chimeric antibodies comprising, for example, a mouse or rat variable region and a human
 constant region. The term "functional fragment" as used herein refers to fragments of the
 antibodies as specified herein which retain or essentially retain the binding specificity of the
 30 antibodies like, separated light and heavy chains, Fab, Fab/c, Fv, Fab', F(ab')₂. The term
 "antibody" also comprises bifunctional (bispecific) antibodies and antibody constructs, like single-
 chain Fvs (scFv) or antibody-fusion proteins. The term "scFv fragment" (single-chain Fv fragment)
 is well understood in the art and preferred due to its small size and the possibility to produce such
 fragments recombinantly. Said antibody or antibody binding portion is a human antibody or a
 35 humanized antibody. The term "humanized antibody" means, in accordance with the present
 invention, an antibody of non-human origin, where at least one complementarity determining region
 (CDR) in the variable regions such as the CDR3 and preferably all 6 CDRs have been replaced by

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CDRs of an antibody of human origin having a desired specificity. Optionally, the non-human constant region(s) of the antibody has/have been replaced by (a) constant region(s) of a human antibody. Methods for the production of humanized antibodies are described in, e.g., EP-A1 0 239 400 and WO90/07861.

5

Optionally effector proteins, variants, fragments, or immunomodulatory domains according to present invention are linked to a linked to a cargo molecule.

10

The term "linked to a cargo molecule" means that the cargo molecule may be connected by any means known to the person skilled in the art to the compounds of the invention (for example covalently, non-covalently etc.). It is envisaged that structures on the surface of the cargo molecule like functional or reactive chemical groups are used to establish a linkage or binding between a compound of the invention and a cargo molecule. "Linked to" also includes that the compounds of the invention and the cargo molecules are expressed/expressible on/from a single nucleic acid as a single continuous region. Fusion proteins consisting of a proteinaceous cargo (polypeptides, antibodies etc.) and the compound(s) of the invention are likewise contemplated. Nucleic acids encoding these fusion proteins, vectors comprising these nucleic acids and pharmaceutical compositions comprising these vectors or nucleic acids are likewise contemplated.

15

The compounds of the present invention may be linked to a cargo by any method known to the person skilled in the art, e.g. by chemical cross-linking, an avidin bridge, a glutathion-S-transferase bridge, a linkage comprising at least one, at least two or at least three disulfide bonds or at least one peptide bond or at least two peptide bonds.

20

Various functional groups, such as hydroxyl, amino or halogen groups present on the cargo may be used as a handle to attach a suitable complexing group. For example, a hydroxyl group may be modified to include an acidic phosphate group. It is also envisaged that the linkage includes a disulfide bond. The linkage may also include a streptavidin-biotin complex. It is envisaged that the delivery peptide, i.e. a compound of the invention is biotinylated and the cargo molecule is avidin labeled. Thus, "linked to" also includes a non-covalent linkage/association of cargo molecules with the compounds of the invention.

25

The linkage between the delivery peptide and the cargo may also be achieved by a peptide bond. Examples including those peptide bonds or linkers are described for example in U. S. Pat. No. 5,073,627.

30

In a preferred embodiment, the cargo is linked via a peptide bond in the form of a protein-protein fusion. In such a protein-protein fusion, the cargo may be separated from the compounds of the invention by an amino acid linker (spacer). Such a linker is preferably of the size of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 to 50 amino acids or of any other suitable size known to the person skilled in the art. The linker may consist of any suitable amino acid known to the person

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skilled in the art. Preferably, a linker comprising the amino acid glycine is used. The linker is not restricted to amino acids but may also comprise other entities/molecules, for example such as poly('hydroxy'methylene) groups.

In a further preferred embodiment, the protein-protein fusion may be in the form of a transcriptional fusion. Suitable transcriptional fusions, as well as suitable methods to generate corresponding constructs, are known to the person skilled in the art.

Furthermore, the compounds of the invention may be linked to a cargo by a cleavable linker.

It is envisaged that the cargo is modified using a number of methods known in the art, either directly, e.g. with a carbodiimide, or via at least one linking moiety. In particular, carbamate, ester, thioether, disulfide, and hydrazone linkages may be formed. Ester and disulfide linkages are envisaged, if the linkage is to be readily degraded in the cytosol, after transport of the cargo across the cell membrane.

In a further embodiment a compound of the invention as described herein above is linked to a cargo via a linkage at the C-terminus or the N-terminus of the compounds of the invention. Preferably, such a linkage at the C-terminus or the N-terminus is a peptide bond, more preferably said linkage is a protein-protein fusion including, for instance, the presence of a spacer or linker of the size of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16 to 50 amino acids or of any other suitable size known to the person skilled in the art. The linker may consist of any suitable amino acid known to the person skilled in the art. Preferably, a linker comprising the amino acid glycine is used.

In a further embodiment, the compound of the invention is biotinylated and the cargo molecule is avidin labeled or vice versa.

The term "cargo" or "cargo molecule", as used herein, refers to any entity (e.g. a small molecule, macromolecule or macromolecular complex), which may be delivered/transferred/is transferable across the membrane of a cell or into the cytosol of a target cell. It is envisaged that a "cargo" or "cargo molecule" is transferable/transferred or delivered across the membrane of a cell or into the cytosol of a target cell, if it is detected/detectable in the cytoplasmic fraction – either associated with vesicles and/or already released into the cytosol, the latter being preferred. The cytoplasmic fraction denotes the interior of a cell.

It is preferred but not exclusive that the "cargo" is heterologous to a compound of the invention. By the term "heterologous" as used herein is meant that it does not come from, is not normally/naturally produced by and/or required for viability of *Salmonella* or *Shigella*. It is also preferred that the cargo is not a component of a type III secretion system.

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It is particularly preferred that the cargo exerts a beneficial effect in a medical context, i.e. the cargo displays therapeutical and/or diagnostic activity/capabilities, following delivery into the cells, ex vivo and/or in vivo. "Therapeutic activity" includes treatment, amelioration and/or prophylaxis of a disease. "Diagnostic activity" includes visualizing, detecting, distinguishing and/or identifying a pathological/medical condition and attributing the deviation to a clinical picture.

Preferably, the term "cargo" includes, but is in no way limited to, a nucleic acid, a polypeptide, an antibody or a functional fragment thereof, an organic molecule, a small organic molecule, a metal, a nanoparticle, a virus, a modified virus, a viral vector, and/or a plasmid.

10

This invention is generally applicable for therapeutic, prophylactic, or diagnostic intracellular delivery of small molecules and of macromolecules, such as proteins, nucleic acids, and/or polysaccharides, that are not inherently capable of entering target cells at a useful rate. It should be appreciated, however, that alternate embodiments of this invention are not limited to clinical applications. This invention may be advantageously applied in medical and biological research. In research applications of this invention, the cargo may be e. g. a drug or a reporter molecule.

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Pharmaceutical compositions of the invention preferably comprise an effector protein, variant, fragment, or immunomodulatory domain as defined above.

20

Preferably effector proteins, variants, fragments, or immunomodulatory domains according to present invention are functional ubiquitin ligases. That means that an effector protein, variant, fragment, or immunomodulatory domain of the invention is able to ubiquitinate itself and/or eukaryotic proteins after it has autonomously penetrated into a eukaryotic cell in an isolated form and in the absence a T3SS-containing bacterium of the genus *Salmonella* or *Shigella*.

25

"Ubiquitinate" as used herein includes monoubiquitination, oligoubiquitination and polyubiquitination. Preferably effector proteins, variants, fragments, or immunomodulatory domains according to present invention polyubiquitinate themselves and/or eukaryotic proteins. Said eukaryotic proteins that are ubiquitinated are preferably involved in pathways of the innate immune system. More preferably said eukaryotic proteins are involved in the regulation or modulation of cytokines and/or cytokine receptors and/or genes which respond to cytokines of eukaryotic. Even more preferred said eukaryotic proteins are involved in the regulation of the expression of cytokines and/or cytokine receptors. In one preferred embodiment the compound of the invention is SspH1, or a variant, fragment or immunomodulatory domain thereof, wherein SspH1, or a variant, fragment or immunomodulatory domain thereof ubiquitinate itself and mammalian PKN1.

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Ubiquitin has seven lysine residues and an N-terminus that may serve as points of ubiquitination, they are K6, K11, K27, K29, K33, K48 and K63.

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Effector proteins, variants, fragments, or immunomodulatory domains according to present invention are preferably able to ubiquitinate by forming K63-linked-ubiquitin-chains and/or K48-linked-ubiquitin chains.

- 5 An effector proteins, variant, fragment, or immunomodulatory domain according to present invention preferably modulates pathway(s) of the innate immune system of eukaryotic cells after said effector proteins, variant, fragment, or immunomodulatory domain has autonomously penetrated into said eukaryotic cells.
- 10 Preferably an effector proteins, variant, fragment, or immunomodulatory domain according to present invention modulates cytokines and/or cytokine receptors and/or genes which respond to cytokines of a eukaryotic cell after said effector proteins, variant, fragment, or immunomodulatory domain has autonomously penetrated into said eukaryotic cell.
- 15 Optionally an effector proteins, variant, fragment, or immunomodulatory domain according to present invention downregulates the expression of cytokines and/or cytokine receptors of a eukaryotic cell after it has autonomously penetrated into said eukaryotic cell.

- 20 Pharmaceutical compositions of the invention preferably comprise an effector protein, variant, fragment, or immunomodulatory domain as defined above.

"Modulate" includes inducing, enhancing, or suppressing cellular pathway of the innate immune system.

- 25 As described above, the present inventors surprisingly found that recombinant SspH1, which so far has not been characterized as a potential immunosuppressive therapeutic, is, once it has integrated into the cell cytosol, capable of effectively downregulating cytokines.

- 30 Cytokines are an essential class of mediators in physiology and pathology. In the context of inflammation and disease cytokines and particularly pro-inflammatory cytokines play a key role in the acceleration and regulation of inflammatory reactions either by direct interactions or by their ability to induce the synthesis of cellular adhesion molecules or of other cytokines in various cell types involved in the immunological defense network. Many cytokines have beneficial as well as deleterious effects for the organism. Thus, a delicate balance between different cytokine groups, in particular between pro-inflammatory, anti-inflammatory and regulatory cytokines has to be maintained and is vital for health. If this balance is disturbed, diseases like inflammatory bowel disease, rheumatoid arthritis, vascular disease or autoimmunity may develop. The unexpected downregulation of cytokines, in particular of pro-inflammatory cytokines, by recombinant SspH1

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that has autonomously penetrated the eukaryotic cell membrane and entered the cytosol, as shown by the present inventors and illustrated in the Examples, converts SspH1 and its derivatives into efficient medical tools for the regulation of inflammatory reactions, for immunomodulation or especially for immunosuppression.

5

The term "cytokines" relates to soluble proteins and peptides that act as humoral regulators, which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues and also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. The term encompasses type 1 cytokines produced by Th1
 10 T-helper, type 2 cytokines produced by Th2 T-helper cells, interleukins, chemokines or interferons, e.g. IL-1ra (antagonist), CNTF, LIF, OSM, Epo, G-CSF, GH, PRL, IP10, I309, IFN-alpha, IFN-beta, IFN-gamma, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL11, IL12 (p35 + p40), IL13, IL14, IL15, IL16, IL17 A-F, IL18, IL19, IL20, IL21, IL22, IL23 (p19 + p40), IL24, IL25, IL26, IL27 (p28 – EBI3), IL28A, IL28B, IL29, IL30, IL31, IL32, IL33, IL35 (p35 – EBI3), LT-alpha, LT-beta, LIGHT, TWEAK,
 15 APRIL, BAFF, TL1A, GITRL, OX40L, CD40L, FASL, CD27L, CD30L, 4-1BBL, TRAIL, RANK, GM-CSF, M-CSF, SCF, IL1-alpha, IL1-beta, aFGF, bFGF, int-2, KGF, EGF, TGF-alpha, TGF-beta, TNF-alpha, TNF-beta, betacellulin, SCDGF, amphiregulin or HB-EGF, as is known to the person skilled in the art and can be derived, for example, from Tato, C.M. & Cua, D.J. (*Cell* 132: 900; *Cell* 132: 500, *Cell* 132: 324, (2008)) or from Cytokines & Cells Online Pathfinder Encyclopaedia
 20 (<http://www.copewith-cytokines.de>). "Pro-inflammatory cytokines" are also contemplated. The term "pro-inflammatory cytokine" means an immunoregulatory cytokines that favours inflammation. Typically, pro-inflammatory cytokines comprise IL-1-alpha, IL-1-beta, IL-6, and TNF-alpha. These pro-inflammatory cytokines are largely responsible for early responses. Other pro-inflammatory mediators include LIF, IFN-gamma, IFN-alpha, OSM, CNTF, TGF-beta, GM-CSF, TWEAK, IL-11,
 25 IL-12, IL-15, IL-17, IL-18, IL-19, IL-20, IL-8, IL-16, IL-22, IL-23, IL-31, and IL-32 (Tato, C.M. & Cua, D.J. *Cell* 132:900; *Cell* 132:500, *Cell* 132, 324 (2008)). These pro-inflammatory cytokines may act as endogenous pyrogens (IL-1, IL-6, TNF-alpha), up-regulate the synthesis of secondary mediators and pro-inflammatory cytokines by both macrophages and mesenchymal cells (including fibroblasts, epithelial and endothelial cells), stimulate the production of acute phase proteins, or
 30 attract inflammatory cells. Preferably, the term "pro-inflammatory cytokine" relates to TNF-alpha, IL-15, IFN-gamma, IFN-alpha, IL-1-beta, IL-8, IL-16 and IL-22.

The term "downregulates" means that the mRNA levels of an expressed gene, e.g. of a cytokine gene, and/or the protein levels expressed by such mRNAs is reduced in the presence of
 35 compound(s) of the invention. The downregulation of mRNA and/or protein expressed by that mRNA in the context of the compounds of the invention can be tested and determined by methods known to a person skilled in the art or by methods exemplified in the appended examples.

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“Downregulating” encompasses that the expression (either on mRNA or protein level) is reduced by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% in comparison to a control, in which the incubation has been carried out without compound(s) of the invention, e.g. solely in the presence of medium. It can be tested
 5 by a method of quantitative RNA analysis, as described in Myers, T.W. and Gelfand, D.H., *Biochem.* 30:7661-7667 (1991);, Krug, M.S. and Berger, S.L., *Methods Enzymol.* 152:316-325 (1987);, Bustin, S.A., *J. Mol. Endocrinol.* 29:169-193 (2000); Bustin, S.A., *J. Mol. Endocrinol.* 25:23-39 (2002); Ståhlberg, A. et al., *Clin. Chem.* 50:509-515 (2004)).

10 A compound of the invention is preferably capable of downregulating pro-inflammatory cytokines. More preferably, it is capable of downregulating any one of pro-inflammatory cytokines described above, preferably TNF-alpha, IL-15, IFN-alpha, IL-1-beta, IL-8, IL-16 and/or IL-22. The term “downregulating” has been described herein above. The downregulation may be tested in a RNA quantification assay or a test known to the person skilled in the art, for example a test as described
 15 in the Examples.

The term “cytokine receptor” refers to any receptor molecule, which is able to bind a cytokine as a ligand. In the context of the present invention, the term preferably relates to any receptor of the cytokines mentioned herein above, more preferably of the pro-inflammatory cytokines described
 20 herein above. The term “downregulates” has been described herein above. The downregulation may be tested in a RNA quantification assay or test known to the person skilled in the art and/ or by a method as described in the Examples.

In a further preferred embodiment the compounds of the invention are capable of downregulating
 25 cytokines, cytokine receptors and/or genes which respond to cytokines i.e. the compounds of the invention comprise in this embodiment the immunomodulatory domain(s) of compounds of the invention, particularly E3 ligase domain(s) and optionally also at least one leucine-rich repeat (LRR), i.e. one, two, three, four, five, six, seven or eight LRRs. The addition of further LRRs is also envisaged. It is likewise envisaged that these compounds of the invention are linked to/attached to
 30 a cargo molecule.

The term “genes which respond to cytokines” refers to any gene, which is regulated, i.e. can be activated or inactivated, or whose transcription can be initiated or stopped by any of the cytokines mentioned herein above. More preferably, it relates to genes that are regulated by TNF-alpha or
 35 IFN. Most preferably, the term relates to genes that are induced by TNF-alpha or induced by IFN. The term “downregulates” has been described herein above. The downregulation may be tested in a RNA quantification assay or test known to the person skilled in the art.

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Pharmaceutical compositions of the invention preferably comprise an effector protein, variant, fragment, or immunomodulatory domain as defined above.

- 5 For modulation of cellular pathway of the innate immune system of a eukaryotic cell of a subject compounds of the invention preferably penetrate into the nucleus of a eukaryotic cell after autopenetration.

- Hence an effector proteins, variant, fragment, or immunomodulatory domain according to present
10 invention optionally penetrates into the nucleus of a eukaryotic cell after said effector protein, variant, fragment, or immunomodulatory domain has autonomously entered said eukaryotic cell.

- Moreover pharmaceutical compositions of the invention optionally comprise an effector proteins, variant, fragment, or immunomodulatory domain of the invention that penetrates into the nucleus of
15 a eukaryotic cell after said effector protein, variant, fragment, or immunomodulatory domain has autonomously entered said eukaryotic cell.

- The term “penetrating the nucleus of a eukaryotic cell” or “entering the cell nucleus” means that a compound of the invention passes across the nuclear membrane of a cell. The capability of a
20 compound of the invention to enter the cell nucleus can be tested by any suitable methods and assays known to the person skilled in the art, preferably by nuclear localization assays as described in Hällbrink M., et al., (2004) (Biochem. Biophys. Acta 1667:222) and Nare B., et al., (1999) (Anal. Biol. 267:390).

- Preferably, the capability of a compound of the invention to enter the cell nucleus is linked to the
25 presence of a nuclear localization sequence (NLS). More preferably, a compound of the invention comprises the NLS of said compound of the invention as known to the person skilled in the art. In a preferred embodiment compound(s) of the invention used in the context of pharmaceutical compositions comprise a NLS sequence.

- 30 Preferably pharmaceutical compositions of the invention lack any auxiliary agent which can cause the penetration of said effector protein into a eukaryotic cell. “Auxiliary agent” refers to any molecule that is able to deliver a cargo molecule into a eukaryotic cell. “Auxiliary agent” does not refer to compounds of the invention. Within the present invention auxiliary agents include but are not limited to non-bacterial cell-penetrating peptides, YopM, YopM fragment and YopM variants,
35 and cell-penetrating nanoparticles. Within the present invention auxiliary agent also refers to functional bacterial secretion systems, preferably to functional T3SS.

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More preferably pharmaceutical compositions of the invention lack any auxiliary agent selected from the group consisting of T3SS-containing bacterium, bacterium of the genus *Salmonella* or *Shigella*, and cell-penetrating molecule, including cell-penetrating protein that is different from an effector protein of the invention and cell-penetrating nanoparticle.

5

Pharmaceutical compositions of the invention preferably comprise an effector protein, variant, fragment, or immunomodulatory domain as defined above.

10 In one embodiment pharmaceutical compositions of the invention are for use in immunomodulatory therapy of animals or humans. Immunomodulatory therapy thereby refers to the treatment of disease by inducing, enhancing, or suppressing an immune response.

In another embodiment pharmaceutical compositions of the invention are for use in immunosuppressive therapy of animals or humans.

15

In another embodiment pharmaceutical compositions of the invention are for use in the regulation of inflammatory reactions of the immune system, treatment of diseases caused by autoimmunity, acute inflammation or chronic inflammation, treatment of inflammatory disorders, and/or for suppressing the immune system.

20

In one aspect the present invention relates to a pharmaceutical composition as defined herein above for immunomodulation, preferably for immunomodulation of inflammatory reactions. The term "immunomodulation" means regulation of reactions of the immune system. The term "immunomodulation of inflammatory reactions" refers to the regulation of inflammatory reactions of the immune system. Such inflammatory reactions are known to the skilled person and can be derived, for example, from Schmidt-Schönbein (*Annu. Rev. Biomed. Eng.* 8: 93–151 (2006)).

25 In a preferred embodiment the pharmaceutical composition as defined herein above is for the treatment of diseases caused by autoimmunity of the host. The term "diseases caused by autoimmunity of the host" means a disease, which is caused by an immune reaction of the host's immune system. Such diseases are known to the person skilled in the art and can be derived, for example, from <http://www.sbi.uni-rostock.de/aidb/home.php>. Preferably, the term relates to acute disseminated encephalomyelitis (ADEM), Addison's disease, ankylosing spondylitis, antiphospholipid antibody syndrome (APS), aplastic anemia, autoimmune hepatitis, autoimmune Oophoritis, celiac disease, Crohn's disease (Morbus Crohn), diabetes mellitus type 1, gestational pemphigoid, goodpasture's syndrome, Graves' disease, Guillain-Barré syndrome (GBS), Hashimoto's disease, idiopathic thrombocytopenic purpura, Kawasaki's disease, lupus erythematosus, Mixed Connective Tissue Disease, multiple sclerosis, myasthenia gravis,

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opsoclonus myoclonus syndrome (OMS), optic neuritis, Ord's thyroiditis, pemphigus, pernicious anaemia, primary biliary cirrhosis, rheumatoid arthritis, Reiter's syndrome, Sjögren's syndrome, Takayasu's arteritis, temporal arteritis, Warm autoimmune hemolytic anemia and Wegener's granulomatosis.

5

In a further preferred embodiment the pharmaceutical composition as defined herein above is for the treatment of "inflammation". The term "inflammation" means a biological response of tissues, e.g. vascular tissues, to harmful stimuli, such as pathogens, damaged cells, or irritants. Such a pathological condition is known to the person skilled in the art and can be derived, for example, from Schmidt-Schönbein (*Annu. Rev. Biomed. Eng.* 8: 93–151 (2006)). Preferably, the term relates to acute inflammation or chronic inflammation. Furthermore, it encompasses inflammatory disorders like asthma, chronic prostatitis, glomerulonephritis, hypersensitivities, inflammatory bowel diseases, pelvic inflammatory disease, reperfusion injury, arthritis, osteoarthritis, (juvenile) chronic arthritis, rheumatoid arthritis, psoriatic arthritis, *A. mutilans*, septic arthritis, infectious arthritis and/or reactive arthritis, transplant rejection or vasculitis. It also encompasses allergic reactions, inflammatory myopathies, atherosclerosis, ischaemic heart disease, gastroenteritis, chronic gastritis, colitis ulcerosa and psoriasis or psoriasis arthritis.

More preferably, the pharmaceutical composition is for the regulation of inflammatory reactions of the immune system, the treatment of diseases caused by autoimmunity of the host, the treatment of inflammation, chronic inflammation, gastroenteritis, chronic gastritis, inflammatory bowel diseases (IBD), colitis ulcerosa, psoriasis, allergic reactions, Morbus Crohn, arthritis, osteoarthritis, (juvenile) chronic arthritis, rheumatoid arthritis, psoriatic arthritis, *A. mutilans*, septic arthritis, infectious arthritis and/or reactive arthritis or for suppressing the immune system.

In another aspect, the present invention relates to the use of compounds of the invention for the preparation of a pharmaceutical composition for immunomodulation of inflammatory reactions, the regulation of inflammatory reactions of the immune system, the treatment of diseases caused by autoimmunity of the host, and/or the treatment of inflammation, chronic inflammation, gastroenteritis, chronic gastritis, inflammatory bowel diseases (IBD), colitis ulcerosa, psoriasis, allergic reactions, Morbus Crohn, arthritis, osteoarthritis, (juvenile) chronic arthritis, rheumatoid arthritis, psoriatic arthritis, *A. mutilans*, septic arthritis, infectious arthritis, and/or reactive arthritis, or for suppressing the immune system.

In another embodiment the present invention relates to a method of treatment of any of the diseases and medical conditions mentioned herein, in particular in the context of the pharmaceutical composition as defined herein, comprising administering at least one compound of the invention, including all modifications as indicated herein (e.g linked cargo; linked cell specific targeting) to a subject. Preferably, the present invention relates to a method of preventing,

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ameliorating and/or treating diseases which were exemplified herein elsewhere. Preferably, the subject to be treated is an animal and more preferably, the subject to be treated is a human being.

Preferably the present invention provides a method of treatment of diseases caused by autoimmunity, treatment of acute inflammation, chronic inflammation, inflammatory disorders, pathogenic inflammatory reactions of the immune system, and/or a method of suppressing the immune system in a subject, comprising administering a therapeutically effective amount of a pharmaceutical composition of the invention to said subject.

The term "therapeutical amount" is described elsewhere herein.

10

The present invention also provides kits or pharmaceutical packages that can be used in the context of the present invention, for example in the context of administration of the pharmaceutical composition. In one embodiment, a kit/package comprises at least one compound of the invention as defined herein, in one or more containers. Optionally, the kit/package further comprises a documentation indicating the treatment regimen, use and/or employment of the kits/package components or the pharmaceutical composition.

15

In one aspect, the present invention relates to the use of an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it

20

- a) is recombinantly produced or chemically synthesized
- b) comprises an E3 ubiquitin ligase domain, preferably an Novel E3 ubiquitin ligase
- c) optionally comprises at least one Leucin-rich repeat;
- d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS;

25

or use of a variant, fragment or immunomodulatory domain of said effector protein, for the preparation of a pharmaceutical composition for regulating inflammatory reactions of the immune system, treating diseases caused by autoimmunity, treating acute inflammation or chronic inflammation, treating of inflammatory disorders, and/or suppressing the immune system.

30

In another aspect, the present invention relates to the use of an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it

- a) is recombinantly produced or chemically synthesized
- b) comprises an E3 ubiquitin ligase domain, preferably an Novel E3 ubiquitin ligase
- c) optionally comprises at least one Leucin-rich repeat;

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d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS;

or use of a variant, fragment of said effector protein for the preparation of a pharmaceutical composition for delivering at least one cargo molecule across the membrane of a eukaryotic cell, wherein the fusion construct of cargo molecule and said effector protein or variant or fragment thereof is a cell-penetrating construct, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

"Cargo molecule" is described above.

10

The term "delivering a cargo molecule" means that said isolated compound of the invention is capable of transporting and delivering a cargo molecule as defined herein, e.g. peptides or proteins, DNA, RNA, carbohydrates, lipids or chemically devised molecules of natural or non-natural origin without accessory factors into higher cells.

15

Compounds of the invention also include polypeptides which comprise non-classical amino acids including D-isomers of the common amino acids, 2,4-diaminobutyric acid, α -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ -Abu, ϵ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoroamino acids, designer amino acids such as β -methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In the context of the present invention the term "compounds of the invention" also includes polypeptides which are differentially modified during or after translation, e. g. by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, or proteolytic cleavage etc.. Any of numerous chemical modifications may be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, acetylation, formylation, oxidation, reduction; or metabolic synthesis in the presence of tunicamycin ; etc..

Additional post-translational modifications encompassed by the invention include, for example N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends, attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of prokaryotic host cell expression.

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The present invention also relates to polypeptides of the invention (compounds of the invention). In one aspect the invention provides an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella* or a variant, fragment or immunomodulatory domain of said effector protein, wherein the compound of the invention is artificially modified. "Artificially modified" refers to man-made modifications, such as labeling, chemical modification or derivatization as described below. By way of example, the invention relates to an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella* or a variant, fragment or immunomodulatory domain of said effector protein, wherein said effector protein, variant, fragment or immunomodulatory domain is linked to a cargo molecule and/or cell-specific targeting agent.

Compounds of the invention may also be modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein, the addition of epitope tagged peptide fragments (e. g., His, FLAG, HA, GST, thioredoxin, maltose binding protein, etc.), attachment of affinity tags such as biotin and/or streptavidin, the covalent attachment of chemical moieties to the amino acid backbone, N-or C-terminal processing of the polypeptides ends (e. g., proteolytic processing), deletion of the N-terminal methionine residue, etc.

Compounds of the invention encompass chemically modified derivatives that may provide additional advantages such as increased solubility, stability, and circulating time of the polypeptide altogether increasing its half-life, or decreased immunogenicity (see U.S. Patent NO: 4,179,337). The chemical moieties for derivatization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethyl cellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties. Preferably, a chemical derivatization is contemplated wherein the chemical is a hydrophilic polymer residue. Exemplary hydrophilic polymers, including derivatives, may be those that include polymers in which the repeating units contain one or more hydroxy groups (polyhydroxy polymers), including, for example, poly (vinyl alcohol); polymers in which the repeating units contain one or more amino groups (polyamine polymers), including, for example, peptides, polypeptides, proteins and lipoproteins, such as albumin and natural lipoproteins; polymers in which the repeating units contain one or more carboxy groups (polycarboxy polymers), including, for example, carboxymethyl cellulose, alginic acid and salts thereof, such as sodium and calcium alginate, glycosaminoglycans and salts thereof, including salts of hyaluronic acid, phosphorylated and sulfonate derivatives of carbohydrates, genetic material, such as interleukin-2 and interferon, and phosphorothioate oligomers; and polymers in

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which the repeating units contain one or more saccharide moieties (polysaccharide polymers), including, for example, carbohydrates.

The molecular weight of the hydrophilic polymers may vary, and is generally about 50 to about 5,000,000, with polymers having a molecular weight of about 100 to about 50,000 being preferred.

5 The polymers may be branched or unbranched. More preferred polymers have a molecular weight of about 150 to about 10,000, with molecular weights of 200 to about 8,000 being even more preferred.

For polyethylene glycol, the preferred molecular weight is between about 1 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh
10 more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e. g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog). Additional preferred polymers, which may be used to derivatize the compounds of the invention,
15 include, for example, poly(ethylene glycol) (PEG), poly(vinylpyrrolidone), polyoxomers, polysorbate and poly(vinyl alcohol), with PEG polymers being particularly preferred. Preferred among the PEG polymers are PEG polymers having a molecular weight from about 100 to about 10,000. More preferably, the PEG polymers have a molecular weight of from about 200 to about 8,000, with PEG 2,000, PEG 5,000 and PEG 8,000, which have molecular weights of 2,000, 5,000 and 8,000,
20 respectively, being even more preferred. Other suitable hydrophilic polymers, in addition to those exemplified above, are known to the person skilled in the art. Generally, the polymers used may include polymers that can be attached to the polypeptides of the invention via alkylation or acylation reactions.

The polyethylene glycol molecules (or other chemical moieties) should be attached to the
25 compounds of the invention with consideration of effects on functional or antigenic domains of the protein. There are a number of attachment methods available to those skilled in the art, e.g. derivable from EP 0 401 384. For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as, a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid
30 residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. Preferred for therapeutic purposes is attachment at an amino group, such as attachment at the N-terminus or lysine group. One may
35 specifically desire proteins chemically modified at the N-terminus.

Using polyethylene glycol as an illustration of the present composition, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of

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polyethylene glycol molecules to protein (polypeptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein.

5 The method of obtaining the N-terminally pegylated preparation (i. e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules.

Selective proteins chemically modified at the N-terminus modification may be accomplished by reductive alkylation that exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminus) available for derivatization in a particular protein. Under the
10 appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved.

As with the various polymers exemplified above, it is contemplated that the polymeric residues may contain functional groups in addition, for example, to those typically involved in linking the polymeric residues to the compound of the invention in accordance with the present invention.
15 Such functionalities include, for example, carboxyl, amine, hydroxy and thiol groups.

In addition to residues of hydrophilic polymers, the chemical used to derivatize the polypeptides of the present invention can be a saccharide residue. Exemplary saccharides which can be derived include, for example, monosaccharides or sugar alcohols, such as erythrose, threose, ribose, arabinose, xylose, lyxose, fructose, sorbitol, mannitol and sedoheptulose, with preferred
20 monosaccharides being fructose, mannose, xylose, arabinose, mannitol and sorbitol; and disaccharides, such as lactose, sucrose, maltose and cellobiose. Other saccharides include, for example, inositol and ganglioside head groups. Other suitable saccharides, in addition to those exemplified above, will be readily apparent to one skilled in the art based on the present disclosure. Generally, saccharides that may be used for derivatization include saccharides that can
25 be attached to the polypeptides of the invention via alkylation or acylation reactions.

Moreover, the invention also encompasses derivatization of the compounds of the invention, for example, with lipids (including cationic, anionic, polymerized, charged, synthetic, saturated, unsaturated, and any combination of the above, etc.) or stabilizing agents. Preferably, the present
30 invention encompasses derivatization of compound of the invention with compounds that may serve a stabilizing function (e.g., to increase the polypeptides half-life in solution, to make the polypeptides more water soluble, to increase the polypeptides hydrophilic or hydrophobic character, etc.). Polymers useful as stabilizing materials may be of natural, semi-synthetic (modified natural) or synthetic origin. Exemplary natural polymers include naturally occurring
35 polysaccharides, such as, for example, arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans (such as, for example, inulin), levan, fucoidan, carrageenan, galatocarlose, pectic acid, pectins, including amylose, pullulan, glycogen, amylopectin, cellulose,

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dextran, dextrin, dextrose, glucose, polyglucose, polydextrose, pustulan, chitin, agarose, keratin, chondroitin, dermatan, hyaluronic acid, alginic acid, xanthin gum, starch and various other natural homopolymer or heteropolymers, such as those containing one or more of the following aldoses, ketoses, acids or amines: erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, 5 glucose, dextrose, mannose, gulose, idose, galactose, talose, erythrulose, ribose, xylulose, psicose, fructose, sorbose, tagatose, mannitol, sorbitol, lactose, sucrose, trehalose, maltose, cellobiose, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, glucuronic acid, gluconic acid, glucaric acid, galacturonic acid, mannuronic acid, glucosamine, galactosamine, and neuraminic acid, and naturally occurring 10 derivatives thereof. Accordingly, suitable polymers include, for example, proteins, such as albumin, polyalginates, and polylactide-coglycolide polymers. Exemplary semi-synthetic polymers include carboxymethylcellulose, hydroxymethyl-cellulose, hydroxypropylmethylcellulose, methylcellulose, and methoxycellulose. Exemplary synthetic polymers include polyphosphazenes, hydroxyapatites, fluoro-apatite polymers, polyethylenes (such as, for example, polyethylene glycol, polyoxyethylene, 15 and polyethylene terephthalate), polypropylenes (such as, for example, polypropylene glycol), polyurethanes (such as, for example, polyvinyl alcohol (PVA), polyvinyl chloride and polyvinylpyrrolidone), polyamides including nylon, polystyrene, polylactic acids, fluorinated hydrocarbon polymers, fluorinated carbon polymers (such as, for example, polytetrafluoroethylene), acrylate, methacrylate, and polymethyl-methacrylate, and derivatives 20 thereof. Methods for the preparation of derivatized polypeptides of the invention which employ polymers as stabilizing compounds will be readily apparent to one skilled in the art, in view of the present disclosure, when coupled with information known in the art, such as that described and referred to in U. S. Pat. No. 5,205,290.

25 The "type III secretion system" (T3SS) is a complex molecular injection machine of many pathogenic gram-negative bacteria (see e.g. Cornelis GR et al., 2006; Büttner D et al., 2006). According to phylogenetic differences in amino acid sequences, T3S systems from animal- and plant-pathogenic or symbiotic bacteria have been classified into different families, including flagellar T3SS, Ysc, Inv-Mxi-Spa, Ssa-Esc, Hrp1, and Hrp2 T3S systems as well as T3SS of the 30 Chlamydiales and Rhizobiales families (Büttner D et al., 2006). Within the present invention the term "T3SS" encompasses all classes of type III secretion systems.

Pathogenic bacteria use the needle-like structure of the T3SS as a sensory probe to detect the presence of eukaryotic organisms and to secrete proteins, called effector proteins directly from the bacterial cell into the eukaryotic host cell. Within the present invention T3SS-containing bacteria 35 include *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Escherichia* spp., *Pseudomonas* spp. and *Chlamydia* spp.. These species include *Salmonella bongori*, *Salmonella enterica*, *Salmonella subterranean*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella enterica* serovar *typhimurium*, *Salmonella enteritidis*, *Salmonella pullorum*, *Salmonella dublin*, *Salmonella arizonae*,

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Salmonella choleraesuis, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella sonnei*, *Shigella boydii*, *Yersinia pestis*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Enteropathogenic Escherichia coli* strains (EPEC), *Pseudomonas aeruginosa*, *Pseudomonas syringae*. Subspecies of species above listed are also encompassed within the present invention.

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Salmonellae are described to contain two type III secretion systems, which are encoded by two distinct gene clusters termed SPI-1 and SPI-2 (for *Salmonella* pathogenicity island) (Hueck CJ, 1998). T3SS-containing *Salmonella* spp. refer herein to *Salmonella* spp. containing a T3SS encoded by SPI-1 and to *Salmonella* spp. containing a T3SS encoded SPI-2.

10

The term "cell" or "eukaryotic cell" means any type of isolated eukaryotic cell, cells in the context of a living organism or in tissue as well as isolated cells/tissues in cell cultures (for example HeLa cells, T84 cells, HL60 cells or XS52 cells etc.). Preferably, the term relates to higher eukaryotic cells, more preferably to animal cells, even more preferably to mammalian cells and most preferred to human cells. Epithelial cells, fibroblasts (for example synovial fibroblasts – see Example 9), primary cells, endothelial cells (for example human intestinal microvascular endothelial cells HIMEC – see Example 8), cells of the immune system like monocytes, dendritic cells, macrophages and/or NK cells are also envisaged.

15

Whether any particular polypeptide is at least 40%, 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the sequence of a compound of the invention as described herein can be determined by any means known to the person skilled in the art, e.g. by using conventionally known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the CLUSTALW computer program (Thompson, J. D., et al., *Nucleic Acids Research*, 2 (22): 4673-4680, (1994)), which is based on the algorithm of Higgins, D. G., et al., *Computer Applications in the Biosciences* (CABIOS), 8 (2): 189-191, (1992). The result of said global sequence alignment is in percent identity. Preferred parameters used in a CLUSTALW alignment of protein sequences to calculate percent identity via pairwise alignments are: Matrix=Gonnet, k-tuple=1, Number of Top Diagonals=5, Gap Penalty=3, Gap Open Penalty 10, Gap Extension Penalty=0. 1, Scoring Method=Percentage, Window Size=5 or the length of the subject polypeptide sequence, whichever is shorter. The pairwise and multiple alignment parameters provided for CLUSTALW above represent the default parameters as provided with the AlignX software program (Vector NTI suite of programs).

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The present invention encompasses the application of a manual correction to the percent identity results, in the instance where the subject sequence is shorter than the query sequence because of

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N-terminal or C-terminal deletions, not because of internal deletions. If only the local pairwise percent identity is required, no manual correction is needed.

However, a manual correction may be applied to determine the global percent identity from a global polypeptide alignment. Percent identity calculations based upon global polypeptide alignments are often preferred since they reflect the percent identity between the polypeptide molecules as a whole (i. e., including any overhangs, not just overlapping regions), as opposed to, only local matching polypeptides. Manual corrections for global percent identity determinations are required since the CLUSTALW program does not account for N-terminal or C-terminal truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the N-terminal or C-terminal ends, relative to the query sequence, the percent identity is corrected by calculating the number of amino acids of the query sequence that are N-terminal or C-terminal of the subject sequence, which are not matched/aligned, as a percent of the total amino acids of the query sequence. Whether an amino acid is matched/aligned is determined by results of the CLUSTALW sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above CLUSTALW program using the specified parameters, to arrive at a final percent identity score. This corrected score may be used for the purposes of the present invention. In addition to the above method of aligning two or more polypeptide sequences to arrive at a percent identity value for the aligned sequences, it may be desirable in some circumstances to use a modified version of the CLUSTALW algorithm which takes into account known structural features of the sequences to be aligned, such as for example, the SWISS-PROT designations for each sequence. The result of such a modified CLUSTALW algorithm may provide a more accurate value of the percent identity for two polypeptide sequences. Support for such a modified version of CLUSTALW is provided within the CLUSTALW algorithm and would be readily appreciated to a person skilled in the art.

25

Polynucleotides encoding compound(s) of the invention may contain alterations in the coding regions, non-coding regions, or both. For example, the polynucleotides encoding compound(s) of the invention may contain alterations, which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded polypeptide. Nucleotide variants produced by silent substitutions due to the degeneracy of the genetic code are preferred.

30

Compounds of the invention are preferably produced recombinantly, for example, by any suitable method known to the person skilled in the art. The present invention, thus, also encompasses methods for the production of polypeptides of the invention. Accordingly, the present invention contemplates polynucleotides encoding polypeptides of the invention and vectors containing said polynucleotides, host cells comprising those polynucleotides and/or vectors, and the production of polypeptides of the invention by recombinant techniques. A suitable vector may be, for example, a phage, plasmid, viral, or retroviral vector. Retroviral vectors may be replication-competent or

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replication-defective. In the latter case, viral propagation generally will occur only in complementing host cells.

Polynucleotides encoding polypeptides of the invention may be joined to a vector containing a selectable marker for propagation in a host.

- 5 As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include, for instance, dihydrofolate reductase, G418, or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin, or ampicillin resistance genes for culturing in *E. coli* and other bacteria.

- 10 Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells (e.g., *Saccharomyces cerevisiae* or *Pichia pastoris*); insect cells such as *Drosophila melanogaster* S2 and *Spodoptera frugiperda* Sf9 cells; animal cells such as CHO, COS, HEK 293, and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above described host cells are known in the art.

- 15 Vectors preferred for use in bacteria are known to the person skilled in the art.

Introduction of the construct into the host cell can be effected by methods described in many standard laboratory manuals, such as Davis et al., (*Basic Methods In Molecular Biology* (1986)).

A polypeptides of the invention can be recovered and purified from recombinant cell cultures by any suitable method known to the person skilled in the art.

- 20 Polypeptides of the invention can also be recovered from: products purified from natural sources, including bodily fluids, tissues and cells, whether directly isolated or cultured; products of chemical synthetic procedures; and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect, and mammalian cells.

- 25 In addition, polypeptides of the invention can be chemically synthesized using techniques known in the art (Creighton, 1983, *Proteins: Structures and Molecular Principles*, W. H. Freeman & Co., N. Y., and Hunkapiller et al., *Nature*, 310: 105-111 (1984)). For example, a polypeptide corresponding to a fragment of a polypeptide sequence of the invention can be synthesized by use of a peptide synthesizer.

30

It is envisaged that pharmaceutical compositions of the invention comprise one or more compounds of the invention which are further modified as explained herein, for example which are linked to a cargo molecule and/or linked to a cell-specific targeting agent. It is likewise envisaged that the pharmaceutical compositions of the present invention comprise the nucleic acids and/or

35 polynucleotides of the present invention. The pharmaceutical composition may optionally comprise a pharmaceutically acceptable carrier.

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A pharmaceutical composition according to the present invention may be administered with the help of various delivery systems known to the person skilled in the art, e. g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e. g., Wu and Wu, J. Biol. Chem. 262: 4429-4432 (1987)),
 5 construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The composition may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e. g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other
 10 biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compounds or compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g.,
 15 by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e. g., in
 20 conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a protein of the invention, care must be taken to use materials to which the protein does not absorb.

25 A preferred method of local administration is by direct injection. Preferably, the compounds of the present invention as well as the nucleic acids/vectors of the present invention is/are complexed with a delivery vehicle to be administered by direct injection into or locally within the area of arteries.

For systemic administration, compounds of the invention can be complexed to a targeted delivery
 30 vehicle.

In another embodiment the pharmaceutical composition may be delivered directly to disease sites in internal organs, body cavities and the like by use of imaging devices used to guide an injecting needle directly to the disease site. The pharmaceutical composition may also be administered to disease sites at the time of surgical intervention.

35 In another embodiment, the pharmaceutical composition can be delivered in a vesicle, in particular a liposome (Langer, R., Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy

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of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327).

- In yet another embodiment, the composition can be delivered in a controlled release system. In one embodiment, a pump may be used (Sefton, *CRC Crit. Ref. Biomed. Eng.* 14: 201 (1987);
- 5 Buchwald et al., *Surgery* 88: 507 (1980); Saudek et al., *N. Engl. J. Med.* 321: 574 (1989)). In another embodiment, polymeric materials can be used (see *Medical Applications of Controlled Release*, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); *Controlled Drug Bioavailability, Drug Product Design and Performance*, Smolen and Ball (eds.), Wiley, New York (1984) ; Ranger and Peppas, J., *Macromol. Sci. Rev. Macromol. Chem.* 23: 61 (1983); see also
- 10 Levy et al., *Science* 228: 190 (1985); During et al., *Ann. Neurol.* 25: 351 (1989); Howard et al., *J. Neurosurg.* 71: 105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i. e., the brain, thus requiring only a fraction of the systemic dose (see, e. g., Goodson, in *Medical Applications of Controlled Release*, *supra*, vol. 2, pp. 115-138 (1984)).
- 15 Preferably the pharmaceutical composition is in a form, which is suitable for oral, local or systemic administration. In a preferred embodiment the pharmaceutical composition is administered locally, orally or systemically.

- The term "pharmaceutically acceptable" means approved by a regulatory agency or other generally
- 20 recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such a carrier is pharmaceutically acceptable, i.e. is non-toxic to a recipient at the dosage and concentration employed. It is preferably isotonic, hypotonic or weakly hypertonic and has a relatively low ionic strength, such as provided by a sucrose solution. Examples of suitable
- 25 pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilised powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent.

- 30 Assays, e.g. those described in the Examples, may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances.
- 35 It is envisaged that pharmaceutical compositions of the invention comprise the compounds of the invention in a therapeutically effective amount.

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The term "administered" means administration of a therapeutically effective dose of the aforementioned composition. By "therapeutically effective amount" is meant a dose that produces the effects for which it is administered, preferably this effect is the downregulation of cytokines, and/or cytokine receptors and/or genes which respond to cytokines. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. As is known in the art and described above, adjustments for systemic versus localized delivery, age, body weight, general health, sex, diet, time of administration, drug interaction and the severity of the condition may be necessary, and will be ascertainable with routine experimentation by those skilled in the art.

10 The pharmaceutical composition may be used in both human therapy and veterinary therapy. The compounds described herein having the desired therapeutic activity may be administered in a physiologically acceptable carrier to a patient, as described herein. The concentration of the therapeutically active compound in the formulation may vary from about 0.01-100 wt %. The agent may be administered alone or in combination with other treatments.

15 A typical dose can be, for example, in the range of 0.001 to 1000 µg; however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors.

It is also envisaged that the pharmaceutical compositions are employed in co-therapy approaches, i.e. in co-administration with other medicaments or drugs, for example other immunosuppressive drugs.

20 Topical administration of the pharmaceutical composition of the present invention is useful when the desired treatment involves areas or organs readily accessible by topical administration. For a topically application, e.g. to the skin, the pharmaceutical composition is preferably formulated with a suitable paste, ointment, lotion, cream, gel or transdermal patches.

In a further embodiment compound(s) of the invention provided in the form of a living therapeutic. The term "living therapeutic" means that said compound(s) of the invention, for example as defined in the context of the pharmaceutical composition, is/are expressed in a live carrier. Accordingly, the present invention relates to polynucleotides encoding compounds of the invention as defined herein above which are suitable for expression in a living cell or carrier. The term "live carrier" relates to any appropriate living host cell or virus known to the person skilled in the art. Representative examples of appropriate hosts include, but are not limited to, bacterial cells such as *Escherichia coli* or *Lactobacillus*, fungal cells, such as yeast cells, protozoa, insect cells, or animal cells. Preferably, the term relates to attenuated bacteria, attenuated fungal cells or attenuated protozoa.

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The present invention also relates to the use of a compound of the invention for the manufacture of a pharmaceutical composition. It is envisaged that compound(s) of the invention are linked/attached to a cargo molecule and/or a cell-specific targeting molecule as described herein. The mentioned pharmaceutical composition is for use in any of the diseases described herein.

5

The terms "Leucin-rich repeat(s)" and "Leucin-rich domain(s)" are used herein interchangeably.

The terms "cell-penetrating peptide", "CPP", "cell-penetrating protein", "cell-permeable peptide", "cell-penetrating protein" and are used herein interchangeably.

10

The terms "protein transduction domain" (PTD) and "cell-penetrating entity" are used herein interchangeably.

15

"Autopenetration" or "cell-penetration" as used herein describes the capability of compounds of the invention to penetrate the cell membrane and to enter the cytosol of a cell without the assistance of exogenous factors, such as a T3SS.

20

The terms "autopenetrating", "autonomously penetrating" and "penetrate/pass/cross the membrane of eukaryotic cells without the assistance of exogenous factors, such as a T3SS" are used herein interchangeably.

The terms "amino acid sequence" and "polypeptide sequence" are used herein interchangeably.

25

It is to be understood that this invention is not limited to the particular methodology, protocols, proteins, bacteria, vectors, reagents etc. described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention that will be limited only by the appended claims.

30

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.), whether supra or infra, is hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

35

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the", include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a polypeptide" includes one or more of such polypeptides, and reference to "the

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method” includes reference to equivalent steps and methods known to those of ordinary skill in the art that could be modified or substituted for the methods described herein.

DESCRIPTION OF THE DRAWINGS

5 **Figure 1** shows a schematic overview of the recombinant SspH1 and its derivative SspH1-Nter. SspH1-Nter lacks the C-terminal domain of SspH1 which encodes an ubiquitin ligase. Proteins were tagged with a C-terminal 6xHis-tag (not shown) for purification.

10 **Figure 2** shows an immunoblot analysis of subcellular fractionations of HeLa cells incubated with 25 µg/ml SspH1 and SspH1-Nter, respectively. In order to assess their purity, membrane and cytosolic fractions were analyzed using antibodies against transferrin receptor (TF-R) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively (left panel). Purity of cytoplasmic and nuclear fractions was determined using antibodies against tubulin and Lysine-specific demethylase 1 (LSD-1), respectively (right panel). SspH1 and SspH1-Nter partition was assessed using anti-FLAG antibody. CF: cytoplasmic fraction MF: membrane fraction NF: nuclear fraction.

20 **Figure 3** shows an immunofluorescence microscopy of HeLa cells incubated with SspH1-Cy3 and SspH1-Nter-Cy3 1 h. DNA was stained with DAPI. Both fluorescence images were merged.

Figure 4 shows a quenched time lapse assay of HeLa cells incubated with YopM-AlexaFluor488, SspH1- AlexaFluor 488 or Tat-GFP-GSK. HeLa cells were incubated with 20 µg/ml protein. Samples were taken from the incubation at different time points. Extracellular fluorescence was quenched with trypan blue (final concentration: 0,2 %) and intracellular fluorescence was measured using a flow cytometer. Data are expressed as geometric means of fluorescence intensities from at least 10,000 cells relative to background fluorescence of untreated cells, and are presented as means ± standard deviations from three independent experiments.

30 **Figures 1 to 4** show that SspH1 and SspH1-Nter translocate into the host cell cytoplasm independently of Salmonella's T3SS.

Figure 5 shows a Membrane integrity assay of HeLa cells incubated with SspH1, YopM and Tat, respectively. Cells were incubated with 20µg/ml protein and co-incubated with 1 µg/ml propidium iodide (PI).

35 **Figure 6** shows Recombinant SspH1 and SspH1-Nter do not have cytotoxic effects on Hela cells. Cells were incubated with SspH1 and SspH1-Nter, respectively, for 1, 6, and 24h and analysed using the CytoTox96® Non-Radioactive Cytotoxicity Assay. LDH: lactate dehydrogensase.

Figure 7 shows *in vitro* ubiquitination assays which were performed in the presence of UbcH5c. Shown are synthesized unanchored polyubiquitin chains detected by anti-ubiquitin western blot. Upon incubation with E1, E2 and ubiquitin, SspH1 was able to remove ubiquitin from the E2
5 enzyme and perform self-ubiquitination

Figure 8 shows that recombinant SspH1 ubiquitinates PKN1 *in vitro*. Shown are immunoblot analysis using anti-HA, anti-PKN1, and anti-His antibodies of reactions performed in the presence of Ub-Ha, E1, E2, Ub-Ha, SspH1, and PKN1. Upon incubation with E1, E2 and ubiquitin, SspH1
10 was able to remove ubiquitin from the E2 enzyme and ubiquitinate PKN1.

Figure 9 shows that recombinant SspH1 co-immunoprecipitates with PKN1 from transfected cells and self-ubiquitinates. HeLa cells were transiently transfected with PKN1-Myc. 24 h later, cells were incubated with FLAG-tagged SspH1 for 3 h. Following SspH1 incubation, PKN1 was
15 immunoprecipitated and the immunoprecipitates and supernatants were immunoblotted using anti-FLAG and anti-Ubiquitin antibodies.

Figure 10 shows the effect of SspH1 on IL-8 mRNA in A549 cells stimulated with IL1 β . A549 cells were incubated with SspH1 for 3 h and subsequently stimulated with 20 ng/ml IL1 β . After RNA
20 isolation and cDNA synthesis, all qRT-PCR data were obtained using SYBR green and IL8-specific primers. The HPRT1 gene (hypoxanthine phosphoribosyl-transferase I), a low abundance housekeeping gene, was used as reference. Data represent means and standard deviations of at least three independent experiments each performed in duplicate * $p < 0.05$.

25 **Figures 6 to 10** show that recombinant SspH1 possesses E3 ubiquitin ligase activity.

Figure 11a to 11e shows an uptake analysis of effector proteins of the LPX family. Upper panel: Confocal laser scanning immunofluorescence microscopy of HeLa cells incubated with IpaH1.4-Cy3, IpaH4.5-Cy3, IpaH7.8-Cy3, IpaH9.8-Cy3, and SlrP-cy3 for 1 h. Actin was counterstained with
30 phalloidin AlexaFluor488 and nuclei with DRAQ5. All three fluorescence images were merged and confocal Z-stack projections are included in all images. The cross hairs show the position of the xy and yz planes. Scale bars: 10 μ m. Lower panel: Quenched time-lapse assay of HeLa cells incubated with different AlexaFluor 488 labeled effector proteins (20 μ g/ml). Samples were taken from the incubation at different time points. Extracellular fluorescence was quenched with trypan
35 blue (final concentration: 0.2 %) and intracellular fluorescence was measured using a flow cytometer. Data are expressed as geometric means of fluorescence intensities from at least

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10,000 cells relative to fluorescence of untreated cells, and are presented as means $\pm$ standard deviations from three independent experiments.

Figure 12 shows a schematic overview of an *in-silico* prediction analysis YopM, SspH1, SspH2, SlrP, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, and IpaH9.8. Boxes indicate sequence segments containing predicted PTD sequences (PTDs of maximal length).

Figure 13. Figure 13a shows an in vitro ubiquitination assay of recombinant SspH1. Upon incubation with E1, E2 and ubiquitin, SspH1 is able to remove ubiquitin from the E2 enzyme and to self-ubiquitinate (see also Figure 7). In the presence of PKN1, SspH1, but not SspH1-Nter, polyubiquitinates the kinase (Figure 13a). Figure 13b shows I κ B mRNA expression in A549 cells following incubation with SspH1 and subsequent stimulation with TNF α .

Figures 14 to 22 shows amino acid sequences of SlrP, SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8 and IpaH9.8. Leucin-rich repeats (called "LRR stretch") are marked in light grey and are non-edged. E3 Ubiquitin ligase domains are marked in dark grey and are edged. Sequence sections within which the protein transduction domains (PTDs) are predicted are bold and underlined. Marked are the maximal sequence sections within the PTD is predicted. PTDs can correspond to the marked sequence sections or to C-terminally and/or N-terminally truncated fragments of the marked sequence sections.

Figure 23 shows a cell fractionation of HeLa cells incubated with recombinant IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, and IpaH9.8, respectively.

Western blot analysis after cell fractionation of HeLa cells which were incubated with the indicated LPX effector protein (25 μ g/ml) for 3 h. Proteins were separated by 12.5 % SDS-PAGE and immobilized on a nitrocellulose membrane by Western blotting. Proteins were detected using an α FLAG-antibody and corresponding PO-conjugated secondary antibody. Purity of both the cytoplasmic (CF) and membrane fraction (MF) were assessed using an α -GAPDH-antibody and α TF-R-antibody and corresponding PO-conjugated secondary antibodies.

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Figure 24 shows a confocal fluorescence microscopy of HeLa cells incubated with recombinant IpaH1.4, IpaH4.5, IpaH7.8, IpaH9.8, or SlrP, concerning co-localization with the endocytic markers Rab5, Rab7, and CD63. HeLa cells were incubated with the indicated recombinant, Cy3-labeled (red) LPX effector proteins (25 μ g/ml) for 1 h (i), 3 h (ii), and 6 h (iii), respectively (A)-(F). The endocytic markers Rab5, Rab7, and CD63 were detected using appropriate primary antibodies and stained via incubation with Cy2-labelled (green) secondary antibodies. DNA was stained using

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Draq5 (blue). Overlays of all three channels are shown in large squares; pictures of the individual channels are shown on the right. Magnification 63x.

Figure 25 shows a flow cytometry analysis of effector proteins of the LPX family. HeLa cells were with endocytic inhibitors cytochalasin D (2.5 μ M), amiloride (3 mM), filipin (7.5 μ M), nocodazole (20 μ M), dynasore (80 μ M), methyl- β -cyclodextrin (5 mM) for 1 h prior to the addition of recombinant LPX effectors. Three hours later the cells were washed with D-PBS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$), trypsinized, resuspended in D-PBS (without $\text{Ca}^{2+}/\text{Mg}^{2+}$), diluted with trypan blue (final concentration 0.2 %) and analyzed by flow cytometry

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Figure 26 shows a membrane integrity assay of HeLa cells incubated with recombinant IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH9.8, or SlrP. HeLa cells were co-incubated with both the indicated LPX effector protein (25 μ g/ml) and propidium iodide (PI) (1 μ g/ml) at 37°C or 4°C for a total duration of 6 h and 3 h, respectively, (A)-(F). Samples were taken from the ongoing incubation at given time points and subjected to FACS analysis. Fluorescence of PI was detected at 617 nm. All samples were measured twice and three independent experiments were carried out for each protein. (A)-(F): Diagrams show the fluorescence intensity of HeLa cells co-incubated with the indicated Alexa Fluor 488-labeled LPX effector protein and PI at different incubation times both at 37°C and 4°C in comparison to HeLa cells which were solely incubated with PI (dashed lines). The geometric mean fluorescence [arbitrary units] (normalized to negative/untreated control cells) is plotted against the incubation time [minutes].

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Figure 27 shows the relative release of LDH of HeLa cells upon incubation with recombinant IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH9.8, or SlrP. HeLa cells were incubated with the indicated LPX effector protein (25 μ g/ml) for a total duration of 24 h, 6 h, and 1 h, respectively, (A)-(F). The release of LDH was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay. Diagrams show the relative amount of released LDH normalized to the amount releases by non-treated (Medium control) which was set equal to 1. Additionally, a LDH positive control was measured. LDH: Lactate dehydrogenase.

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Figure 28 shows an in vitro ubiquitination assay using either recombinant IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH9.8, or SlrP. Western blot analysis of in vitro ubiquitination assays which were performed in 40 μ l reaction mixtures containing (+) or not containing (-) the indicated protein (4 μ g), HA-tagged ubiquitin (2 μ g), E2 (UbcH5b)(2 μ g), and E1 (0.5 μ g). Reactions were stopped by the addition of 4 x SDS sample buffer without DTT. Samples were subjected to 10 % SDS-PAGE and immobilized on a Nitrocellulose membrane by Western blotting. Ubiquitin was detected

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using an α -HA antibody and a corresponding PO-conjugated secondary antibody. Different Western blots are separated by dashed lines.

DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO: 1: Amino acid sequence of SlrP from *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. 14028S

SEQ ID NO: 2: Amino acid sequence of SspH1 from *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. 14028S

SEQ ID NO: 3: Amino acid sequence of SspH2 from *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. 14028S

SEQ ID NO: 4: Amino acid sequence of IpaH1.4 from *Shigella flexneri*

SEQ ID NO: 5: Amino acid sequence of IpaH2.5 from *Shigella flexneri*

SEQ ID NO: 6: Amino acid sequence of IpaH3 from *Shigella flexneri*

SEQ ID NO: 7: Amino acid sequence of IpaH4.5 from *Shigella flexneri*

SEQ ID NO: 8: Amino acid sequence of IpaH7.8 from *Shigella flexneri*

SEQ ID NO: 9: Amino acid sequence of IpaH9.8 from *Shigella flexneri*

SEQ ID NO: 10: Nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 1

SEQ ID NO: 11: Nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 2

SEQ ID NO: 12: Nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 3

SEQ ID NO: 13: Nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 4

SEQ ID NO: 14: Nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 5

SEQ ID NO: 15: Nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 6

SEQ ID NO: 16: Nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 7

SEQ ID NO: 17: Nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 8

SEQ ID NO: 18: Nucleotide sequence encoding the amino acid sequence shown in SEQ ID NO: 9

SEQ ID NOs: 19 to 27: Ubiquitin ligase domains of SEQ ID NOs: 1 to 9 as indicated in Figures 14-22

SEQ ID NOs: 28 to 36: Leucine rich repeat stretches or areas of comprising Leucine rich repeat stretches SEQ ID NOs: 1 to 9 as indicated in Figures 14-22

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SEQ ID NOs: 37 to 65 and 550: Maximal protein-transduction domains of SEQ ID NOs: 1 to 9 as indicated in Figures 14-22

SEQ ID NOs: 66 to 529: Protein-transduction domains of SEQ ID NOs: 1 to 9 as determined in Example 5 and indicated in Example 9

SEQ ID NOs: 530 to 549: Oligonucleotide sequences as indicated in Table 3.3

EXAMPLES

The following examples are for illustrative purposes only and are not intended to limit the scope of the claims.

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EXAMPLE 1

SspH1 can translocate into the host cell cytoplasm independently of Salmonella's T3SS

The present inventors constructed and recombinantly expressed SspH1, an LPX effector protein of *Salmonella enterica* serovar *typhimurium*. Potential T3SS-independent translocation of this effector protein was analyzed by cell fractionation of HeLa cells, immunofluorescence microscopy, and FACS.

To enable the expression and purification of SspH1, recombinant proteins tagged with a C-terminal 6xHis-tag were constructed. The C-terminal region of SspH1 encodes an ubiquitin ligase that might interfere with potential T3SS-independent uptake of SspH1 due to size and structure. Therefore, a truncated derivative of SspH1 was constructed in addition to the full length SspH1 protein. The N-terminal construct SspH1-Nter comprises the N-terminal α -helical domain as well as the Leucine-rich repeats (LRRs), lacking the enzymatic domain (Fig. 1). In order to initially analyse whether the recombinant proteins SspH1 and SspH1-Nter are able to translocate into the cytoplasm of eukaryotic host cells, cell fractionation of HeLa cells was performed.

The results of the cell fractionation indicate that recombinant SspH1 is able to translocate across eukaryotic plasma membranes in a T3SS-independent manner and is taken up into the host cell cytoplasm. Furthermore, the N-terminal construct of SspH1 (SspH1-Nter), comprising the N-terminal α -helical domain and the LRRs, was also detected in the cytoplasmic fraction after 1 h of incubation of HeLa cells with the recombinant protein (Fig. 2). Internalization of the N-terminal construct indicates that T3SS-independent uptake of recombinant SspH1 depends on the N-terminal domain of the protein. The enzymatic C-terminal domain of SspH1 does not seem to interfere with membrane penetration. During infection, T3SS-translocated SspH1 has been shown to localize to the host cell nucleus (Haraga & Miller, 2003). In order to analyze localization of recombinant SspH1 to the nucleus, nuclear fractionation was performed. SspH1 as well as SspH1-Nter were detected in the nuclear fraction of HeLa cells after 1 h of incubation (Fig. 2), indicating

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that recombinant SspH1 is able to translocate to the nucleus following internalization by a yet unknown mechanism.

The uptake of recombinant SspH1 was further characterized by immunofluorescence microscopy of HeLa cells incubated with the recombinant protein. In order to allow detection of SspH1 and SspH1-Nter by immunofluorescence microscopy, the proteins were labeled with the fluorescent dye Cy3 (GE Healthcare, Braunschweig). Both proteins, Cy3-labeled SspH1 and SspH1-Nter are clearly detected in the HeLa cell cytoplasm indicating uptake of SspH1 (Fig. 3). The proteins show no obvious difference in their intracellular localization. Both proteins are equally distributed throughout the cytoplasm following internalization (Fig. 3).

For quantitative analysis of cell-penetration by SspH1, a flow cytometry-based quenched time-lapse assay was performed and compared to uptake efficiencies of YopM and the Tat-GFP-GSK fusion protein (Fig. 4). The results indicate a significantly higher uptake of the bacterial proteins YopM and SspH1 compared to the Tat-GFP-GSK fusion protein. After 3 h of incubation, the relative fluorescence intensity of cells incubated with AF488-labeled SspH1 and YopM, respectively is about 3.5 to 4 times higher than of cells incubated with Tat-GFP-GSK. These results correlate with the data obtained from fluorescence microscopy, where less Tat-GFP-GSK was detected within the cells compared to YopM.

Taken together, these experiments show that both bacterial effector proteins YopM and SspH1 are efficient cell-penetrating effector proteins (CPE), which share the ability to enter host cells independently of T3SS.

EXAMPLE 2

Recombinant SspH1 Affects Membrane Integrity but is not Cytotoxic

HeLa cells were incubated with the recombinant proteins SspH1-AF488, YopM-AF488 and Tat-GFP-GSK and co-incubated with 1 µg/ml PI during the ongoing incubation (Fig. 5). Samples were taken from the culture at defined time points and directly subjected to FACS analysis. In addition to fluorescence of PI, the fluorescence of the recombinant proteins was measured to monitor uptake of the peptides. As a negative control, HeLa cells were incubated with PI but without addition of recombinant proteins. Untreated control cells were used to select viable cells for data acquisition before the samples were measured in triplicate with 10,000 events per measurement.

In order to monitor potential peptide-induced membrane lysis, the fluorescence intensity of PI of cells incubated with the recombinant proteins YopM-AF488, SspH1-AF488 and Tat-GFP-GSK, respectively, was measured. Membrane disruption is indicated by an increase of PI fluorescence due to increasing permeability of the plasma membrane for PI. Cells incubated with the

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recombinant proteins were compared to control cells which were also incubated with PI but not with the proteins.

Incubation of HeLa cells with SspH1 results in the highest increase of PI fluorescence indicating massive membrane disruption by the recombinant protein. The PI fluorescence intensity of cells incubated with SspH1 is 4 to 5 times higher during the course of incubation compared to the control cells. However, SspH1-induced membrane disruption does not seem to lead to cell lysis since only viable cells were detected for data acquisition. Cells incubated with the bacterial CPP YopM and the well described Tat peptide show an approximately 2-fold increase in PI fluorescence intensity compared to the control cells. These data indicate some effect on the membrane integrity by YopM and Tat though compared to SspH1, the effects are much lower. However, both proteins have been suggested to be internalised by endocytic uptake mechanisms which per se do not imply disruption of the plasma membrane. Thus, the present results indicate that accumulation of both proteins at the plasma membrane might cause destabilisation of the plasma membrane. Whether the observed membrane disruption leads to a potentially direct uptake of the peptides cannot be concluded from the present results. As for cells incubated with SspH1, effects of YopM and Tat-GFP-GSK do not lead to complete cell lysis as cells were still detected as viable.

In order to further exclude that the observed effect of SspH1 on membrane integrity result from cell lysis, a cytotoxicity assay was performed (Fig. 6). Therefore, HeLa cells were incubated with 25 – 50 µg/ml SspH1 and SspH1-Nter, respectively for 1, 6 and 24 h. Cytotoxicity was measured using the CytoTox96® Non-Radioactive Cytotoxicity Assay (Promega). In this assay, the amount of lactate dehydrogenase (LDH) released by the cells into the supernatant is measured. An increase in LDH release indicates cytotoxic effects of the proteins. Following incubation with SspH1 and SspH1-Nter, no increase in LDH release was observed when compared to untreated control cells even after 24 h of incubation, indicating that the proteins do not have any cytotoxic effects on the HeLa cells.

The strong effects of SspH1 on the membrane integrity of HeLa cells with no effects on cell viability, suggest a potential direct uptake mechanism by the formation of a transient membrane pore.

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EXAMPLE 3

Functionality of the bacterial effector protein SspH1

SspH1 belongs to the family of proteins containing the LRR motif and comprises 8 LRRs. The C-terminal domain of the protein resembles that of the type III secreted IpaH proteins from *Shigella flexneri* which possess E3 ubiquitin ligase activity. Ubiquitin ligases mediate the transfer of ubiquitin to target proteins. Ubiquitination is a process generally occurring in all eukaryotic cells that is involved in protein degradation, signal transduction as well as cell cycle regulation. It has

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been shown that the C-terminal domain of SspH1 is indeed an E3 ubiquitin ligase domain (Quezada et al, 2009) that uses ubiquitin as well as protein kinase 1 (PKN1) as substrates for ubiquitination (Rohde et al, 2007). Furthermore, it has been suggested that this interaction is involved in downregulation of expression of pro-inflammatory cytokines by SspH1.

5 To test if recombinant SspH1 is also functional and could polyubiquitinate proteins, in vitro ubiquitination assays were carried out in a 40 µl reaction mixture containing ubiquitination buffer (25 mM Tris•HCl [pH 7.5], 50 mM NaCl, 5 mM ATP, 10 mM MgCl₂, 0.1 mM DTT), 2 µg Ha-ubiquitin, 0.5 µg E1, and 2 µg E2 (UbcH5B) in the presence or absence of 1 µg recombinant SspH1. Ubiquitination analysis of PKN1 was performed in the presence or absence of additional
10 0.4 µg GST-tagged PKN1. Reactions were incubated at 37°C for 1 h and stopped by the addition of Laemmli sample buffer with or without 100 mM DTT.

Western blot analysis showed that also recombinant SspH1 has the ability to remove ubiquitin from ubiquitinated UbcH5B, to autoubiquitinate, and to polyubiquitinate Ha-tagged ubiquitin (Fig. 7). Additionally, the inventors also tested ubiquitination of PKN1 by SspH1. When
15 the reaction was performed in the presence of SspH1 and PKN1 in vitro, anti-PKN1 antibodies detected an additional species migrating at a size >148 kDa (Fig. 8). Furthermore, the inventors wanted to confirm interaction of SspH1 with PKN1 and its Ubiquitination also in vivo (Fig. 9). Therefore, HeLa cells were transiently transfected with a PKN1 expression vector and subsequently incubated with recombinant FLAG-tagged SspH1 and Ssph1-Nter, respectively. The
20 proteins were then immunoprecipitated with anti-PKN1-agarose antibody conjugate. As shown in figure 9, Ssph1-FLAG co-immunoprecipitated with PKN1. Furthermore, using anti-ubiquitin antibodies, the inventors could confirm self-ubiquitination of SspH1 in HeLa cells.

To determine whether cytokine production is also down-regulated by recombinant SspH1, the levels of IL-8 mRNA of A549 cells after stimulation with IL1β in the presence or absence of
25 SspH1 were analyzed. As shown in Fig. 10, cells which have been pre-incubated with SspH1 for 3 h produced significantly less IL-8 mRNA upon IL-1β stimulation than untreated cells.

Taken together, the present inventors showed that the SspH1 effector protein of *Salmonella enterica serovar typhimurium* is able to translocate into eukaryotic cells without a requirement for
30 additional factors. Furthermore, the inventors demonstrated that recombinant SspH1 is functional as an E3 ubiquitin ligase that uses PKN1 as substrate after penetrating the host cells and that is able to reduce the expression of Interleukin 8 in IL1β stimulated cells, but not in TNFα-stimulated cells (Figure 13b)

EXAMPLE 4

35 **Analysis of further effector proteins of the LPX family**

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The inventors identified SspH1 as a bacterial cell-penetrating protein. Subsequently the inventors investigated whether there is a general concept of T3SS-independent translocation by LPX effector proteins. For this, the effector proteins of the IpaH subset from *Shigella flexneri* as well as SlrP from *Salmonella enterica serovar typhimurium* have been cloned with a C-terminal 6xHis-tag for
 5 purification. After purification, the different effector proteins were labeled with the fluorescent dyes Cy3 (IF) or AlexaFluor488 (FACS) for subsequent uptake analysis by immunofluorescence microscopy and FACS (Fig. 11a-e).

Fluorescence microscopy analysis revealed that the effector proteins of the IpaH subset (IpaH1.4-
 10 Cy3, IpaH4.5-Cy3, IpaH7.8-Cy3, IpaH9.8-Cy3), as well as Cy3-labeled SlrP have entered cells after 1 h incubation (Fig. 11a-e, upper panel). Although the intracellular amount of the different effector proteins appeared to differ, all proteins are distributed throughout the cytoplasm and show only little difference in their intracellular localization (Fig. 11a-e, upper panel). Additionally, after the proteins have been conjugated to an AlexaFluor 488 fluorescent dye, a flow cytometry based
 15 quenched time lapse assay was performed for quantitative analysis of uptake efficiency (Fig. 11a-e, lower panel). In all cases, an increase of relative fluorescence intensity was monitored for HeLa cells after incubation with the recombinant proteins, indicating efficient internalization. The increase of fluorescence intensities appeared different for the shown effector proteins. However, comparison of the curves over the entire time course shows a considerable increase of intracellular
 20 fluorescence intensity for incubation times of up to 180 min for IpaH1.4, IpaH4.5, IpaH7.8, IpaH9.8, and SlrP respectively, indicating an efficient internalization (Fig. 11a-e, lower panel).

In summary, the experiments of the inventors demonstrate that the LPX-family from *S. flexneri* and *S. enterica serovar typhimurium* share the ability of YopM and SspH1 to enter the cytosol
 25 independently of the T3SS. These results strengthen the hypothesis of a new general concept for internalization of these effector proteins.

EXAMPLE 5

In-silico prediction of cell-penetrating properties of LPX family members

To identify putative protein transduction domains (PTD) within the amino acid sequences of the
 30 LPX family members, the inventors used *in-silico* prediction of cell-penetrating properties based on a method developed by Stephen White's laboratory shareware (<http://blanco.biomol.uci.edu>, (Jaysinghe S., 2009). This approach utilizes the Wimley-White hydrophobicity scales (White & Wimley, 1999), reflecting the ability of binding and insertion into lipid bilayers. Hence, available amino acid sequences of SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, IpaH9.8,
 35 SlrP, YopM were screened for putative PTD with the help of CEPEP company (Ülo Langel, Schweden). The results of this screen are described in Example 5 and summarized in Figure 12.

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The computational analysis of the YopM amino acid sequence from *Y. enterocolitica* (pYV8081) identified a PTD within the N-terminal domain of the protein (Fig. 12). This sequence overlaps with the previous experimentally identified transport domain of YopM (Rüter et al., 2010), indicating that the *in-silico* prediction of cell-penetrating properties is a usable tool to identify potential PTDs within the amino acid sequences of CPEs. Several unknown PTDs have been predicted for the rest of the family members. Obviously, all effector proteins of the IpaH subset share two potential PTDs in their C-terminal region (Fig. 12). Furthermore, IpaH4.5 harbors two additional PTDs and IpaH7.8 as well as IpaH9.8 only one additional transport domain. Similarly to the IpaH proteins of *Shigella*, several PTDs were also identified within the sequence of the effector proteins from *Salmonella*. While three unknown PTDs were identified within the sequence of SlrP, both SspH effector proteins (SspH1&2) showed in total twelve PTDs (six each) with different sequences to the previously reported.

Taken together, the *in-silico* PTD-prediction analysis of SspH1, SspH2, SlrP, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, and IpaH9.8 in compare to YopM revealed that they harbor several unknown PTDs, which might be responsible for T3SS-independent uptake of the effector proteins into eukaryotic cells.

EXAMPLE 6

Methods and Materials of Experiments 1 to 5 and Figures 1 to 22

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1 Construction of 6xHis-tagged recombinant proteins

1.1 Restriction Free (RF) Cloning by Linear Amplification

Restriction Free (RF) cloning is an alternative method to insert a DNA fragment into a desired location within the vector (Chen et al. 2000, van den Ent & Löwe, 2006) Using primers that contain a 3' target-specific sequence and a 5' extension that is complementary to the desired insertion site within the vector, a double-stranded PCR product is generated, containing the target sequence and the sequence of the insertion site at both the 3' and 5' end. This PCR product is then used as a pair of mega-primers in a second amplification reaction. Both strands of the PCR product anneal to the insertion site of the vector and are extended in a linear amplification reaction resulting in a nicked double-stranded plasmid.

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The PCR reaction for target DNA amplification was carried out as listed in Tables 1 & 2.

Table 1: PCR reaction RF cloning

Template DNA	~ 1-200 ng
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dNTP mix (10 mM each)	1 µl (final concentration 100 µM)
Oligonucleotide primers	0.1 µg (each)
Phusion Polymerase buffer	1 x
Phusion Polymerase	1 unit
	Add H ₂ O to 50 µl

Table 2: PCR cycling conditions

Reaction step	Temperature	Duration	Cycles
I Initial denaturation	98°C	1 min	1x
II Denaturation	98°C	30 s	
III Annealing	T _m	30 s	35x
IV Elongation	72°C	15-30 s/kb	
V Final elongation	72°C	10 min	1x

- 5 Linear amplification of vector and insert using the double-stranded PCR product from the first PCR as megaprimers was performed according to Unger *et al.* (2010) using the reaction mixture listed in Table 3 and carried out in a PCR thermal cycler with the cycling conditions listed in Table 4.

Table 3: PCR reaction linear amplification

10	Template DNA	20 ng
	dNTP mix (10 mM each)	1 µl (final concentration 200 µM)
	PCR product (mega-primers)	100 ng
	Phusion Polymerase buffer	1 x
	Phusion Polymerase	1 unit
		Add H ₂ O to 50 µl

Table 4: PCR cycling conditions for linear amplification

Reaction step	Temperature	Duration	Cycles
I Initial denaturation	98°C	30 s	1x

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II Denaturation	98°C	30 s	
III Annealing	60°C	1 min	30x
IV Elongation	72°C	5 min	
V Final elongation	72°C	7 min	1x

Following the amplification reaction, 0.2 U/ μ l DpnI were added for 2 h at 37°C to eliminate parental plasmid DNA. 10 μ l of the DpnI treated reaction mixture were then used for transformation of competent *E. coli* DH5 α cells.

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2 Expression and Purification of Recombinant Protein

2.1 Expression of Recombinant Protein in *E. coli*

For expression of recombinant proteins, the pET24b(+) expression vector was chosen which provides the coding sequence for a C-terminal 6 x His-tag. All plasmids used in this study are derivatives of pET24b(+).

For protein expression, the recombinant plasmids carrying the respective coding sequences were transformed into *E. coli* BL21(DE3) cells. Expression of the target proteins was carried out in 500 ml of Standard I medium containing 50 μ g/ μ l kanamycin. The culture was inoculated 1:100 with an overnight culture and incubated at 37°C and 180 rpm until an OD₆₀₀ of 0.6-0.8 was reached. Then expression of the recombinant proteins was induced by adding IPTG to a final concentration of 1 mM before the culture was incubated for an additional 4 h. Cells were harvested by centrifugation at 3,000 x g and 4°C for 15 min and the cell pellet was stored at -20°C until further usage.

20

2.2 Preparation of Cleared *E. coli* Lysates

The bacterial pellets were thawed on ice and resuspended in 10 ml of lysis buffer. Cleared lysates were prepared by sonication (Branson Sonifier 250; 4 x 30 s, level 4, 50% cycle, on ice). Bursts were followed by 15 s breaks. The cellular debris was removed by centrifugation (7,200 x g, 15 min, 4°C) and the supernatant containing the recombinant protein was subjected to affinity chromatography.

Heterologous expression of recombinant proteins can lead to formation of inclusion bodies; aggregates of the overexpressed protein that remain insoluble. These can be solubilised by addition of anionic detergents such as N-lauroylsarcosine sodium salt. Where the desired protein was detected in the insoluble fraction, 2% (w/v) N-lauroylsarcosine sodium salt were added following sonication and incubated on a rotary shaker (15 rpm, 4°C) for 1 h before centrifugation as mentioned above.

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Lysis buffer

Tris-HCl, pH 8.0	50 mM
NaCl	500 mM
Imidazole	10 mM
Glycerol	10% (v/v)
Triton X-100	0.1% (v/v)

N-Lauroylsarcosine sodium salt

Tris-HCl, pH 8.0	25 mM
NaCl	100 mM
N-Lauroylsarcosine sodium salt	10% (w/v)

2.3 Purification of Recombinant Protein

Protein purification was performed by nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography (Qiagen, Hilden) under native conditions according to "The QIAexpressionist" handbook (Qiagen, Hilden).

To enable binding of the 6 x His-tagged recombinant protein to the Ni-NTA resin, 1 ml of Ni-NTA Agarose was added to the cleared lysate and mixed by shaking on a rotary shaker (15 rpm, 4°C) for 1 h. The mixture was centrifuged (800 x g, 2 min, 4°C) and the supernatant was discarded. Three wash steps were carried out (800 x g, 2 min, 4°C) using 10 ml of wash buffer each before the mixture was loaded into a column. Generally, wash buffer I was used for purification. Wash buffer II was employed for purification of proteins that have previously been solubilised using N-lauroylsarcosine sodium salt. The protein was eluted in 500 µl aliquots using 5 ml of elution buffer. All fractions were stored at 4°C for SDS-PAGE analysis.

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Wash buffer I

Tris-HCl, pH 8.0	50 mM
NaCl	300 mM
Imidazole	20 mM
Glycerol	10% (v/v)
Triton X-100	0.1% (v/v)

Wash buffer II

Tris-HCl, pH 8.0	50 mM
NaCl	300 mM
Imidazole	20 mM
Glycerol	10% (v/v)
Triton X-100	2% (v/v)

Elution buffer

Tris-HCl, pH 8.0	50 mM
NaCl	300 mM
Imidazole	200 mM
Glycerol	10% (v/v)

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Triton X-100 0.1% (v/v)

2.4 Protein Dialysis and Concentration

After purification, fractions of highest purity were pooled and dialysed in PBS. The protein solution was applied to a dialysis tube (pore size: 6-10 kDa, Roth, Karlsruhe) and dialysed overnight in 2 l PBS at 4°C with gentle stirring. After dialysis, the protein was concentrated to a final volume of 2 ml using Amicon® centrifugal filters (Millipore, Eschborn) at 500 x g and 4°C.

2.5 Protein Labelling with Fluorescent Dyes

In order to detect the recombinant proteins used in this study by fluorescence microscopy and FACS analysis, the proteins were labelled using fluorescent dyes.

Protein Labelling with Alexa Fluor®488

For FACS analysis, the recombinant proteins YopM and SspH1 were labelled with the green fluorescent dye Alexa Fluor®488 using the Alexa Fluor®488 Protein labelling kit (Invitrogen, Karlsruhe). Fluorescence of proteins labelled with Alexa Fluor® 488 can be excited at 494 nm and emission is detected at 519 nm.

Labelling of recombinant YopM and SspH1 with Alexa Fluor® 488 was performed according to the manufacturer's recommendations.

Protein Labelling with Cy3

For subsequent fluorescence microscopy, recombinant proteins were labelled with the orange fluorescent cyanine dye Cy3 using the Cy3 Ab labelling kit (Amersham Biosciences, Freiburg). Fluorescence of Cy3-labelled proteins can be excited at 550 nm and emission is detected at 570 nm.

3 Cell Fractionation of Eukaryotic Cells

Cell fractionation of eukaryotic cells allows separation of soluble cytoplasmic proteins from insoluble membrane proteins (Behrens, 1938). In this study, cell fractionation was used to check for autointegration of the recombinant proteins into the cytoplasm (Kenny und Finlay, 1997; Gauthier *et al.*, 2000).

The cells were cultured to confluency in a 10 cm culture dish and subsequently incubated with recombinant protein (25 µg/ml) in 10 ml culture medium for 1 h. Cells were washed with ice cold D-PBS (with Ca²⁺ and Mg²⁺) 3 x for 15 min before the cells were quenched with acid buffer wash for 5 min. After an additional wash step with D-PBS (with Ca²⁺ and Mg²⁺), the cells were scraped from the surface and resuspended in 1 ml sonication buffer (supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Roche Biochemicals, Mannheim) prior to use). The cells

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were permeabilised by sonication (ultrasound water bath, 4 x 1 s, level 4, 4°C) followed by centrifugation (108,000 x g, 15 min, 4°C). The supernatant containing the cytoplasmic proteins was collected and saved as cytoplasmic fraction (CF) until further usage. The insoluble pellet was washed once in 1 ml sonication buffer before it was resuspended in 1 ml Triton buffer (supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Roche Biochemicals, Mannheim) prior to use) and incubated on a rotary shaker (15 rpm, 4°C) for 30 min. The cell lysate was centrifuged (108,000 x g, 15 min, 4°C) again and the supernatant containing membrane proteins soluble in Triton X-100 was collected as the membrane fraction (MF). Cytoplasmic and membrane fraction were precipitated using trichloroacetic acid and subsequently analysed by Western blotting.

Sonication Buffer

Tris-HCl	50 mM
NaCl	150 mM
EDTA	1 mM
EGTA	1 mM
Glycerol	30% (v/v)
NaVO ₄	0.4 mM
NaF	1 mM

Triton Buffer

Sonication Buffer	1 l
Triton X-100	1% (v/v)

Acid Buffer Wash

Glycine	0.2 g
D-PBS	Add to 100 ml
pH 2.0	

4 Nuclear Fractionation of Eukaryotic Cells

Nuclear fractionation allows separation of cytoplasmic and nuclear protein fractions. In this study nuclear fractionation was performed to verify cell penetration by recombinant proteins and to check for a potential nuclear localisation of these recombinant proteins. Therefore, the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, USA) was used which is based on stepwise cell lysis and centrifugal separation of cytoplasmic and nuclear protein fractions.

Cells grown to confluency in a 10 cm culture dish were incubated with recombinant protein (25 µg/ml) for 1 h. Following incubation, the cells were washed with D-PBS (without Ca²⁺ and Mg²⁺) and subsequently detached with trypsin and centrifuged at 500 x g for 5 min. The cells were washed with D-PBS, transferred to a microcentrifuge tube and centrifuged again (500 x g, 3 min). All buffers were included in the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Rockford, USA) and used at the recommended volumes. Buffers CER I and NER were

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supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Roche Biochemicals, Mannheim) prior to use.

5 Immunofluorescence Microscopy

5 Recombinant proteins used for immunofluorescence analyses in this study were either GFP fusion proteins or labelled with the fluorescent dye Cy3.

In order to detect proteins by immunofluorescence analysis, cells were cultured on cover slips in 24-well plates and subsequently incubated with recombinant protein (25 µg/ml). Cells were washed with D-PBS (with Ca²⁺ and Mg²⁺) three times to remove non-integrated proteins. A different experimental setup included trypsinization after HeLa cells were incubated with the proteins. This procedure is supposed to be even more efficient in removing cell-surface bound proteins (Richard *et al.*, 2003). After trypsinization, HeLa cells were seeded on cover slips in 24-well plates and incubated overnight to allow cell recovery. Afterwards cells were fixed using 500 µl 4% PFA (w/v) per well and washed with D-PBS for 5 min. Subsequently, the cells were quenched with 0.2% glycine for 20 min and washed again. Cells were permeabilized using 0.2% Triton X-100 for 4 min followed by an additional wash step. DNA was selectively stained using Hoechst 33 258 (DAPI) diluted 1:1000 in D-PBS for 7.5 min followed by three wash steps with D-PBS for 5 min each. The preparations were embedded in Moviol/ DABCO and analyzed by fluorescence microscopy using appropriate filters.

20

0.2% Triton X-100

Triton X-100	200 µl
D-PBS	Add to 100 ml

0.2% Glycine

Glycine	0.2 g
D-PBS	Add to 100 ml

4% PFA

Paraformaldehyde	4 g
D-PBS	Add to 100 ml

6 Fluorescence Activated Cell Sorting (FACS)

Internalisation of YopM, SspH1 and Tat-GFP-GSK into HeLa cells was monitored by fluorescence activated cell sorting (FACS). FACS is a specialised type of flow cytometry which allows separation of cells labelled with fluorescent markers and measurement of fluorescence intensity (Bonner *et al.*, 1972).

25

In this study, FACS analyses were performed to confirm and further investigate uptake of YopM and SspH1 in comparison to the Tat-GFP-GSK construct, a derivative of the well characterised CPP Tat. Uptake of the recombinant proteins was examined by determining the

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fluorescence intensity of HeLa cells. Therefore recombinant YopM and SspH1 were labelled with the fluorescent dye Alexa Fluor®488.

HeLa cells were grown to confluency in 175 cm² culture flasks and subsequently detached from the surface by adding trypsin. The cells were centrifuged and resuspended in D-PBS (without Ca²⁺ and Mg²⁺) and preheated to 37°C prior to incubation with the recombinant proteins (20 µg/ml). To assure that only viable cells are detected, the FACS cytometer was adjusted using control cells that were not incubated with protein. Dead cells and cellular debris can be excluded from acquisition by measuring forward and side scatter which display cell size and granularity, respectively. Dead cells have lower forward scatter and higher side scatter which allows distinction from living cells. First, the control cells were applied to the cytometer and viable cells were gated due to side and forward scatter and thus selected for acquisition. The cytometer (BD Biosciences, Heidelberg) was set to acquire 10,000 events from the gated cell population per run, every event being a single cell. In case of internalisation of the proteins tagged with the fluorescent markers GFP and Alexa Fluor®488, respectively, fluorescence of the cells can be measured. However, even cells without labelling show fluorescence, so-called autofluorescence which is caused by components of the cell like pyridinic (NADPH) and flavin coenzymes (Monici, 2005). To subtract the autofluorescence from the total fluorescence intensity, the fluorescence intensity of HeLa cells which were not incubated with the fluorescent labelled proteins was measured.

20 **6.1 Quenched Time-Lapse Uptake and CPP-induced Membranolysis Assay**

In this study, the uptake kinetics of the recombinant proteins YopM, SspH1 and Tat-GFP-GSK were monitored in two parallel experiments that are based on an ongoing incubation and allow measurements from the same incubation at different time points (Florén *et al.*, 2011).

The first approach is based on addition of trypan blue (TB) to the samples prior to FACS analysis to measure the protein uptake rate. TB is a quencher that is not able to enter the cells and efficiently eliminates fluorescence (Sahlin *et al.*, 1983, Hed *et al.*, 1987). Thus extracellular fluorescence can be excluded from the measurement, ensuring that only intracellularly located proteins are detected which are inaccessible to TB.

The second assay allows monitoring of potential lytic effects of the recombinant proteins on membrane integrity. Protein-induced membranolysis can be assessed by the addition of propidium iodide (PI). PI is a fluorescent DNA intercalating agent that is not able to cross membranes of intact cells. PI can only enter cells with disrupted membranes and hence accumulates in dead cells. Thus, protein-induced membrane lysis can be monitored by the detection of PI fluorescence (Radošević *et al.*, 1990).

In order to measure the fluorescence intensity of the samples, the detectors were tuned to the appropriate wavelength of emission of the used fluorescent marker. As mentioned above, YopM and SspH1 were labelled with Alexa Fluor®488 which can be excited at 494 nm and emits

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light at 519 nm. Fluorescence intensity of cells incubated with Tat-GFP-GSK depends on fluorescence of GFP which is excited at 488 nm and emission was detected at 510 nm. For monitoring membranolytic effects of the proteins, 1 µg/ml PI was added to the medium. Fluorescence of PI is excited at 536 nm and can be detected at 617 nm. Samples for measuring
 5 intracellular accumulation of the proteins were diluted 1:1 with TB (0.4% (w/v)) prior to FACS analysis. The samples were applied to the cytometer and fluorescence emitted by the cells was measured. In addition, side and forward scatter were measured for each cell crossing the laser beam. The obtained data were analysed using CellQuest™ Pro (BD Biosciences, Heidelberg).

10 **7 *In vitro* Ubiquitination Assay**

In vitro ubiquitination experiments were performed in 40 µl reaction buffer (50 mM Tris-HCl, pH7.4, 10 mM MgCl₂, 0.1 mM DTT and 2 mM ATP) containing 0.5 µg E1, 2 µg UbcH5b (E2), 2 µg HA-ubiquitin, 2 µg SspH1/ SspH1-Nter in the presence or absence of 0.4 µg PKN1. Reactions were incubated at 37°C for 1h and stopped by addition of SDS sample buffer, with or without 100 mM
 15 DTT. Samples were separated by SDS-PAGE and subjected to western blotting using anti-ubiquitin, anti-His and anti-PKN1 antibodies.

8 Immunoprecipitation (IP)

20 HeLa cells were grown to 80% confluency in 10 cm dishes and subsequently transfected with pCMVEntry-PKN1-Myc. 24 h post transfection, cells were incubated with 25 µg/ml FLAG-tagged SspH1 and SspH1-Nter, respectively for 3 h. Cells were washed with D-PBS (with Ca²⁺ and Mg²⁺) (3 x 5 min), before they were scraped from the surface and resuspended in 800 µl IP lysis buffer (supplemented with Complete EDTA-free Protease Inhibitor Cocktail (Roche Biochemicals,
 25 Mannheim) prior to use). The cells were permeabilised by sonication (3 x 20 s, 4°C) and subsequently incubated on a rotary shaker for 30 min at 4°C. Lysates were cleared by centrifugation (16,000 x g, 30 min, 4°C) and a sample of the lysate was taken and stored at -20°C until further usage. 30 µl protein A/G agarose beads (Santa Cruz, sc-2003) were incubated with 5 µg α-PKN1 antibody (BD Biosciences) on ice for 5 min before the lysate was added to the mix and
 30 incubated on a rotary shaker overnight at 4°C. The beads were pelleted by centrifugation (1000 x g, 3 min, 4°C) and a sample of the supernatant was collected and stored until further usage. The beads were washed with 600 µl IP lysis buffer three times and centrifuged as mentioned above. The supernatant was discarded and 30 µl 4 x SDS sample buffer were added to the beads, heated at 95°C for 5 min and subsequently centrifuged at 16,000 x g for 5 min. The supernatant along with
 35 the samples of the lysate and the unbound protein were subjected to immuno blot analysis.

IP lysis Buffer

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Tris-HCl pH 7.4	50 mM
NaCl	150 mM
EDTA	2 mM

9 Non-radioactive Cytotoxicity Assay

In order to analyse a potential cytotoxic effect of the recombinant proteins on the eukaryotic cells, the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) was used. HeLa cells were grown in 96 well plates and incubated with 25- 50 µg/ml SspH1 and SspH1-Nter, respectively for different time points (1, 6 & 24 h). Following incubation, the assay was performed according to the manufacturer's recommendations.

10 Analysis of Eukaryotic Gene Expression

In order to analyse the effect of recombinant SspH1 on cytokine and chemokine expression, qRT-PCR was performed. Therefore, A459 cells were grown in 6 well plates to confluency and incubated with SspH1 for 3 h. The cells were subsequently stimulated with 20 ng/ml Il1β, before RNA was isolated according to the manufacturer's recommendations using the RNeasy Mini Kit (Qiagen, Hilden).

15

10.1 cDNA Synthesis

The RNA was then used for cDNA synthesis using the Transcriptor Reverse Transcriptase-Kit (Roche, Mannheim) according to the manufacturer's recommendations. First, primers were annealed to the RNA, before the cDNA synthesis was initiated by addition of the RT mix.

20

Step	Reaction mixture	Concentration	Program
I.	RNA T7 Oligo (dT) ₂₄ Primer DNA / RNA free H ₂ O	3-5 µg 10 pmol ad 13 µl	10 min, 65°C -> 4°C
II.	5 x RT-Puffer dNTPS RNase Inhibitor (40 U/µl) Reverse Transcriptase (20 U/µl)	4 µl 2 µl 0,5 µl 0,5 µl	30 min, 55°C 5 min, 85°C -> 4°C

cDNA was synthesised in a PCR thermo cycler and stored at -20°C until further usage.

25 10.2 Quantitative Real Time PCR

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The qRT-PCR enables quantification of PCR products by measuring the fluorescent intensities of a DNA-intercalating fluorescent dye. In this study, qRT-PCR was performed using the LightCycler1.5 (Roche, Mannheim). Data was analysed using the LightCycler Data Analysis 5.3.2 software (Roche, Mannheim). Values for each sample were normalised for a low abundance Housekeeping gene (here *HPRT*: Hypoxanthin-Phosphoribosyl-Transferase I) (Vandesompele *et al.*, 2002).
 5 qRT-PCR was performed using the LightCycler® Fast Start DNA Master^{PLUS} SYBR Green I kit (Roche, Mannheim) which contains the reaction buffer, the dNTP mix, the SYBR Green I dye and MgCl₂. The hot start Taq polymerase is added to the mix and heat-activated at 95°C for 15 min before the PCR reaction. Different dilutions of the cDNA were prepared (1:10, 1:100, 1:1000) in
 10 sterile dH₂O and used as a template for the qRT-PCR.

2 µl	cDNA
2 µl	LightCycler® Fast Start DNA Master ^{PLUS} SYBR Green I
2 µl	Primer Mix
4 µl	Sterile dH ₂ O

qRT-PCR was performed using the following cycling conditions.

Reaction Step	Temperature	Time	Cycles
I. Denaturation and DNA Polymerase Activation	95°C	15 min	1 x
II.			
1. Denaturation	95°C	12 s	
2. Annealing	60°C	10 s	35-55 x
3. Elongation	72°C	12 s	
III. Melting	60-95°C	stepwise	1 x

15

Statistical analysis of the obtained data was performed using Prism 4 (GraphPad Software). The statistical significance of differences in gene expression was analysed using the unpaired student t-test and values $p < 0.05$ were regarded as significant.

20 3 Material

3.1 Bacterial Strains

Bacterial strains used in this study are listed in Table 3.1.

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Table 3.1 Bacterial strains

Strain	Relevant Characteristics	Reference
<i>E. coli</i> DH5 α	F ⁻ , <i>endA1</i> , <i>recA1</i> , <i>hsdR17</i> (r _K ⁻ M _K ⁺) <i>deoR</i> , <i>thi-1</i> , <i>supE44</i> , <i>gyrA96</i> , Δ (<i>lacZYA-argF</i>) U169 (Φ 80d <i>lacZ</i> Δ M15)	Hanahan <i>et al.</i> , 1991
<i>E. coli</i> BL21 (DE3)	F ⁻ , <i>hsdS_B</i> (r _B ⁻ m _B ⁻), <i>dcm</i> , <i>gal</i> , <i>ompT</i> , (λ DE3)	Studier & Moffatt, 1986

3.4 Plasmids and Oligonucleotides

- 5 Plasmids used in this study are listed in Table 3.3.

Table 3.2: Plasmids

Plasmid	Relevant Characteristics	Reference
pET24b(+)	Expression vector, Kan ^R	Novagen
pET-YopM	Nucleotides 1-1101 of <i>yopM</i> from <i>Y. enterocolitica</i> O:8 JB580v (NheI/XhoI) in pET24b(+)	Heusipp <i>et al.</i> , 2006
pET-SspHI	Nucleotides 1-2103 of <i>sspHI</i> of <i>S. enterica</i> subspec. <i>enterica</i> serovar Typhimurium 14928S (NheI/XhoI) in pET24b(+)	Lubos, M.-L.
pET-SspHI-Nter	Nucleotides 1-1161 of <i>sspHI</i> of <i>S. enterica</i> subspec. <i>enterica</i> serovar Typhimurium 14928S (NheI/XhoI) in pET24b(+)	Lubos, M.-L.
pET:Tat-GFP-GSK	Coding sequence of the Tat CPP with 3' GFP coding sequence and GSK-3 β tag coding sequence in pET24b(+)	Lubos, M.-L.
pET:ipaH1.4	<i>ipaH1.4</i> of <i>S. flexneri</i> in pET24b+	S. Norkowski
pET:ipaH2.5	<i>ipaH2.5</i> of <i>S. flexneri</i> in pET24b+	S. Norkowski
pET:ipaH3	<i>ipaH3</i> of <i>S. flexneri</i> in pET24b+	S. Norkowski
pET:ipaH4.5	<i>ipaH4.5</i> of <i>S. flexneri</i> in pET24b+	S. Norkowski
pET:ipaH7.8	<i>ipaH7.8</i> of <i>S. flexneri</i> in pET24b+	S. Norkowski
pET:ipaH9.8	<i>ipaH9.8</i> of <i>S. flexneri</i> in pET24b+	S. Norkowski
pET:SspH2	<i>SspH2</i> of <i>S. typhimurium</i> in pET24b+	S. Norkowski

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pET:SlrP	<i>slrP</i> of <i>S. typhimurium</i> in pET24b+	S. Norkowski
pCMVEntry-PKN1	Myc-DDK-tagged Human <i>pkn1</i> transcript variant 1 in pCMV6-Entry, RC215735	Origene

Synthetic oligonucleotides used for DNA amplification are listed in Table 3.4. All primers were purchased from MWG Biotech AG (Ebersberg).

5 **Table 3.3: Oligonucleotide sequences for DNA amplification by PCR (restriction sites are underlined)**

Oligonucleotide	Sequence (5'→3')
F-SspHI (NheI) SEQ ID NO: 530	CTA <u>GCT AGC</u> GTT ACC GAT AAA TAA TAA CTT
R-SspHI (XhoI) SEQ ID NO: 531	CGC <u>CTC GAG</u> TGA ATG GTG CAG TTG TGA GCC
R-SspHI-Nter (XhoI) SEQ ID NO: 532	CCG <u>CTC GAG</u> CCG TGG GCC GTG GTA GTC CGG
F-Tat (NdeI) SEQ ID NO: 533	TAT GAT GTG CGG CCG TAA GAA ACG TCG CCA GCG TCG CCG TCC GCC GCA ATG CG
R-Tat (NheI) SEQ ID NO: 534	CTA GCG CAT TGC GGC GGA CGG CGA CGC TGG CGA CGT TTC TTA CGG CCG CAC AGC A
F-IpaH1.4 SEQ ID NO: 535	<u>GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG ATT</u> AAA TCA ACC AAT ATA CAG
R-IpaH1.4 SEQ ID NO: 536	<u>CTT ATC GTC GTC ATC CTT GTA ATC GCT AGC TGC</u> GAT ATG ATT TGA GCC GTT TTC AGA CAA
F-IpaH2.5/IpaH4.5 SEQ ID NO: 537	<u>GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG ATT</u> AAA TCA ACA AAT ATA CAG GTA ATC GGT
R-IpaH2.5 SEQ ID NO: 538	<u>CTT ATC GTC GTC ATC CTT GTA ATC GCT AGC GGC</u> CAG TAC CTC GTC AGT CAA CTG ACG GTA
F-IpaH3 SEQ ID NO: 539	<u>GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG TTA</u> CCG ATA AAT AAT AAC TTT TCA TTG TCC
R-IpaH3 SEQ ID NO: 540	<u>CTT ATC GTC GTC ATC CTT GTA ATC GCT AGC GTC</u> AGC TGA CGG TAA ATC TGC TGT TAC AGT
F-IpaH4.5 SEQ ID NO: 541	<u>GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG AAA</u> CCG ATC AAC AAT CAT TCT TTT TTT CGT
F-IpaH7.8 SEQ ID NO: 542	<u>GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG TTC</u> TCT GTA AAT AAT ACA CAC TCA TCA GTT
R-IpaH7.8 SEQ ID NO: 543	<u>CTT ATC GTC GTC ATC CTT GTA ATC GCT AGC TGA</u> ATG GTG CAG TCG TGA GCC GTT TTC AGA

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F-lpaH9.8 SEQ ID NO: 544	<u>GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG TTA</u> CCG ATA AAT AAT AAC TTT TCA TTG CCC
R-lpaH9.8 SEQ ID NO: 545	<u>CTT ATC GTC GTC ATC CTT GTA ATC GCT AGC TGA</u> ATG GTG CAG TTG TGA GCC GTT TTC AAA
F-SspH2 SEQ ID NO: 546	<u>GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG CCC</u> TTT CAT ATT GGA AGC GGA TGT CTT CCC
R-SspH2 SEQ ID NO: 547	<u>CTT ATC GTC GTC ATC CTT GTA ATC GCT AGC GTT</u> ACG ACG CCA CTG AAC GTT CAG ATA GCT
F-SlrP SEQ ID NO: 548	<u>GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG TTT</u> AAT ATT ACT AAT ATA CAA TCT ACG GCA
R-SlrP SEQ ID NO: 549	<u>CTT ATC GTC GTC ATC CTT GTA ATC GCT AGC TCG</u> CCA GTA GGC GCT CAT GAG CGA GCT CAC

3.6 Antibodies

Primary and secondary antibodies used for Western blot analysis and fluorescent dyes for immunofluorescence studies are listed in Tables 3.6 and 3.7.

5

Table 3.4: Primary antibodies used for Western blot analyses

Antibody	Dilution	Characteristics	Reference
α -Penta-His	1:1000	Mouse monoclonal antibody against His ₆ -epitope, isotype IgG	Quiagen (Hilden)
Anti- α -Tubulin	1:5000	Mouse monoclonal antiserum against human α -tubulin	Sigma-Aldrich (München)
α -LSD1 (C69G12)	1:1000	Rabbit monoclonal antibody against human lysine-specific demethylase 1 (LSD1)	Cell Signaling (Danvers, USA)
α -GAPDH	1:200	Rabbit polyclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Santa Cruz Biotechnology (Heidelberg)
α -Transferrin receptor	1:500	Mouse monoclonal antibody against human transferrin receptor	Invitrogen (Karlsruhe)
α -FLAG	1:1000	Mouse monoclonal antibody against DYKDDDDK -tag epitope, Clone M2	Sigma-Aldrich (München)
α -PKN1	1:1000	Monoclonal mouse antibody against human protein kinase N1	BD Bioscience (Heidelberg)

Table 3.5: Secondary antibodies used for Western blot (WB) analyses and fluorescent dyes for immunofluorescence (IF) analyses

Antibody	Dilution	Characteristics	Reference
GAM-PO	(WB) 1:10000	Peroxidase (PO) conjugated goat monoclonal antibody against mouse-IgG	Dianova (Hamburg)
GAR-PO	(WB) 1:10000	Peroxidase (PO) conjugated goat monoclonal antibody against rabbit-IgG	Dianova (Hamburg)
Hoechst 33258 (DAPI)	(IF) 1:1000	Selective DNA dye	Sigma Aldrich (Taufkirchen)

5 3.7 Kits

Kits used in this study are listed in Table 3.6.

Table 3.6: Kits

Kit	Supplier
Zyppy™ Plasmid Miniprep Kit	Zymo Research (Irvine, USA)
Zymoclean™ Gel DNA Recovery Kit	Zymo Research (Irvine, USA)
Cy3 Ab Labelling Kit PA 33000	GE Healthcare (Braunschweig)
Alexa Fluor® 488 Protein Labelling Kit	Invitrogen (Karlsruhe)
NE-PER Nuclear and Cytoplasmic Extraction Reagents	Thermo Scientific (Rockford, USA)
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega

10

References (Materials and Methods of Examples 1 to 5):

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20 **Methods of Examples 7 to 20**

The experiments described in Examples 7 to 20 and shown in Figures 23 to 28 may be carried out in various ways well-known to one skilled in the art. For example, according to the methods indicated above or described in Examples 7 to 20.

25 Selected methods are described below in more detail. Methods well-known to one skilled in the art have not been described in detail in order to not unnecessarily obscure the present invention.

Cell Fractionation of Eukaryotic Cells

30 Cellular uptake of proteins was assessed by cell fractionation of eukaryotic cells. This method allows separation of soluble cytoplasmic proteins from insoluble membrane proteins (Behrens, 1938).

35 Therefore, HeLa cells were seeded in 10 cm cell-culture dishes and grown to 80% confluence. Upon protein treatment, the dishes were transferred to ice and the cells were washed twice with ice-cold D-PBS (with Ca²⁺/Mg²⁺). An additional wash step with acid buffer for 5 min was used to remove any residual surface-bound proteins (Langel, Ü. (ed) (2005). Handbook of cell-penetrating

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peptides. CRC Press, Taylor and Francis Group). After washing the cells again with D-PBS (with Ca²⁺/Mg²⁺), cells were collected using a cell scraper, transferred to a reaction tube on ice and resuspended in 1 ml sonication buffer (supplemented with Complete EDTA-free Protease Inhibitor Cocktail). The suspension was applied to sonication (ultrasound water bath, 4 x 1 sec, level 4, 4°C) in order to permeabilize the cells. Subsequently, the suspension was centrifuged (108,000 x g, 15 min, 4°C) and the supernatant was taken as the cytoplasmic fraction (CF). After the insoluble pellet was washed once with 1 ml sonication buffer (108,000 x g, 15 min, 4°C), it was resuspended in 1 ml Triton buffer (supplemented with Complete EDTA-free Protease Inhibitor Cocktail) and incubated on a rotary shaker at 15 rpm and 4°C for 1 h or overnight. Subsequently, the cell lysate was centrifuged (108,000 x g, 30 min, 4°C) and the supernatant was taken as the membrane fraction (MF).

Both the cytoplasmic and the membrane fraction were precipitated using trichloroacetic acid. Subsequently, the samples were subjected to SDS-PAGE and analyzed by Western blotting.

Acid Buffer		Sonication buffer	
Glycine	62.5 mM	1 mM Tris-HCl, pH 7.8	50 mM
in PBS, pH 2.0		NaCl	150 mM
		EDTA	1 mM
Triton buffer		EGTA	1 mM
Triton X-100	1 % (v/v)	Glycerol	30 % (v/v)
in Sonication buffer		NaVO ₄	0.4 mM
		NaF	1 mM

15

Membranolysis Assay

For analysis of effects on membrane integrity induced by LPX effector proteins, a FACS-based membranolysis assay following the 'CPP-induced Membranolysis Assay' (Florén et al., 2011) was performed.

20

HeLa cells were cultured and prepared as described above. For monitoring membranolytic effects of the proteins, HeLa cells were incubated with the respective protein and co-incubated with 1 µg/ml PI. After defined time points samples were taken and applied to the FACS analysis. Each sample was measured in duplicates. Fluorescence of PI is excited at 536 nm and can be detected at 617 nm. The obtained data were analyzed using the CellQuestTM Pro software.

25

Lactate Dehydrogenase Assay

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In order to assess cytotoxicity and potential lytic effects of recombinant proteins, the release of lactate dehydrogenase (LDH) can be measured and used as a parameter for membrane integrity. Cytotoxicity and potential lytic effects of recombinant proteins were measured using CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions.

5

HeLa cells were seeded in 96-well plates and grown to 80 % confluence. Upon incubation with recombinant proteins for 24, 6, and 1 h in 100 µl culture medium, the plate was centrifuged (400 x g, 4 min, RT) and 50 µl of the supernatant from each well of the assay plate were transferred to the corresponding well of a new 96-well plate. In addition, 50 µl of a LDH positive control were added to separate wells in order to verify that the assay is functioning properly. HeLa cells contained in the remaining 50 µl were lysed by adding 5.5 µl Lysis Solutions (10 x) for 30 min. Afterwards, 50 µl of the reconstituted Substrate Mix were added to each well of the two plates and both plates were incubated for 30 min at RT, protected from light. Finally, 50 µl of the Stop Solution were added to each well of the plates and the absorbance at 490 nm was recorded. All buffers used for this procedure were provided by the CytoTox 96 Non-Radioactive Cytotoxicity Assay-Kit.

10

15

In Vitro Ubiquitination Assay

In order to verify the functionality of recombinant LPX effectors as proposed E3 ubiquitin ligases, in vitro ubiquitination assays were performed. Upon incubation with ubiquitin-activating enzymes E1, ubiquitin-conjugating enzymes E2, and ubiquitin, LPX effector proteins were tested whether they were able to remove ubiquitin from the E2 enzyme and catalyze the formation of poly-ubiquitin chains.

20

In vitro ubiquitination assays were performed in a volume of 40 µl at 37°C for 1 h. The reaction mixture was composed as shown below. The reaction was stopped by adding of 10 µl of 4 x SDS sample buffer without dithiothreitol (DTT). The samples were prepared for subsequent SDS-PAGE analysis by incubation at 95°C for 10 min

25

Reaction mixture of in vitro ubiquitination assay:

Component	Amount
E1	0,5 µg
E2 (UbcH5b)	2 µg
Ubiquitin-HA	2 µg
Putative E3	4 µg

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Component	Amount
Ubiquitin reaction buffer	ad to 40 µl

Ubiquitination reaction buffer		4 x SDS sample buffer without DTT	
Tris-HCl, pH 7.5	25 mM	Tris-HCl, pH 6.8	30 mM
NaCl	50 mM	Glycerol	10 %(v/v)
ATP	5 mM	SDS	1,5 % (v/v)
MgCl ₂	10 mM	Bromophenol blue	Spatula tip
DTT	0.1 mM		

EXAMPLE 7**Functional domains of LPX family members**

- 5 Functional domains of SlrP, SspH1, SspH2, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8 and IpaH9.8 are shown in Figures 14 to 22.

Sequence segment comprising Leucin-rich repeats (called "LRR stretch") are marked in light grey and are non-edged. E3 Ubiquitin ligase domains are marked in dark grey and are edged.

- 10 Sequence sections within which the protein transduction domains (PTDs) are predicted are bold and underlined. Marked are the maximal sequence sections within the PTD is predicted. PTDs can correspond to the marked sequence sections or to c-terminally and/or N-terminally truncated fragments of the marked sequence sections.

EXAMPLE 8

- 15 **Corresponding DNA sequences of the proteins of the invention analyzed in the examples above**

SlrP

SEQ ID NO: 10

- 20 >gb|CP001363.1|:867285-869582 *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. 14028S, complete genome

SspH1

SEQ ID NO: 11

- 25 >gb|CP001363.1|:1332051-1334153 *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. 14028S

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SspH2

SEQ ID NO: 12

>gb|CP001363.1|:2392438-2394804 *Salmonella enterica* subsp. *enterica* serovar
Typhimurium str. 14028S, complete genome

5

IpaH1.4

SEQ ID NO: 13

>gi|12329037:206811-208538 *Shigella flexneri* virulence plasmid pWR100:
from 1 to 213494

10

IpaH2.5

SEQ ID NO: 14

>gi|12329037:43257-44948 *Shigella flexneri* virulence plasmid pWR100:
from 1 to 213494

15

IpaH3

SEQ ID NO: 15

>ENA|EID62303|EID62303.1 *Shigella flexneri* 5a str. M90T invasion plasmid antigen: Location:
1..1752

20

IpaH4.5

SEQ ID NO: 16

>gi|12329037:66187-67911 *Shigella flexneri* virulence plasmid pWR100:
from 1 to 213494

25

IpaH7.8

SEQ ID NO: 17

>gi|12329037:64062-65759 *Shigella flexneri* virulence plasmid pWR100:
from 1 to 213494

30

IpaH9.8

SEQ ID NO: 18

>gi|12329037:174343-175980 *Shigella flexneri* virulence plasmid pWR100:
from 1 to 213494

35

EXAMPLE 9**Protein transduction domains determined in Example 5****IpaH1.4**

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SEQ ID NO: 4

>tr|Q9AJU5|Q9AJU5_SHIFL Putative uncharacterized protein ipaH1.4
OS=Shigella flexneri GN=ipaH1.4 PE=4 SV=1

5 Predicted PTD sequences:

519	SQRVADRLKA	SEQ ID NO: 361	371	RVALTWNNLRKTL	SEQ ID NO: 367
520	QRVADRLKAS	SEQ ID NO: 362	371	RVALTWNNLRKTLL	SEQ ID NO: 368
521	RVADRLKASG	SEQ ID NO: 363	370	DRVALTWNNLRKTLL	SEQ ID NO: 369
520	QRVADRLKASG	SEQ ID NO: 364	371	RVALTWNNLRKTLLV	SEQ ID NO: 370
521	RVADRLKASGL	SEQ ID NO: 365			
520	QRVADRLKASGL	SEQ ID NO: 366			

IpaH2.5

SEQ ID NO: 5

10 >gi|12329051|emb|CAC05782.1| IpaH2.5, member of the IpaH family,
probably secreted by the Mxi-Spa machinery, function unknown [Shigella
flexneri 5a str. M90T]

Predicted PTD sequences:

15

519	SQRVADRLKA	SEQ ID NO: 371	371	RVALTWNNLRKTL	SEQ ID NO: 377
520	QRVADRLKAS	SEQ ID NO: 372	371	RVALTWNNLRKTLL	SEQ ID NO: 378
521	RVADRLKASG	SEQ ID NO: 373	370	DRVALTWNNLRKTLL	SEQ ID NO: 379
520	QRVADRLKASG	SEQ ID NO: 374	371	RVALTWNNLRKTLLV	SEQ ID NO: 380
521	RVADRLKASGL	SEQ ID NO: 375			
520	QRVADRLKASGL	SEQ ID NO: 376			

IpaH3

SEQ ID NO: 6

20 >tr|I0VDT7|I0VDT7_SHIFL Invasion plasmid antigen OS=Shigella flexneri 5a
str. M90T GN=ipaH_3 PE=4 SV=1

Predicted PTD sequences:

511	PQRVADRLKA	SEQ ID NO: 381	513	RVADRLKASGL	SEQ ID NO: 386
512	QRVADRLKAS	SEQ ID NO: 382	363	RVALTWNNLRKTL	SEQ ID NO: 387
513	RVADRLKASG	SEQ ID NO: 383	511	PQRVADRLKASGL	SEQ ID NO: 388
511	PQRVADRLKAS	SEQ ID NO: 384	363	RVALTWNNLRKTLL	SEQ ID NO: 389

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512 QRVADRLKASG SEQ ID NO: 385 362 DRVALTWNNLRKTLL SEQ ID NO: 390
363 RVALTWNNLRKTLLV SEQ ID NO: 391

IpaH4.5

SEQ ID NO: 7

5 >gi|12329057|emb|CAC05788.1| IpaH4.5, member of the IpaH family,
probably secreted by the Mxi-Spa secretion machinery, function unknown
[Shigella flexneri 5a str. M90T]

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Predicted PTD sequences:

48	NRIQAVRLLK	SEQ ID NO: 392	204	SLKYLKVGENQLRRL	SEQ ID NO: 437
49	RIQAVRLLKI	SEQ ID NO: 393	205	LKYLKVGENQLRRLS	SEQ ID NO: 438
50	IQAVRLLKIC	SEQ ID NO: 394	208	LKVGENQLRRLSRLP	SEQ ID NO: 439
51	QAVRLLKICL	SEQ ID NO: 395	204	SLKYLKVGENQLRRL	SEQ ID NO: 440
54	RLLKICLDTR	SEQ ID NO: 396	205	LKYLKVGENQLRRLS	SEQ ID NO: 441
208	LKVGENQLRR	SEQ ID NO: 397	208	LKVGENQLRRLSRLP	SEQ ID NO: 442
209	KVGENQLRRL	SEQ ID NO: 398	214	QLRRLSRLPQELLAL	SEQ ID NO: 443
530	SQRVADRLKA	SEQ ID NO: 399	381	DRVALTWNNLRKTLL	SEQ ID NO: 444
531	QRVADRLKAS	SEQ ID NO: 400	382	RVALTWNNLRKTLLV	SEQ ID NO: 445
532	RVADRLKASG	SEQ ID NO: 401	48	NRIQAVRLLKICLDTR	SEQ ID NO: 446
47	ENRIQAVRLLK	SEQ ID NO: 402	49	RIQAVRLLKICLDTRE	SEQ ID NO: 447
48	NRIQAVRLLKI	SEQ ID NO: 403	203	QSLKYLKVGENQLRRL	SEQ ID NO: 448
49	RIQAVRLLKIC	SEQ ID NO: 404	208	LKVGENQLRRLSRLPQ	SEQ ID NO:
208	LKVGENQLRRL	SEQ ID NO: 405	449		
531	QRVADRLKASG	SEQ ID NO: 406	213	NQLRRLSRLPQELLAL	SEQ ID NO:
532	RVADRLKASGL	SEQ ID NO: 407	450		
46	TENRIQAVRLLK	SEQ ID NO: 408	47	ENRIQAVRLLKICLDTR	SEQ ID NO: 451
47	ENRIQAVRLLKI	SEQ ID NO: 409	48	NRIQAVRLLKICLDTRE	SEQ ID NO:
48	NRIQAVRLLKIC	SEQ ID NO: 410	452		
49	RIQAVRLLKICL	SEQ ID NO: 411	205	LKYLKVGENQLRRLSRL	SEQ ID NO:
52	AVRLLKICLDTR	SEQ ID NO: 412	453		
216	RRLSRLPQELLA	SEQ ID NO: 413	204	SLKYLKVGENQLRRLSRL	SEQ ID NO:
531	QRVADRLKASGL	SEQ ID NO: 414	454		
46	TENRIQAVRLLKI	SEQ ID NO: 415	39	WAREGTTTENRIQAVRLLK	SEQ ID NO:
47	ENRIQAVRLLKIC	SEQ ID NO: 416	455		
48	NRIQAVRLLKICL	SEQ ID NO: 417	203	QSLKYLKVGENQLRRLSRL	SEQ ID
49	RIQAVRLLKICLD	SEQ ID NO: 418	NO: 456		
51	QAVRLLKICLDTR	SEQ ID NO: 419	208	LKVGENQLRRLSRLPQELL	SEQ ID NO:
208	LKVGENQLRRLSR	SEQ ID NO: 420	457		
209	KVGENQLRRLSRL	SEQ ID NO: 421	209	KVGENQLRRLSRLPQELLA	SEQ ID
215	LRRLSRLPQELLA	SEQ ID NO: 422	NO: 458		
216	RRLSRLPQELLAL	SEQ ID NO: 423	39	WAREGTTTENRIQAVRLLKI	SEQ ID NO:
382	RVALTWNNLRKTL	SEQ ID NO: 424	459		
47	ENRIQAVRLLKICL	SEQ ID NO: 425	208	LKVGENQLRRLSRLPQELLA	SEQ ID
48	NRIQAVRLLKICLD	SEQ ID NO: 426	NO: 460		
49	RIQAVRLLKICLDT	SEQ ID NO: 427	209	KVGENQLRRLSRLPQELLAL	SEQ ID
50	IQAVRLLKICLDTR	SEQ ID NO: 428	NO: 461		
205	LKYLKVGENQLRRL	SEQ ID NO: 429	205	LKYLKVGENQLRRLSRLPQEL	SEQ ID
208	LKVGENQLRRLSRL	SEQ ID NO: 430	NO: 462		
214	QLRRLSRLPQELLA	SEQ ID NO: 431	206	KYLKVGENQLRRLSRLPQELL	SEQ ID
215	LRRLSRLPQELLAL	SEQ ID NO: 432	NO: 463		
382	RVALTWNNLRKTLL	SEQ ID NO: 433	208	LKVGENQLRRLSRLPQELLAL	SEQ ID
46	TENRIQAVRLLKICL	SEQ ID NO: 434	NO: 464		
48	NRIQAVRLLKICLDT	SEQ ID NO: 435	54	RLLKICLDTREPVNLNLSLLKLR	SEQ ID NO:

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49	RIQAVRLLKICLDTR	SEQ ID NO: 436	465	
			205	LKYLKVGGENQLRRLSRLPQELL SEQ ID NO: 466
			206	KYLKVGGENQLRRLSRLPQELLA SEQ ID NO: 467
			207	YLKVGGENQLRRLSRLPQELLAL SEQ ID NO: 468
			205	LKYLKVGGENQLRRLSRLPQELLA SEQ ID NO: 469
			206	KYLKVGGENQLRRLSRLPQELLAL SEQ ID NO: 470
			204	SLKYLKVGGENQLRRLSRLPQELLA SEQ ID NO: 471
			205	LKYLKVGGENQLRRLSRLPQELLAL SEQ ID NO: 472
			206	KYLKVGGENQLRRLSRLPQELLALD SEQ ID NO: 473
			51	QAVRLLKICLDTREPVLNLSLLKLR SEQ ID NO: 474
			203	QSLKYLKVGGENQLRRLSRLPQELLA SEQ ID NO: 475
			204	SLKYLKVGGENQLRRLSRLPQELLAL SEQ ID NO: 476
			205	LKYLKVGGENQLRRLSRLPQELLALD SEQ ID NO: 477
			49	RIQAVRLLKICLDTREPVLNLSLLKL SEQ ID NO: 478
			50	IQAVRLLKICLDTREPVLNLSLLKLR SEQ ID NO: 479
			203	QSLKYLKVGGENQLRRLSRLPQELLAL SEQ ID NO: 480
			49	RIQAVRLLKICLDTREPVLNLSLLKLR SEQ ID NO: 481
			48	NRIQAVRLLKICLDTREPVLNLSLLKLR SEQ ID NO: 482
			49	RIQAVRLLKICLDTREPVLNLSLLKLRS SEQ ID NO: 483
			47	ENRIQAVRLLKICLDTREPVLNLSLLKLR SEQ ID NO: 484
			49	RIQAVRLLKICLDTREPVLNLSLLKLRSL SEQ ID NO: 485
			48	NRIQAVRLLKICLDTREPVLNLSLLKLRSL SEQ ID NO: 486

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IpaH7.8

SEQ ID NO: 8

>gi|12329056|emb|CAC05787.1| IpaH7.8, member of the IpaH family,
secreted by the Mxi-Spa secretion machinery, function unknown [Shigella
5 flexneri 5a str. M90T]

Predicted PTD sequences:

238	TRVLQSLQRL	SEQ ID NO: 487	510	RVADRLKASGL	SEQ ID NO: 493
239	RVLQSLQRLT	SEQ ID NO: 488	509	QRVADRLKASGL	SEQ ID NO: 494
508	SQRVADRLKA	SEQ ID NO: 489	360	RVALTWNNLRKTL	SEQ ID NO: 495
509	QRVADRLKAS	SEQ ID NO: 490	360	RVALTWNNLRKTLL	SEQ ID NO: 496
510	RVADRLKASG	SEQ ID NO: 491	359	DRVALTWNNLRKTLL	SEQ ID NO: 497
509	QRVADRLKASG	SEQ ID NO: 492	360	RVALTWNNLRKTLLV	SEQ ID NO: 498

IpaH9.8

10 SEQ ID NO: 9

>gi|12329122|emb|CAC05853.1| IpaH9.8, secreted by the Mxi-Spa secretion
machinery, function unknown [Shigella flexneri 5a str. M90T]

Predicted PTD sequences:

155	LPQALKNLRA	SEQ ID NO: 499	154	SLPQALKNLRATR	SEQ ID NO: 515
157	QALKNLRATR	SEQ ID NO: 500	155	LPQALKNLRATR	SEQ ID NO: 516
158	ALKNLRATR	SEQ ID NO: 501	158	ALKNLRATR	SEQ ID NO: 517
488	PQRVADRLKA	SEQ ID NO: 502	340	RVALTWNNLRKTL	SEQ ID NO: 518
489	QRVADRLKAS	SEQ ID NO: 503	488	PQRVADRLKASGL	SEQ ID NO: 519
490	RVADRLKASG	SEQ ID NO: 504	153	PSLPQALKNLRATR	SEQ ID NO: 520
156	PQALKNLRATR	SEQ ID NO: 505	154	SLPQALKNLRATR	SEQ ID NO: 521
488	PQRVADRLKAS	SEQ ID NO: 506	157	QALKNLRATR	SEQ ID NO: 522
489	QRVADRLKASG	SEQ ID NO: 507	340	RVALTWNNLRKTLL	SEQ ID NO: 523
490	RVADRLKASGL	SEQ ID NO: 508	152	LPSLPQALKNLRATR	SEQ ID NO: 524
155	LPQALKNLRATR	SEQ ID NO: 509	153	PSLPQALKNLRATR	SEQ ID NO: 525
489	QRVADRLKASGL	SEQ ID NO: 510	155	LPQALKNLRATR	SEQ ID NO: 526
151	SLPSLPQALKNLRATR	SEQ ID NO: 511	156	PQALKNLRATR	SEQ ID NO: 527
152	LPSLPQALKNLRATR	SEQ ID NO: 512			
154	SLPQALKNLRATR	SEQ ID NO: 513			
155	LPQALKNLRATR	SEQ ID NO: 514			

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339 DRVALTWNNLRKTLL SEQ ID
NO: 528
340 RVALTWNNLRKTLLV SEQ ID
NO: 529

SspH1

SEQ ID NO: 2

5 >gi|267993082|gb|ACY87967.1| SspH1 [Salmonella enterica subsp. enterica
serovar Typhimurium str. 14028S]

Predicted PTD sequences:

372	LSVRTLQALR	SEQ ID NO: 152	693	LTARWRLN	SEQ ID NO: 155
70	ARLKALTFFA	SEQ ID NO: 153	319	LQKLWAYNNRL	SEQ ID NO: 156
626	RFNALREKQI	SEQ ID NO: 154	476	ALRAKTFAMAT	SEQ ID NO: 157

10 SspH2

SEQ ID NO: 3

>gi|267994325|gb|ACY89210.1| leucine-rich repeat-containing protein
[Salmonella enterica subsp. enterica serovar Typhimurium str. 14028S]

15 Predicted PTD sequences:

185	SRGRAAVVQK	SEQ ID NO: 158	627	QIAREKVRTL	SEQ ID NO: 260
186	RGRAAVVQKM	SEQ ID NO: 159	628	IAREKVRTLA	SEQ ID NO: 261
187	GRAAVVQKMR	SEQ ID NO: 160	629	AREKVRTLAL	SEQ ID NO: 262
188	RAAVVQKMRA	SEQ ID NO: 161	630	REKVRTLALV	SEQ ID NO: 263
187	GRAAVVQKMRA	SEQ ID NO: 162	648	YQNKLKKS LG	SEQ ID NO: 264
188	RAAVVQKMRAC	SEQ ID NO: 163	649	QNK LK KSLGL	SEQ ID NO: 265
620	FRLGKLEQIAR	SEQ ID NO: 164	709	RKAPERVNAL	SEQ ID NO: 266
624	KLEQIAREKVR	SEQ ID NO: 165	710	KAPERVNALR	SEQ ID NO: 267
627	QIAREKVRTLA	SEQ ID NO: 166	646	LAYQNK LK KSL	SEQ ID NO: 268
628	IAREKVRTLAL	SEQ ID NO: 167	647	AYQNK LK KSLG	SEQ ID NO: 269
629	AREKVRTLALV	SEQ ID NO: 168	714	RVNALREKQIS	SEQ ID NO: 270
185	SRGRAAVVQKMR	SEQ ID NO: 169	12	ATISNRRRIYRIA	SEQ ID NO: 271
186	RGRAAVVQKMRA	SEQ ID NO: 170	619	MFRLGKLEQIAR	SEQ ID NO: 272
188	RAAVVQKMRACL	SEQ ID NO: 171	621	RLGKLEQIAREK	SEQ ID NO: 273
177	RRAAPAEESRGRA	SEQ ID NO: 172	623	GKLEQIAREKVR	SEQ ID NO: 274
185	SRGRAAVVQKMRA	SEQ ID NO: 173	627	QIAREKVRTLAL	SEQ ID NO: 275
186	RGRAAVVQKMRAC	SEQ ID NO: 174	628	IAREKVRTLALV	SEQ ID NO: 276
188	RAAVVQKMRACLN	SEQ ID NO: 175	646	LAYQNK LK KSLG	SEQ ID NO: 277

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620	FRLGKLEQIAREK	SEQ ID NO: 176	647	AYQNKLLKKSGL	SEQ ID NO: 278
621	RLGKLEQIAREKV	SEQ ID NO: 177	751	RTIGARAMESAKK	SEQ ID NO: 279
622	LGKLEQIAREKVR	SEQ ID NO: 178	177	RRAAPAEESRGRAA	SEQ ID NO:
627	QIAREKVRTLALV	SEQ ID NO: 179	280		
645	WLAYQNKLLKKSGL	SEQ ID NO: 180	184	ESRGRAAVVQKMRA	SEQ ID NO:
646	LAYQNKLLKKSGL	SEQ ID NO: 181	281		
705	RVLERKAPERVNAL	SEQ ID NO: 182	186	RGRAAVVQKMRACL	SEQ ID NO:
706	VLERKAPERVNALR	SEQ ID NO: 183	282		
710	KAPERVNALREKQI	SEQ ID NO: 184	619	MFRLGKLEQIAREK	SEQ ID NO: 283
751	RTIGARAMESAKKT	SEQ ID NO: 185	620	FRLGKLEQIAREKV	SEQ ID NO: 284
616	GREMFRLGKLEQIAR	SEQ ID NO:	621	RLGKLEQIAREKVR	SEQ ID NO: 285
186			644	VWLAYQNKLLKKSGL	SEQ ID NO:
619	MFRLGKLEQIAREKV	SEQ ID NO:	286		
187			645	WLAYQNKLLKKSGL	SEQ ID NO: 287
620	FRLGKLEQIAREKVR	SEQ ID NO: 188	177	RRAAPAEESRGRAAV	SEQ ID NO:
751	RTIGARAMESAKKTF	SEQ ID NO: 189	288		
621	RLGKLEQIAREKVRT	SEQ ID NO: 190	185	SRGRAAVVQKMRACL	SEQ ID NO:
644	VWLAYQNKLLKKSGL	SEQ ID NO:	289		
191			186	RGRAAVVQKMRACLN	SEQ ID NO:
645	WLAYQNKLLKKSGLT	SEQ ID NO:	290		
192			176	WRAAPAEESRGRAAV	SEQ ID NO:
706	VLERKAPERVNALREK	SEQ ID NO:	291		
193			177	RRAAPAEESRGRAAVV	SEQ ID NO:
751	RTIGARAMESAKKTFL	SEQ ID NO:	292		
194			182	AEESRGRAAVVQKMRA	SEQ ID NO:
176	WRAAPAEESRGRAAVV	SEQ ID NO:	293		
195			186	RGRAAVVQKMRACLN	SEQ ID NO:
177	RRAAPAEESRGRAAVVQ	SEQ ID NO:	294		
196			619	MFRLGKLEQIAREKVR	SEQ ID NO:
178	RAAPAEESRGRAAVVQK	SEQ ID NO:	295		
197			620	FRLGKLEQIAREKVRT	SEQ ID NO:
706	VLERKAPERVNALREKQ	SEQ ID NO:	296		
198			621	RLGKLEQIAREKVRTL	SEQ ID NO:
176	WRAAPAEESRGRAAVVQ	SEQ ID	297		
NO: 199			609	LAALVATGREMFRLGKL	SEQ ID NO:
177	RRAAPAEESRGRAAVVQK	SEQ ID NO:	298		
200			614	ATGREMFRLGKLEQIAR	SEQ ID NO:
615	TGREMFRLGKLEQIAREK	SEQ ID NO:	299		
201			616	GREMFRLGKLEQIAREK	SEQ ID NO:
619	MFRLGKLEQIAREKVRTL	SEQ ID NO:	300		
202			617	REMFRLGKLEQIAREKV	SEQ ID NO:
620	FRLGKLEQIAREKVRTLA	SEQ ID NO:	301		
203			618	EMFRLGKLEQIAREKVR	SEQ ID NO:
621	RLGKLEQIAREKVRTLAL	SEQ ID NO:	302		
204			619	MFRLGKLEQIAREKVRT	SEQ ID NO:

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175	AWRRAAPAEESRGRAAVVQ	SEQ ID	303		
NO: 205			620	FRLGKLEQIAREKVRTL	SEQ ID NO:
176	WRRAPAEESRGRAAVVQK	SEQ ID	304		
NO: 206			621	RLGKLEQIAREKVRTL	SEQ ID NO:
177	RRAAPAEESRGRAAVVQKM	SEQ ID	305		
NO: 207			706	VLERKAPERVNALREKQI	SEQ ID NO:
178	RAAPAEESRGRAAVVQKMR	SEQ ID	306		
NO: 208			707	LERKAPERVNALREKQIS	SEQ ID NO:
175	AWRRAAPAEESRGRAAVVQK	SEQ ID	307		
NO: 209			617	REMFRLGKLEQIAREKVRT	SEQ ID NO:
176	WRRAPAEESRGRAAVVQKM	SEQ ID	308		
NO: 210			618	EMFRLGKLEQIAREKVRTL	SEQ ID NO:
177	RRAAPAEESRGRAAVVQKMR	SEQ ID	309		
NO: 211			619	MFRLGKLEQIAREKVRTL	SEQ ID NO:
178	RAAPAEESRGRAAVVQKMRA	SEQ ID	310		
NO: 212			620	FRLGKLEQIAREKVRTLAL	SEQ ID NO:
705	RVLERKAPERVNALREKQIS	SEQ ID NO:	311		
213			621	RLGKLEQIAREKVRTLALV	SEQ ID NO:
751	RTIGARAMESAKKTFDGLR	SEQ ID	312		
NO: 214			611	ALVATGREMFRLGKLEQIAR	SEQ ID
174	SAWRRAPAEESRGRAAVVQK	SEQ ID	NO: 313		
NO: 215			615	TGREMFRLGKLEQIAREKVR	SEQ ID
175	AWRRAAPAEESRGRAAVVQKM	SEQ ID	NO: 314		
NO: 216			616	GREMFRLGKLEQIAREKVRT	SEQ ID
176	WRRAPAEESRGRAAVVQKMR	SEQ ID	NO: 315		
NO: 217			617	REMFRLGKLEQIAREKVRTL	SEQ ID NO:
177	RRAAPAEESRGRAAVVQKMRA	SEQ ID	316		
NO: 218			618	EMFRLGKLEQIAREKVRTL	SEQ ID NO:
610	AALVATGREMFRLGKLEQIAR	SEQ ID	317		
NO: 219			619	MFRLGKLEQIAREKVRTLAL	SEQ ID NO:
612	LVATGREMFRLGKLEQIAREK	SEQ ID	318		
NO: 220			620	FRLGKLEQIAREKVRTLALV	SEQ ID NO:
614	ATGREMFRLGKLEQIAREKVR	SEQ ID	319		
NO: 221			621	RLGKLEQIAREKVRTLALVD	SEQ ID NO:
616	GREMFRLGKLEQIAREKVRTL	SEQ ID	320		
NO: 222			705	RVLERKAPERVNALREKQIS	SEQ ID NO:
617	REMFRLGKLEQIAREKVRTL	SEQ ID	321		
NO: 223			751	RTIGARAMESAKKTFDGLR	SEQ ID
618	EMFRLGKLEQIAREKVRTLAL	SEQ ID	NO: 322		
NO: 224			174	SAWRRAPAEESRGRAAVVQK	SEQ ID
619	MFRLGKLEQIAREKVRTLALV	SEQ ID	NO: 323		
NO: 225			175	AWRRAAPAEESRGRAAVVQKM	SEQ ID
620	FRLGKLEQIAREKVRTLALVD	SEQ ID	NO: 324		
NO: 226			176	WRRAPAEESRGRAAVVQKMR	SEQ ID
621	RLGKLEQIAREKVRTLALVD	SEQ ID NO:	NO: 325		

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227			177	RRAAPAEESRGRAAVVQKMRA	SEQ ID
175	AWRRAAPAEESRGRAAVVQKMR	SEQ ID	NO: 326		
NO: 228			610	AALVATGREMFRLGKLEQIAR	SEQ ID
176	WRRAPAEESRGRAAVVQKMRA	SEQ ID	NO: 327		
NO: 229			612	LVATGREMFRLGKLEQIAREK	SEQ ID
177	RRAAPAEESRGRAAVVQKMRAC	SEQ ID	NO: 328		
NO: 230			614	ATGREMFRLGKLEQIAREKVR	SEQ ID
609	LAALVATGREMFRLGKLEQIAR	SEQ ID	NO: 329		
NO: 231			614	ATGREMFRLGKLEQIAREKVR	SEQ ID
611	ALVATGREMFRLGKLEQIAREK	SEQ ID	NO: 330		
NO: 232			616	GREMFRLGKLEQIAREKVRTL	SEQ ID
614	ATGREMFRLGKLEQIAREKVRT	SEQ ID	NO: 331		
NO: 233			617	REMFRLGKLEQIAREKVRTLA	SEQ ID
615	TGREMFRLGKLEQIAREKVRTL	SEQ ID	NO: 332		
NO: 234			618	EMFRLGKLEQIAREKVRTLAL	SEQ ID
616	GREMFRLGKLEQIAREKVRTLA	SEQ ID	NO: 333		
NO: 235			619	MFRLGKLEQIAREKVRTLALV	SEQ ID
617	REMFRLGKLEQIAREKVRTLAL	SEQ ID	NO: 334		
NO: 236			620	FRLGKLEQIAREKVRTLALVD	SEQ ID
618	EMFRLGKLEQIAREKVRTLALV	SEQ ID	NO: 335		
NO: 237			172	WWSAWRRAAPAEESRGRAAVVQK	SEQ
619	MFRLGKLEQIAREKVRTLALVD	SEQ ID	ID NO: 336		
NO: 238			174	SAWRRAAPAEESRGRAAVVQKMR	SEQ
173	WSAWRRAAPAEESRGRAAVVQKMR	SEQ	ID NO: 337		
ID NO: 239			175	AWRRAAPAEESRGRAAVVQKMRA	SEQ
174	SAWRRAAPAEESRGRAAVVQKMRA	SEQ	ID NO: 338		
ID NO: 240			176	WRRAPAEESRGRAAVVQKMRAC	SEQ
176	WRRAPAEESRGRAAVVQKMRACL	SEQ	ID NO: 339		
ID NO: 241			177	RRAAPAEESRGRAAVVQKMRACL	SEQ ID
177	RRAAPAEESRGRAAVVQKMRACLN	SEQ	NO: 340		
ID NO: 242			610	AALVATGREMFRLGKLEQIAREK	SEQ ID
609	LAALVATGREMFRLGKLEQIAREK	SEQ ID	NO: 341		
NO: 243			612	LVATGREMFRLGKLEQIAREKVR	SEQ ID
610	AALVATGREMFRLGKLEQIAREKV	SEQ ID	NO: 342		
NO: 244			614	ATGREMFRLGKLEQIAREKVRTL	SEQ ID
611	ALVATGREMFRLGKLEQIAREKVR	SEQ ID	NO: 343		
NO: 245			615	TGREMFRLGKLEQIAREKVRTLA	SEQ ID
614	ATGREMFRLGKLEQIAREKVRTLA	SEQ ID	NO: 344		
NO: 246			616	GREMFRLGKLEQIAREKVRTLAL	SEQ ID
615	TGREMFRLGKLEQIAREKVRTLAL	SEQ ID	NO: 345		
NO: 247			617	REMFRLGKLEQIAREKVRTLALV	SEQ ID
616	GREMFRLGKLEQIAREKVRTLALV	SEQ ID	NO: 346		
NO: 248			171	AWWSAWRRAAPAEESRGRAAVVQKMR	
617	REMFRLGKLEQIAREKVRTLALVD	SEQ ID	SEQ ID NO: 347		
NO: 249			172	WWSAWRRAAPAEESRGRAAVVQKMRA	

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608	DLAALVATGREMFRLGKLEQIAREKVR	SEQ ID NO: 250	609	LAALVATGREMFRLGKLEQIAREKVR	SEQ ID NO: 348
609	LAALVATGREMFRLGKLEQIAREKVRT	SEQ ID NO: 251	610	AALVATGREMFRLGKLEQIAREKVRT	SEQ ID NO: 349
610	AALVATGREMFRLGKLEQIAREKVRTL	SEQ ID NO: 252	611	ALVATGREMFRLGKLEQIAREKVRTL	SEQ ID NO: 350
611	ALVATGREMFRLGKLEQIAREKVRTLA	SEQ ID NO: 253	612	LVATGREMFRLGKLEQIAREKVRTLA	SEQ ID NO: 351
612	LVATGREMFRLGKLEQIAREKVRTLAL	SEQ ID NO: 254	613	VATGREMFRLGKLEQIAREKVRTLAL	SEQ ID NO: 352
614	ATGREMFRLGKLEQIAREKVRTLALVD	SEQ ID NO: 255	614	ATGREMFRLGKLEQIAREKVRTLALV	SEQ ID NO: 353
607	NDLAALVATGREMFRLGKLEQIAREKVRTL	SEQ ID NO: 256	171	AWWSAWRRAAPAEESRGRAAVVQKMRA	SEQ ID NO: 354
608	DLAALVATGREMFRLGKLEQIAREKVRTLA	SEQ ID NO: 257	607	NDLAALVATGREMFRLGKLEQIAREKVR	SEQ ID NO: 355
609	LAALVATGREMFRLGKLEQIAREKVRTLAL	SEQ ID NO: 258	608	DLAALVATGREMFRLGKLEQIAREKVRT	SEQ ID NO: 356
610	AALVATGREMFRLGKLEQIAREKVRTLALV	SEQ ID NO: 259	609	LAALVATGREMFRLGKLEQIAREKVRTL	SEQ ID NO: 357
			610	AALVATGREMFRLGKLEQIAREKVRTLA	SEQ ID NO: 358
			611	ALVATGREMFRLGKLEQIAREKVRTLAL	SEQ ID NO: 359
					SEQ ID NO: 360

SlrP

SEQ ID NO: 1

>gi|267992540|gb|ACY87425.1| leucine-rich repeat-containing protein
5 [Salmonella enterica subsp. enterica serovar Typhimurium str. 14028S]

Predicted PTD sequences:

593	SLAREKVKRL	SEQ ID NO: 66	173	RDCLKNNKTELRLKILG	SEQ ID NO: 110
175	CLKNNKTELRLKI	SEQ ID NO: 67	175	CLKNNKTELRLKILGT	SEQ ID NO: 111
176	LKNNKTELRLKIL	SEQ ID NO: 68	172	MRDCLKNNKTELRLKILG	SEQ ID NO: 112
177	KNNKTELRLKILG	SEQ ID NO: 69	173	RDCLKNNKTELRLKILGL	SEQ ID NO: 113
175	CLKNNKTELRLKIL	SEQ ID NO: 70	386	LPAALQIMQASRNNLVRL	SEQ ID NO: 114
176	LKNNKTELRLKILG	SEQ ID NO: 71	585	IFRLEQIESLAREKVKRL	SEQ ID NO: 115
177	KNNKTELRLKILGL	SEQ ID NO: 72	171	RMRDCLKNNKTELRLKILG	SEQ ID NO: 116
389	ALQIMQASRNNLVR	SEQ ID NO: 73	172	MRDCLKNNKTELRLKILGL	SEQ ID NO: 117
388	AALQIMQASRNNLVR	SEQ ID NO: 74	173	RDCLKNNKTELRLKILGLT	SEQ ID NO: 118
389	ALQIMQASRNNLVRL	SEQ ID NO: 75	171	RMRDCLKNNKTELRLKILGL	SEQ ID NO: 119

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175 CLKNNKTELRLKILGL SEQ ID NO: 76
 176 LKNNKTELRLKILGLT SEQ ID NO: 77
 388 AALQIMQASRNNLVRL SEQ ID NO: 78
 587 RLEQIESLAREKVKRL SEQ ID NO: 79
 581 AGREIFRLEQIESLAREKVKR SEQ ID NO:
 80
 582 GREIFRLEQIESLAREKVKRL SEQ ID NO:
 81
 167 EAVQMRDCLKNNKTELRLKIL SEQ ID
 NO: 82
 168 AVQMRDCLKNNKTELRLKILG SEQ ID
 NO: 83
 169 VQMRDCLKNNKTELRLKILGL SEQ ID
 NO: 84
 170 QMRDCLKNNKTELRLKILGLT SEQ ID
 NO: 85
 171 RMRDCLKNNKTELRLKILGLTT SEQ ID
 NO: 86
 580 MAGREIFRLEQIESLAREKVKR SEQ ID
 NO: 87
 581 AGREIFRLEQIESLAREKVKRL SEQ ID
 NO: 88
 167 EAVQMRDCLKNNKTELRLKILGL SEQ ID
 NO: 89
 168 AVQMRDCLKNNKTELRLKILGLT SEQ ID
 NO: 90
 170 QMRDCLKNNKTELRLKILGLTTI SEQ ID
 NO: 91
 578 LIMAGREIFRLEQIESLAREKVKR SEQ ID
 NO: 92
 579 IMAGREIFRLEQIESLAREKVKRL SEQ ID
 NO: 93
 578 LIMAGREIFRLEQIESLAREKVKRL SEQ ID
 NO: 94
 164 NREEAVQMRDCLKNNKTELRLKILG SEQ
 ID NO: 95
 165 REEAVQMRDCLKNNKTELRLKILGL SEQ
 ID NO: 96
 168 AVQMRDCLKNNKTELRLKILGLTTI SEQ ID
 NO: 97
 161 EAANREEAVQMRDCLKNNKTELRLKIL
 SEQ ID NO: 98
 162 AANREEAVQMRDCLKNNKTELRLKILG
 SEQ ID NO: 99
 163 ANREEAVQMRDCLKNNKTELRLKILGL

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172 MRDCLKNNKTELRLKILGLT SEQ ID NO: 120
 173 RDCLKNNKTELRLKILGLTT SEQ ID NO: 121
 168 AVQMRDCLKNNKTELRLKIL SEQ ID NO: 122
 169 VQMRDCLKNNKTELRLKILG SEQ ID NO:
 170 QMRDCLKNNKTELRLKILGL SEQ ID NO:
 171 RMRDCLKNNKTELRLKILGLT SEQ ID NO: 125
 173 RDCLKNNKTELRLKILGLTTI SEQ ID NO: 126
 167 EAVQMRDCLKNNKTELRLKILG SEQ ID NO:
 127
 168 AVQMRDCLKNNKTELRLKILGL SEQ ID NO:
 128
 169 VQMRDCLKNNKTELRLKILGLT SEQ ID NO:
 129
 170 QMRDCLKNNKTELRLKILGLTT SEQ ID NO:
 130
 171 RMRDCLKNNKTELRLKILGLTTI SEQ ID NO:
 131
 579 IMAGREIFRLEQIESLAREKVKR SEQ ID NO:
 132
 580 MAGREIFRLEQIESLAREKVKRL SEQ ID NO:
 581 AGREIFRLEQIESLAREKVKRLF SEQ ID NO:
 134
 167 EAVQMRDCLKNNKTELRLKILGL SEQ ID NO:
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 168 AVQMRDCLKNNKTELRLKILGLT SEQ ID NO:
 136
 165 REEAVQMRDCLKNNKTELRLKILG SEQ ID NO:
 137
 167 EAVQMRDCLKNNKTELRLKILGLT SEQ ID NO:
 138
 168 AVQMRDCLKNNKTELRLKILGLTT SEQ ID NO:
 139
 171 RMRDCLKNNKTELRLKILGLTTIPA SEQ ID NO:
 140
 162 AANREEAVQMRDCLKNNKTELRLKIL SEQ ID
 NO: 141
 163 ANREEAVQMRDCLKNNKTELRLKILG SEQ ID
 NO: 142
 164 NREEAVQMRDCLKNNKTELRLKILGL SEQ ID
 NO: 143
 165 REEAVQMRDCLKNNKTELRLKILGLT SEQ ID
 NO: 144

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SEQ ID NO: 100	157	APAKEAANREEAVQRMRDCLKNNKTELRLK SEQ
164 NREEAVQRMRDCLKNNKTELRLKILGLT	ID NO: 145	
SEQ ID NO: 101	158	PAKEAANREEAVQRMRDCLKNNKTELRLKI SEQ
165 REEAVQRMRDCLKNNKTELRLKILGLTT SEQ	ID NO: 146	
ID NO: 102	159	AKEAANREEAVQRMRDCLKNNKTELRLKIL SEQ
168 AVQRMRDCLKNNKTELRLKILGLTTIPA SEQ	ID NO: 147	
ID NO: 103	160	KEAANREEAVQRMRDCLKNNKTELRLKILG SEQ
157 APAKEAANREEAVQRMRDCLKNNKTELRL	ID NO: 148	
SEQ ID NO: 104	161	EAANREEAVQRMRDCLKNNKTELRLKILGL
159 AKEAANREEAVQRMRDCLKNNKTELRLKI	SEQ ID NO: 149	
SEQ ID NO: 105	162	AANREEAVQRMRDCLKNNKTELRLKILGLT SEQ
161 EAANREEAVQRMRDCLKNNKTELRLKILG	ID NO: 150	
SEQ ID NO: 106	163	ANREEAVQRMRDCLKNNKTELRLKILGLTT SEQ
162 AANREEAVQRMRDCLKNNKTELRLKILGL	ID NO: 151	
SEQ ID NO: 107		
163 ANREEAVQRMRDCLKNNKTELRLKILGLT		
SEQ ID NO: 108		
165 REEAVQRMRDCLKNNKTELRLKILGLTTI		
SEQ ID NO: 109		

EXAMPLE 13

Analysis of T3SS- independent Internalisation of Bacterial Effector Proteins of the LPX Subtype

- 5 Bacterial pathogens including *Salmonella*, *Yersinia* and *Shigella* spp. express effector proteins of the LPX subtype of leucine-rich repeat (LRR) proteins that are translocated into the host cell via a type three secretion system (T3SS) during infection.
- Previous studies identified the LPX effector protein YopM of *Yersinia enterocolitica* as a novel bacterial cell-penetrating protein. YopM's ability to translocate across the host cell plasma
- 10 membrane independently of *Yersinia*'s T3SS is mediated by it's two N-terminal α -Helices.
- The inventors therefore constructed and recombinantly expressed LPX effector proteins of *Shigella flexneri* and *Salmonella typhimurium*. Potential T3SS-independent translocation of these proteins was analysed by cell fractionation of HeLa cells, immunofluorescence microscopy and FACS analyses. Functionality of the recombinant proteins was assessed by in vitro ubiquitination assays.
- 15 Additionally, an effect of the recombinant proteins on the expression of pro-inflammatory cytokines was analysed by quantitative real time PCR.
- The inventors could show that the SspH1 effector protein of *Salmonella typhimurium* is able to translocate into eukaryotic cells without a requirement for additional factors. Furthermore the inventors could show that recombinant SspH1 is a functional E3 ubiquitin ligase that is able to
- 20 reduce the expression of Interleukin 8 in IL1 β stimulated cells.

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These results show that SspH1 is a novel bacterial cell-penetrating protein and, together with YopM, a hint for a general concept of T3SS-independent translocation by LPX effector proteins.

EXAMPLE 14

Group 1:

Leucin-rich repeats of SspH1, wherein SspH1 has the amino acid sequence indicated as SEQ ID NO: 2.

- LRR1 (identification number 1): AA217-238 of SEQ ID NO: 2.
- 10 LRR2 (identification number 2): AA239-257 of SEQ ID NO: 2.
- LRR3 (identification number 3): AA258-279 of SEQ ID NO: 2.
- LRR4 (identification number 4): AA280-297 of SEQ ID NO: 2.
- LRR5 (identification number 5): AA 298-319 of SEQ ID NO: 2.
- LRR6 (identification number 6): AA320-337 of SEQ ID NO: 2.
- 15 LRR7 (identification number 7): AA338-360 of SEQ ID NO: 2.
- LRR8 (identification number 8): AA361-381 of SEQ ID NO: 2.

Group 2:

- 20 Leucin-rich repeats of SspH2, wherein SspH2 has the amino acid sequence indicated as SEQ ID NO: 3.

- LRR1 (identification number 1): AA223-242 of SEQ ID NO: 3.
- LRR2 (identification number 2): AA243-264 of SEQ ID NO: 3.
- 25 LRR3 (identification number 3): AA265-282 of SEQ ID NO: 3.
- LRR4 (identification number 4): AA283-302 of SEQ ID NO: 3.
- LRR5 (identification number 5): AA303-324 of SEQ ID NO: 3.
- LRR6 (identification number 6): AA325-342 of SEQ ID NO: 3.
- LRR7 (identification number 7): AA343-364 of SEQ ID NO: 3.
- 30 LRR8 (identification number 8): AA365-382 of SEQ ID NO: 3.
- LRR9 (identification number 9): AA383-404 of SEQ ID NO: 3.
- LRR10 (identification number 10): AA405-422 of SEQ ID NO: 3.
- LRR11 (identification number 11): AA423-445 of SEQ ID NO: 3.
- LRR12 (identification number 12): AA446-466 of SEQ ID NO: 3.

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Group 3:

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Leucin-rich repeats of Slrp, wherein Slrp has the amino acid sequence indicated as SEQ ID NO: 1.

LRR1 (identification number 1): AA200-219 of SEQ ID NO: 1.

LRR2 (identification number 2): AA221-242 SEQ ID NO: 1.

5 LRR3 (identification number 3): AA243-262 SEQ ID NO: 1.

LRR4 (identification number 4): AA263-284 SEQ ID NO: 1.

LRR5 (identification number 5): AA285-305 SEQ ID NO: 1.

LRR6 (identification number 6): AA306-325 SEQ ID NO: 1.

LRR7 (identification number 7): AA326-346 SEQ ID NO: 1.

10 LRR8 (identification number 8): AA347-368 SEQ ID NO: 1.

LRR9 (identification number 9): AA369-389 SEQ ID NO: 1.

LRR10 (identification number 10): AA390-410 SEQ ID NO: 1.

15 **Group 4:**

Predicted Leucin-rich repeats of IpaH1.4, wherein IpaH1.4 has the amino acid sequence indicated as SEQ ID NO: 4.

LRR1 (identification number 1): AA92 – 113 of SEQ ID NO: 4.

LRR2 (identification number 2): AA132 – 153 of SEQ ID NO: 4.

LRR3 (identification number 3): AA172 – 191 of SEQ ID NO: 4.

LRR4 (identification number 4): AA192 – 213 of SEQ ID NO: 4.

Group 5:

25 Predicted Leucin-rich repeats of IpaH2.5, wherein IpaH2.5 has the amino acid sequence indicated as SEQ ID NO: 5.

LRR1 (identification number 1): AA92 – 113 of SEQ ID NO: 5

LRR2 (identification number 2): AA132 – 153 of SEQ ID NO: 5

LRR3 (identification number 3): AA172 - 191 of SEQ ID NO: 5

30 LRR4 (identification number 4): AA192 - 213 of SEQ ID NO: 5

Group 6:

35 Predicted Leucin-rich repeats of IpaH3, wherein IpaH3 has the amino acid sequence indicated as SEQ ID NO: 6.

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LRR1 (identification number 1): AA80 – 99 of SEQ ID NO: 6
 LRR2 (identification number 2): AA100 – 121 of SEQ ID NO: 6
 LRR3 (identification number 3): AA140 – 161 of SEQ ID NO: 6
 LRR4 (identification number 4): AA162 – 179 of SEQ ID NO: 6
 LRR5 (identification number 5): AA180 – 201 of SEQ ID NO: 6
 LRR6 (identification number 6): AA220 – 241 of SEQ ID NO: 6

Group 7:

Leucin-rich repeats of IpaH4.5, wherein IpaH4.5 has the amino acid sequence indicated as SEQ ID
 5 NO: 7.

LRR1 (identification number 1): AA63-82 of SEQ ID NO: 7.
 LRR2 (identification number 2): AA83-104 of SEQ ID NO: 7.
 LRR3 (identification number 3): AA105-122 of SEQ ID NO: 7.
 10 LRR4 (identification number 4): AA123-143 of SEQ ID NO: 7.
 LRR5 (identification number 5): AA144-165 of SEQ ID NO: 7.
 LRR6 (identification number 6): AA 166-183 of SEQ ID NO: 7.
 LRR7 (identification number 7): AA 184-205 of SEQ ID NO: 7.
 LRR8 (identification number 8): AA206-223 of SEQ ID NO: 7.
 15 LRR9 (identification number 9): AA224-246 of SEQ ID NO: 7.
 LRR10 (identification number 10): AA247-270 of SEQ ID NO: 7.

Group 8:

Leucin-rich repeats of IpaH7.8, wherein IpaH7.8 has the amino acid sequence indicated as SEQ ID
 20 NO: 8.

LRR1 (identification number 1): AA58-79 of SEQ ID NO: 8.
 LRR2 (identification number 2): AA80-97 of SEQ ID NO: 8.
 LRR3 (identification number 3): AA98-119 of SEQ ID NO: 8.
 25 LRR4 (identification number 4): AA120-137 of SEQ ID NO: 8.
 LRR5 (identification number 5): AA138-157 of SEQ ID NO: 8.
 LRR6 (identification number 6): AA158-179 of SEQ ID NO: 8.
 LRR7 (identification number 7): AA180-199 of SEQ ID NO: 8.
 LRR8 (identification number 8): AA202-223 of SEQ ID NO: 8.
 30 LRR9 (identification number 9): AA225-248 of SEQ ID NO: 8.

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Group 9:

Leucin-rich repeats of IpaH9.8, wherein IpaH9.8 has the amino acid sequence indicated as SEQ ID NO: 9.

- 5 LRR1 (identification number 1): AA57-77 of SEQ ID NO: 9.
- LRR2 (identification number 1): 78-99 of SEQ ID NO: 9.
- LRR3 (identification number 1): 100-117 of SEQ ID NO: 9.
- LRR4 (identification number 1): 118-139 of SEQ ID NO: 9.
- LRR5 (identification number 1): 140-157 of SEQ ID NO: 9.
- 10 LRR6 (identification number 1): 158-179 of SEQ ID NO: 9.
- LRR7 (identification number 1): 182-203 of SEQ ID NO: 9.
- LRR8 (identification number 1): 205-228 of SEQ ID NO: 9.

EXAMPLE 15

- 15 The uptake of recombinant LPX effector proteins was further analyzed by sub-cellular fractionation of eukaryotic HeLa cells. By this method, the internalization of putative CPPs can be assessed due to the separation of soluble cytoplasmic and insoluble membrane proteins (Behrens, 1938; Rüter *et al.*, 2010). HeLa cells which were grown to 80 % confluence were incubated with the recombinant proteins (25 µg/ml) for 3 h. After isolation of cytoplasmic and membrane fractions, proteins were
- 20 separated by SDS-PAGE and subsequently immobilized on a nitrocellulose membrane by Western blotting. For detection of the recombinant protein, an α -FLAG-antibody was used as a primary antibody. In case of internalization, proteins were expected to be detected in the cytoplasmic fraction. Since recombinant LPX effector proteins harboring only a single FLAG-tag were not detectable in the HeLa cell background at all (data not shown), constructs with 3xFLAG-tags were
- 25 chosen for this assay due to their improved detectability (Terpe, 2003).

- Recombinant IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, and IpaH9.8 could be detected in the cytoplasmic fraction after 3 h incubation of HeLa cells, indicating successful internalization in a T3SS-independent manner (Figure 23). However, all proteins were also detected in the membrane
- 30 fraction in comparable amounts. Upon incubation with IpaH4.5 and IpaH7.8 the amounts found in the membrane fraction appear to be even slightly higher. Both the cytoplasmic and the membrane fraction appear to be free of contamination since the membrane marker transferring receptor (TF-R) is solely detectable in the membrane fraction, whereas a prominent band for GAPDH can be observed in all cytoplasmic but not in the membrane fractions.

35

EXAMPLE 16**Analysis of Intracellular Trafficking of Recombinant LPX Effector Proteins by Co-localization with Endocytic Markers**

For investigations of possible endocytic uptake mechanisms of recombinant LPX effector proteins, co-localization studies with endocytic markers were performed. Appropriate markers for this purpose are regulatory proteins which are involved in intracellular membrane trafficking between different sub-cellular compartments. These include both vesicle formation and movements as well as membrane fusion. Rab5 served as marker for early endosomes, whereas Rab7 was used as an indicator for maturation into late endosomes. In addition, CD63 served as marker for trafficking from late endosomes to lysosomes.

HeLa cells were incubated with recombinant Cy3-labeled LPX effector proteins for 1 h, 3 h, and 6 h. Following incubation, cells were washed, fixed and permeabilized. Cell-compartment specific markers including Rab5, Rab7, and CD63 were stained using specific fluorescent antibodies. In addition, the nucleus was stained using Draq5. Finally, the co-localization with the Cy3-labeled LPX effector proteins was analyzed by confocal fluorescence microscopy. As depicted in Figure 24 all tested LPX effector proteins were found to be partially co-localized with endosomal compartments. For each marker protein, representative pictures which revealed the highest amount of co-localization are shown; in case of Rab5, pictures taken after 1 h incubation of HeLa cells with labeled proteins show on average the highest degrees of co localization, whereas for Rab7 and CD63 maximal co-localizations were observed only after longer incubation times of 3 h and 6 h.

All tested LPX effector proteins were found to be partially associated with early endosomes (i) since co-localization with the early endosomal marker Rab5 can be detected to some extent. After internalization, the proteins seem to remain in the endosomal compartments; so all shown LPX effector proteins co-localize with the late endosomal marker protein Rab7 (ii) after 3 h incubation. In addition, the overlay images reveal co-localization with CD63 (iii) after 6 h incubation.

In summary, fluorescence microscopy studies reveal partial co-localization with the endosomal markers Rab5, Rab7, and CD63 indicating that endocytic mechanisms seem to be involved in the T3SS-independent uptake of the recombinant LPX effectors IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH9.8, and SlrP into HeLa cells.

EXAMPLE 16

Role of different endocytic pathways in HeLa cell uptake of recombinant LPX effector proteins

HeLa cells were with endocytic inhibitors cytochalasin D (2.5 μ M), amiloride (3 mM), filipin (7.5 μ M), nocodazole (20 μ M), dynasore (80 μ M), methyl- β -cyclodextrin (M β CD) (5 mM) for 1 h prior to the addition of recombinant LPX effectors. Three hours later the cells were washed with D-PBS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$), trypsinized, resuspended in D-PBS (without $\text{Ca}^{2+}/\text{Mg}^{2+}$), diluted with trypan blue (final concentration 0.2 %) and analyzed by flow cytometry. Fluorescence of Alexa Fluor[®] 488 was detected at 510 nm. For exclusion of dead cells and cellular debris, forward and side scatter were set up appropriately and scaled to the living cell population before starting the incubation. Each sample was measured in duplicate counting 10,000 cells in every run. Three independent assays were performed for each protein.

Figure 25 summarizes the results of FACS-based uptake analysis of recombinant LPX effectors in the presence of different endocytic inhibitors. It can be recognized that the different LPX effectors act similar in response to a certain inhibitor. Especially the presence of cytochalasin D which effectively blocks actin-mediated endocytosis, of amiloride which inhibits macropinocytotic events as well as of methyl- β -cyclodextrin which serves as an inhibitor of "lipid-raft"-mediated endocytosis, leads to an enormous reduction of cellular internalization of all tested recombinant LPX effectors. These results indicate that especially the aforementioned endocytic pathways might act synergistically in the cellular transduction of LPX effectors.

20 EXAMPLE 18

For investigation of possible membranolytic effects of recombinant LPX effectors, the FACS-based membranolysis assay was performed (Florén *et al.*, 2011).

HeLa cells were co-incubated with 1 μ g/ml PI and different Alexa Fluor 488-labeled proteins. At given time points, samples were taken from the ongoing incubation and subjected to FACS analysis. Fluorescence of PI was detected at 617 nm. For exclusion of dead cells and cellular debris, forward and side scatter were set up appropriately and scaled to the living cell population before starting the incubation. Each sample was measured in duplicate counting 10,000 cells in every run. Three independent assays were performed for each protein. As a control, HeLa cells were incubated solely with PI under equal conditions.

Figure 26 summarizes the results of the membranolysis assays of HeLa cells which were incubated with recombinant LPX effector proteins. Disruption of the membrane leads to increased fluorescence intensities of PI due to increased permeability. Indeed, for all tested LPX effector proteins an increase of PI fluorescence intensity over the entire time is visible when HeLa cells were incubated at 37°C. However, this increase appears to slightly differ for the tested LPX

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effectors. In direct comparison to non-treated control cells (represented by dashed-lined curves), the strongest increase of PI fluorescence is induced by incubation with IpaH2.5, whereas upon incubation with IpaH9.8 almost no difference to non-treated cells is detectable. In contrast, co-incubation of HeLa cells with LPX effectors and PI at 4°C does not result in increased levels of PI fluorescence intensity and is almost comparable to non-treated control cells.

Example 19

Quantification of Lactate Dehydrogenase (LDH) Release Induced by Recombinant LPX Effector Proteins

For the evaluation of putative cytotoxic effects of recombinant LPX effector proteins on HeLa cells, the amount of released lactate dehydrogenase (LDH) was taken as a parameter for membrane integrity and measured colorimetrically using the CytoTox 96 Non-Radioactive Cytotoxicity Assay. For that, HeLa cells were cultured in 96-well plates and incubated with different recombinant LPX effector proteins (25 or 50 µg/ml) for 24 h, 6 h, and 1 h. The amounts of released LDH of both culture supernatants and lysates were determined by measuring the absorbance at 490 nm. The quotient of both was calculated and normalized to non-treated cells.

Figure 27 shows the relative LDH release of HeLa cells upon incubation with recombinant IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH9.8, or SlrP in comparison to non-treated cells and a LDH positive control. The measured amounts of LDH released upon incubation with the specific LPX effector proteins are comparable to basal LDH release displayed by HeLa cells incubated in culture medium (Medium Control). This appears to be independent of the protein concentration (25 µg/ml or 50 µg/ml) or the incubation time (24 h, 6 h, or 1 h). The large amounts of LDH measured for the positive control ensure that the assay was functional.

Based on the amounts of LDH released by HeLa cells upon incubation with recombinant IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH9.8, or SlrP potential cytotoxic effects of these LPX effector proteins can be excluded.

Example 20

The proteins of the IpaH subset as well as the LPX effector SlrP and the SspH proteins of *Salmonella* harbor an enzymatic domain that was shown to possess an E3 ubiquitin ligase activity (Rohde *et al.*, 2007; Singer *et al.*, 2008; Zhu *et al.*, 2008). In order to test whether recombinant LPX effector proteins are enzymatically functional as ubiquitin E3 ligases an *in vitro* ubiquitination assay was carried out. The assay was performed in a 40 µl reaction mixture containing ubiquitin reaction buffer, 2 µg of HA-tagged ubiquitin, 0.5 µg of E1 and 2 mg of E2 (UbcH5b) in the presence or

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absence of 4 µg of recombinant IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH9.8, SlrP, or BSA as a negative control. As further controls, the mixtures were prepared without the E2 (UbcH5b) enzyme. Reactions were incubated for 1 h at 37°C before they were stopped by the addition of 4 x SDS sample buffer without DTT.

5

The results of Western blot analysis which are depicted in Figure 28 reveal a ladder of ubiquitin chains from ~40 to 200 kDa in reactions performed in the presence of LPX effectors. In reactions performed in the absence of E2 (UbcH5b), if anything, only ubiquitinated E1 at a size of >110 kDa was detected. In the presence of E2 (UbcH5b) but in the absence of any LPX effector proteins
10 (see lane 1+2) both E1 (>110 kDa) and E2 (~30 kDa) were found to be ubiquitinated.

The results of the in vitro ubiquitination assay show that recombinant IpaH1.4, IpaH2.5, IpaH4.5, IpaH7.8, IpaH9.8, and SlrP have the ability to remove ubiquitin from ubiquitinated UbcH5b in order to form poly-ubiquitin chains of the HA-tagged ubiquitin. These findings confirm that the indicated
15 LPX effector proteins are indeed enzymatically functional in vitro.

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CLAIMS

1. A pharmaceutical composition, comprising an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it
- 5 a) is recombinantly produced or chemically synthesized,
- b) comprises an E3 ubiquitin ligase domain,
- c) optionally comprises at least one Leucin-rich repeat, and
- d) is a cell-penetrating protein, which translocates into eukaryotic cells without the
- 10 requirement of a bacterial T3SS.
2. A pharmaceutical composition, comprising a variant, fragment, or immunomodulatory domain of an effector protein according to claim 1.
- 15 3. The pharmaceutical composition of claim 2, wherein said variant, fragment, or immunomodulatory domain is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.
4. The pharmaceutical composition of claim 2 or 3, wherein said variant, fragment, or
- 20 immunomodulatory domain comprises an E3 ubiquitin ligase domain and optionally at least one Leucin-rich repeat.
5. The pharmaceutical composition of claims 1 to 4, wherein said type III secretion system (T3SS)-containing bacterium is classified as *Salmonella bongori*, *Salmonella enterica*, *Salmonella* subterranean, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella enterica* serovar typhimurium, *Salmonella enteritidis*, *Salmonella pullorum*, *Salmonella dublin*, *Salmonella arizonae*, *Salmonella choleraesuis*, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella sonnei*, or *Shigella boydii*.
- 25
6. The pharmaceutical composition of claims 1 to 5, wherein the E3 ubiquitin ligase domain is
- 30 classified as Novel E3 Ligase.
7. The pharmaceutical composition of claims 1 to 6, wherein the E3 ubiquitin ligase domain is closer to the C-terminus of said effector protein than to the N-terminus of said effector protein.

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8. The pharmaceutical composition of claims 1 to 7, wherein the Leucin-rich repeat(s) is/are a Leucin-rich repeat of the LPX-subtype.
9. The pharmaceutical composition of claims 1 to 8, wherein the Leucin-rich repeat(s) is/are closer to the N-terminus of said effector protein than to the C-terminus of said effector protein.
10. The pharmaceutical composition of claims 1 to 9, wherein said effector protein is a bacterial effector protein of the LPX-Subtype.
11. The pharmaceutical composition of claims 1 to 10, wherein said effector protein is SspH1, SspH2, SlrP, IpaH1.4, IpaH2.5, IpaH3, IpaH4.5, IpaH7.8, and IpaH9.8.
12. The pharmaceutical composition of claims 1 to 11, wherein said effector protein has an amino acid sequence selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9.
13. The pharmaceutical composition of claims 1 to 12, wherein said effector protein is encoded by a polynucleotide selected from the group consisting of SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, or SEQ ID NO: 18.
14. The pharmaceutical composition of claims 1 to 13, wherein said effector protein, variant, fragment or immunomodulatory domain comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, or SEQ ID NO: 27.
15. The pharmaceutical composition of claims 1 to 14, wherein said effector protein comprises at least one Leucine-rich repeat, preferably at least one Leucine-rich repeat that is comprised in an amino acid sequence selected from the group consisting of SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, or SEQ ID NO: 36.

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16. The pharmaceutical composition of claims 1 to 15, wherein said effector protein, variant, fragment or immunomodulatory domain comprises at least one amino acid sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, or SEQ ID NO: 550, and/or at least one amino acid sequence that corresponds to a C-terminally or N-terminally truncated fragment of an amino acid sequence selected from the group consisting of SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64, SEQ ID NO: 65, or SEQ ID NO: 550.

17. The pharmaceutical composition of claims 1 to 16, wherein the composition comprises no auxiliary agent which can cause the penetration of said effector protein into a eukaryotic cell.

18. The pharmaceutical composition of claims 1 to 17, wherein the composition comprises no auxiliary agent selected from the group consisting of T3SS-containing bacterium, bacterium of the genus Salmonella or Shigella, and cell-penetrating molecule, including cell-penetrating protein that is different from an effector protein according to claim 1 and cell-penetrating nanoparticle.

19. The pharmaceutical composition of claims 1 to 18, wherein said effector protein or variant, fragment or immunomodulatory domain thereof is linked to a cell-specific targeting agent.

20. The pharmaceutical composition of claims 1 to 19, wherein said effector protein or variant, fragment or immunomodulatory domain thereof is linked to a cargo molecule.

30

21. The pharmaceutical composition of claim 20, wherein said cargo molecule displays a therapeutic and/or diagnostic activity.

22. The pharmaceutical composition of claim 20 or 21, wherein said cargo molecule comprises at least one compound selected from the group consisting of nucleic acids, polypeptides, organic

35

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molecules, small organic molecules, metals, nanoparticles, viruses, modified viruses, viral vectors, antibodies and/or plasmids.

23. The pharmaceutical composition of claims 1 to 22, wherein said effector protein or variant,
5 fragment or immunomodulatory domain thereof ubiquitinates itself and/or eukaryotic proteins after it has autonomously penetrated into a eukaryotic cell.

24. The pharmaceutical composition of claims 1 to 23, wherein said effector protein or variant,
fragment or immunomodulatory domain thereof modulates cellular pathway(s) of the innate
10 immune system of eukaryotic cells after it has autonomously penetrated into said eukaryotic cells.

25. The pharmaceutical composition of claim 1 to 24, wherein said effector protein or variant,
fragment or immunomodulatory domain thereof modulates cytokines and/or cytokine receptors of
eukaryotic cells and/or eukaryotic genes which respond to cytokines of eukaryotic cells after said
15 effector protein or variant, fragment or immunomodulatory domain has autonomously penetrated into said eukaryotic cells.

26. The pharmaceutical composition of claims 1 to 25, wherein said effector protein or variant,
fragment or immunomodulatory domain thereof downregulates the expression of cytokines and/or
20 cytokine receptors of eukaryotic cells after it has autonomously penetrated into said eukaryotic cells.

27. The pharmaceutical composition of claims 1 to 26 for use in immunomodulatory therapy of
animals or humans.
25

28. The pharmaceutical composition of claims 1 to 27 for use in immunosuppressive therapy of
animals or humans.

29. The pharmaceutical composition of claims 1 to 28 for use in the regulation of inflammatory
30 reactions of the immune system, treatment of diseases caused by autoimmunity, acute inflammation or chronic inflammation, treatment of inflammatory disorders, and/or for suppressing the immune system.

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30. The pharmaceutical composition of claims 1 to 25, for use in delivering cargo molecules across the membrane of a eukaryotic cell.
31. A method of treatment of diseases caused by autoimmunity, treatment of acute inflammation, chronic inflammation, inflammatory disorders, pathogenic inflammatory reactions of the immune system, and/or a method of suppressing the immune system in a subject, comprising administering a therapeutically effective amount of a pharmaceutical composition according to any one of the preceding claims to said subject.
32. A kit comprising the pharmaceutical composition of any one of the preceding claims.
33. Use of an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it
- a) is recombinantly produced or chemically synthesized
 - b) comprises an E3 ubiquitin ligase domain, preferably an Novel E3 ubiquitin ligase
 - c) optionally comprises at least one Leucin-rich repeat;
 - d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS;
- or use of a variant, fragment or immunomodulatory domain of said effector protein, for the preparation of a pharmaceutical composition for regulating inflammatory reactions of the immune system, treating diseases caused by autoimmunity, treating acute inflammation or chronic inflammation, treating of inflammatory disorders, and/or suppressing the immune system.
34. Use of an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it
- a) is recombinantly produced or chemically synthesized
 - b) comprises an E3 ubiquitin ligase domain, preferably an Novel E3 ubiquitin ligase
 - c) optionally comprises at least one Leucin-rich repeat;
 - d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS;

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or use of a variant, fragment of said effector protein for the preparation of a pharmaceutical composition for delivering at least one cargo molecule across the membrane of a eukaryotic cell, wherein the fusion construct of cargo molecule and said effector protein or variant or fragment thereof is a cell-penetrating construct, which translocates into eukaryotic cells without the
 5 requirement of a bacterial T3SS.

35. Use of an isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella*, wherein the effector protein is characterized in that it
- 10 a) is recombinantly produced or chemically synthesized,
 - b) optionally comprises an E3 ubiquitin ligase domain,
 - c) optionally comprises at least one Leucin-rich repeat,
 - d) is a cell-penetrating protein, which translocates into eukaryotic cells without the requirement of a bacterial T3SS, and
 - 15 e) is linked to at least one cargo molecule,

for delivering the at least one cargo molecule across the membrane of a eukaryotic cell, wherein the fusion construct of cargo molecule(s) and said effector protein is a cell-penetrating construct, which translocates into eukaryotic cells without the requirement of a bacterial T3SS.

- 20 36. The use of claim 34 or 35, wherein said cargo molecule comprises at least one compound selected from the group consisting of nucleic acids, polypeptides, organic molecules, small organic molecules, metals, dye, nano-particles, viruses, modified viruses, viral vectors, antibodies and/or plasmids.

- 25 37. An isolated effector protein of a type III secretion system (T3SS)-containing bacterium of the genus *Salmonella* or *Shigella* or a variant, fragment or immunomodulatory domain of said effector protein, wherein said effector protein, variant, fragment or immunomodulatory domain is linked to a cargo molecule and/or cell-specific targeting agent.

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FIGURE 1

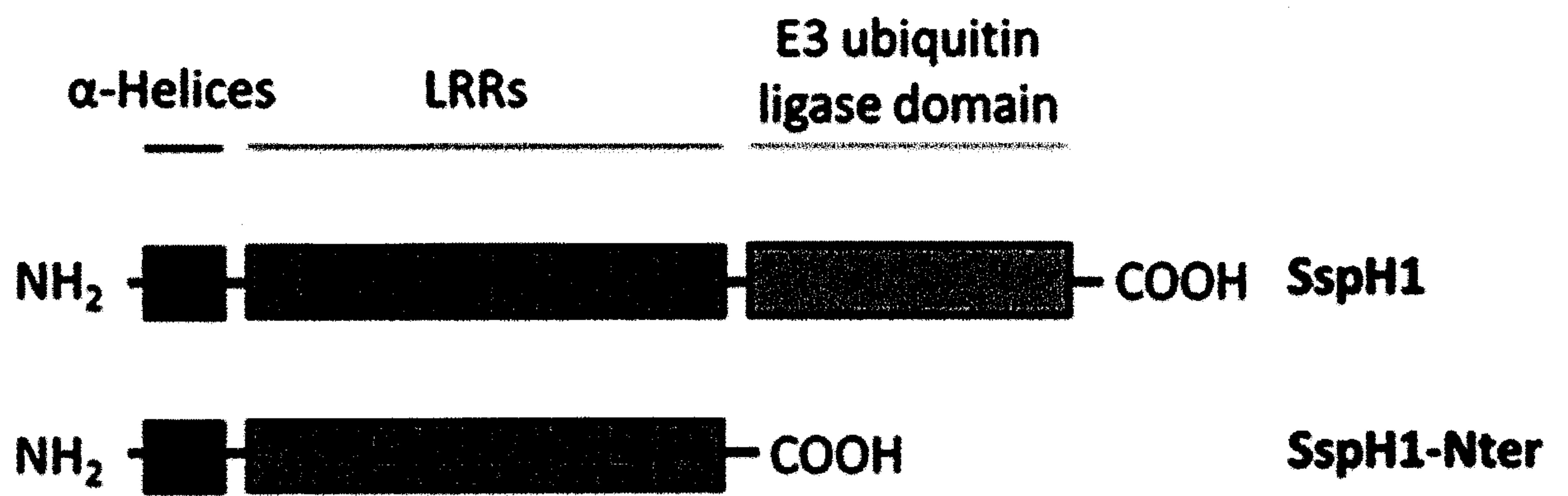
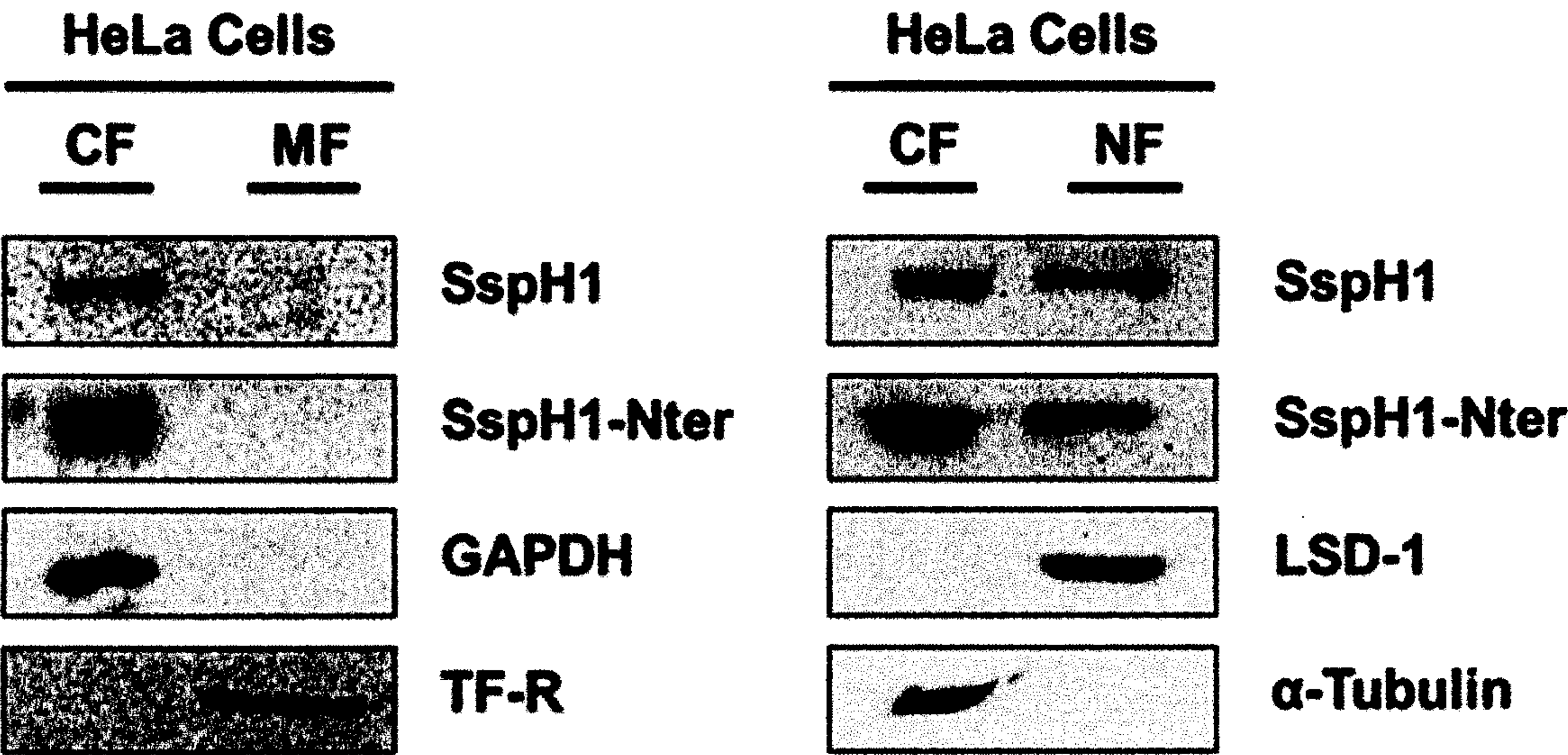


FIGURE 2



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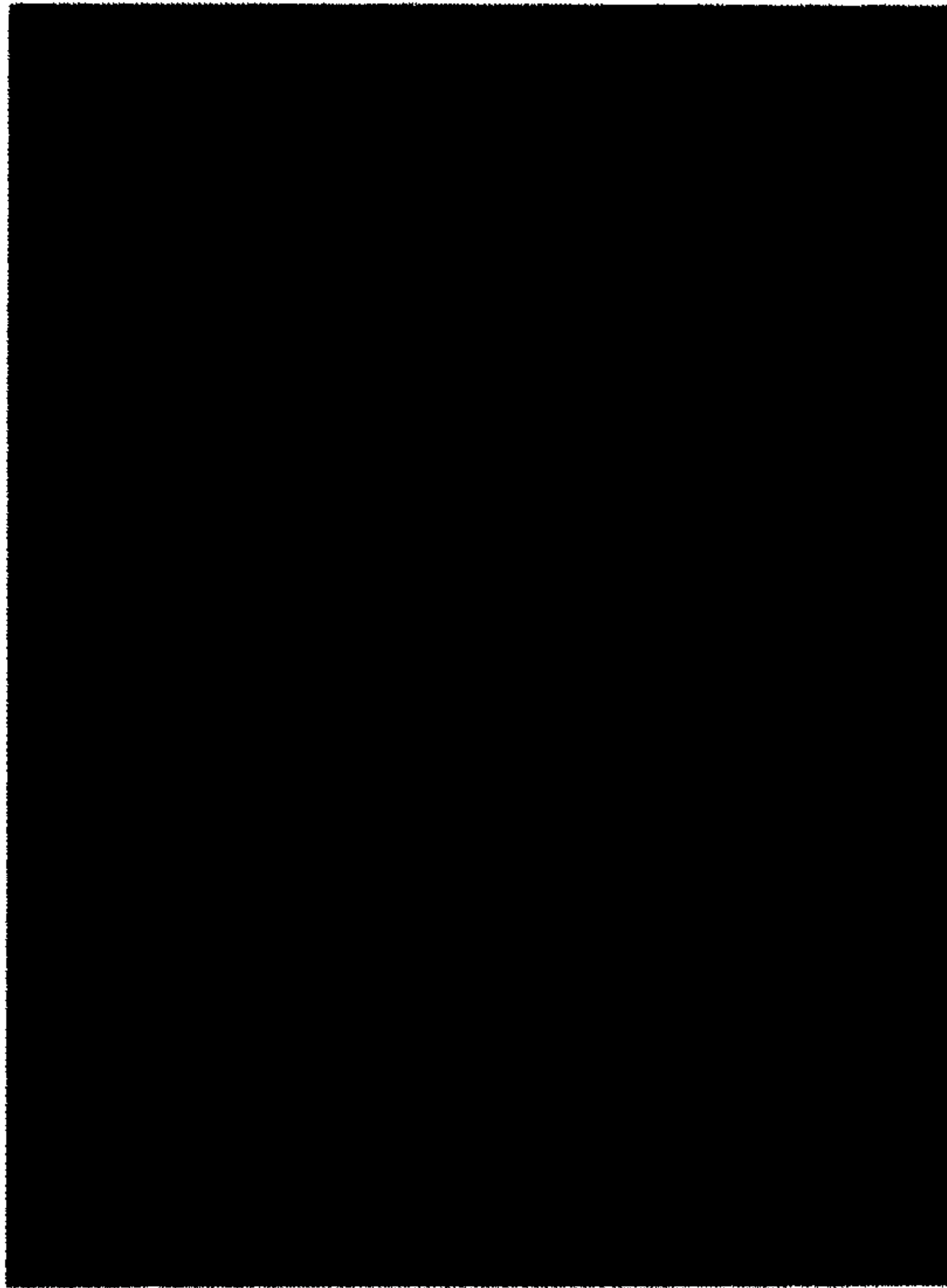
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FIGURE 3

SspH1-Nter

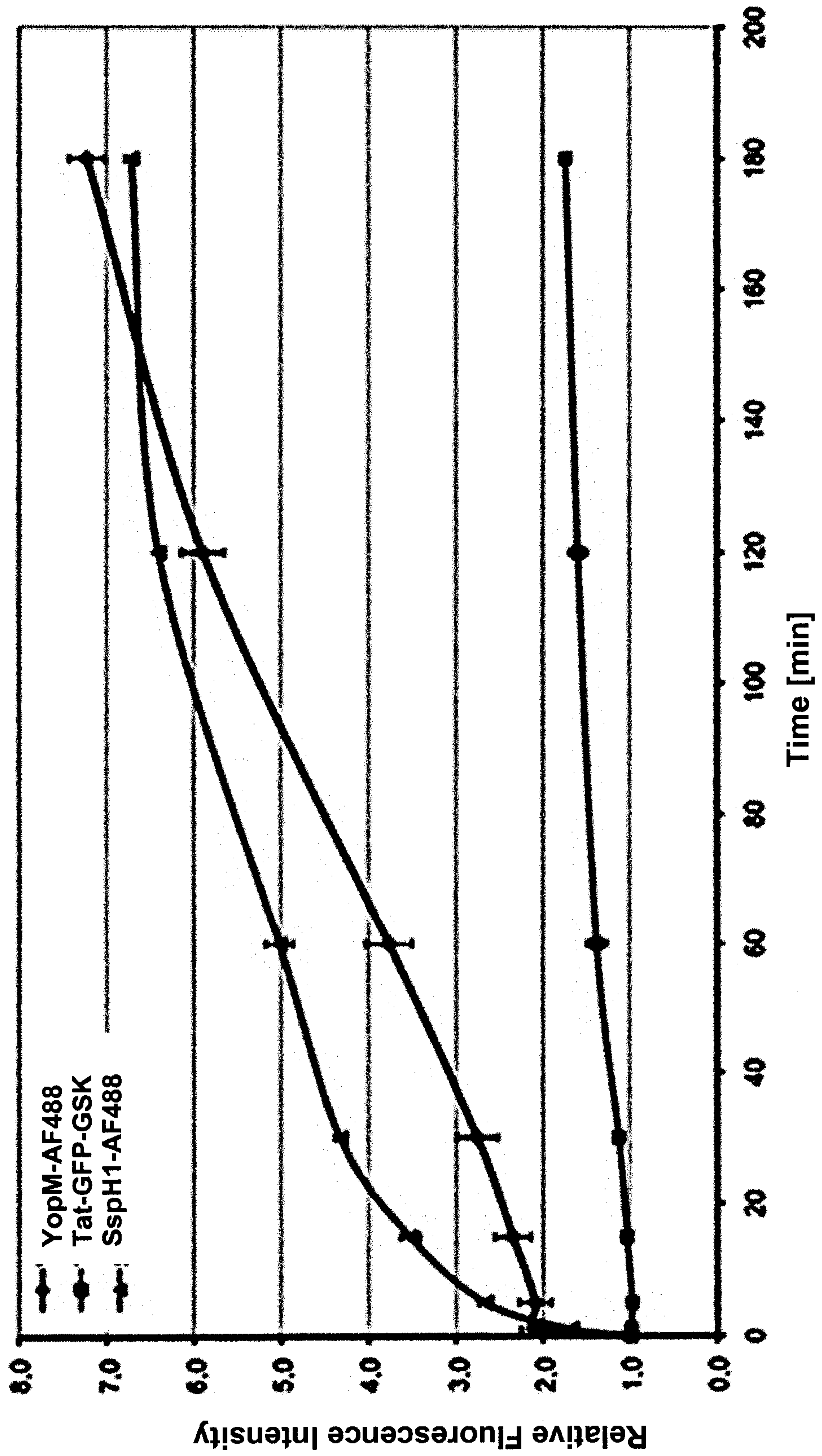


SspH1



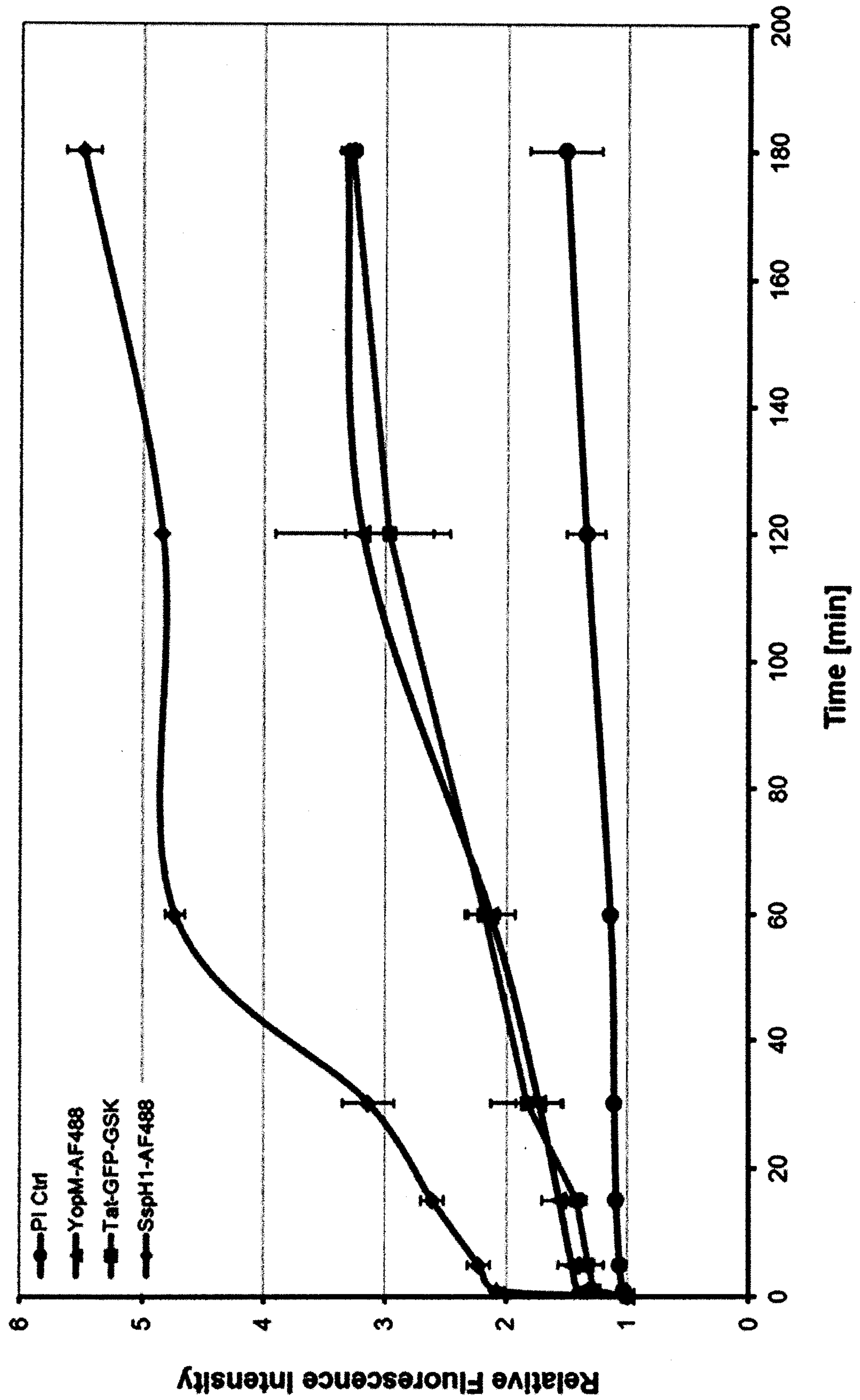
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FIGURE 4



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FIGURE 5



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FIGURE 6

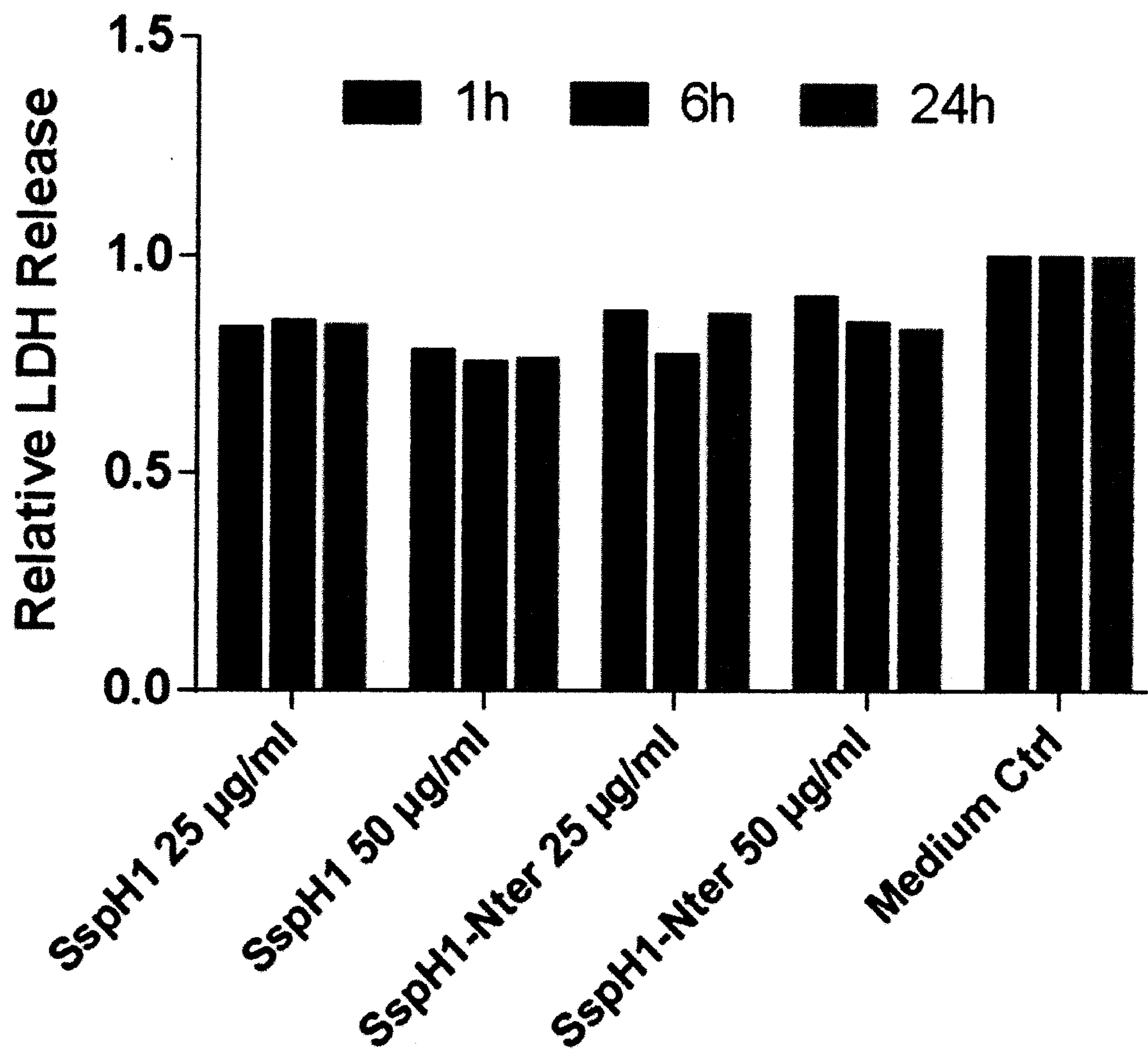
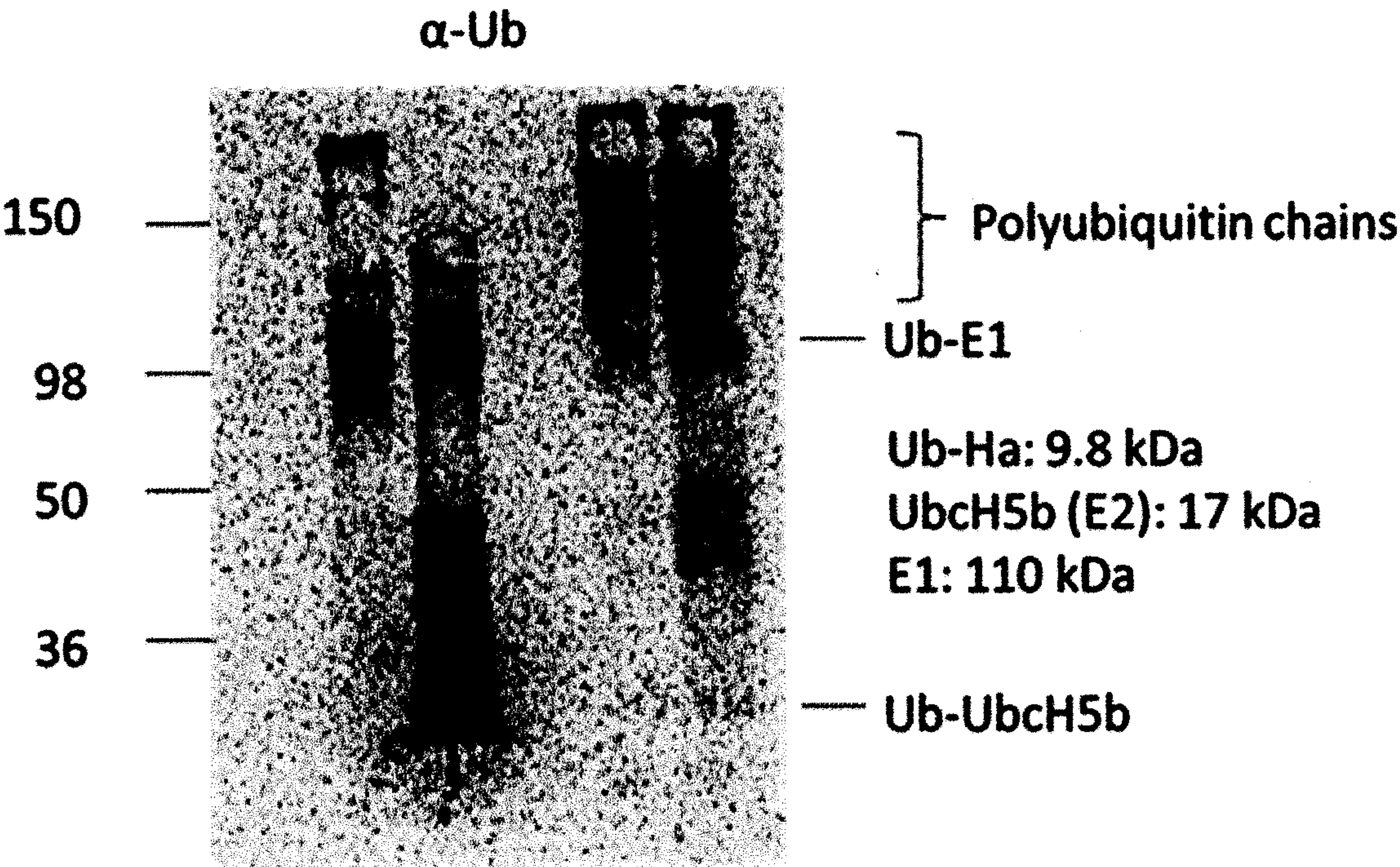
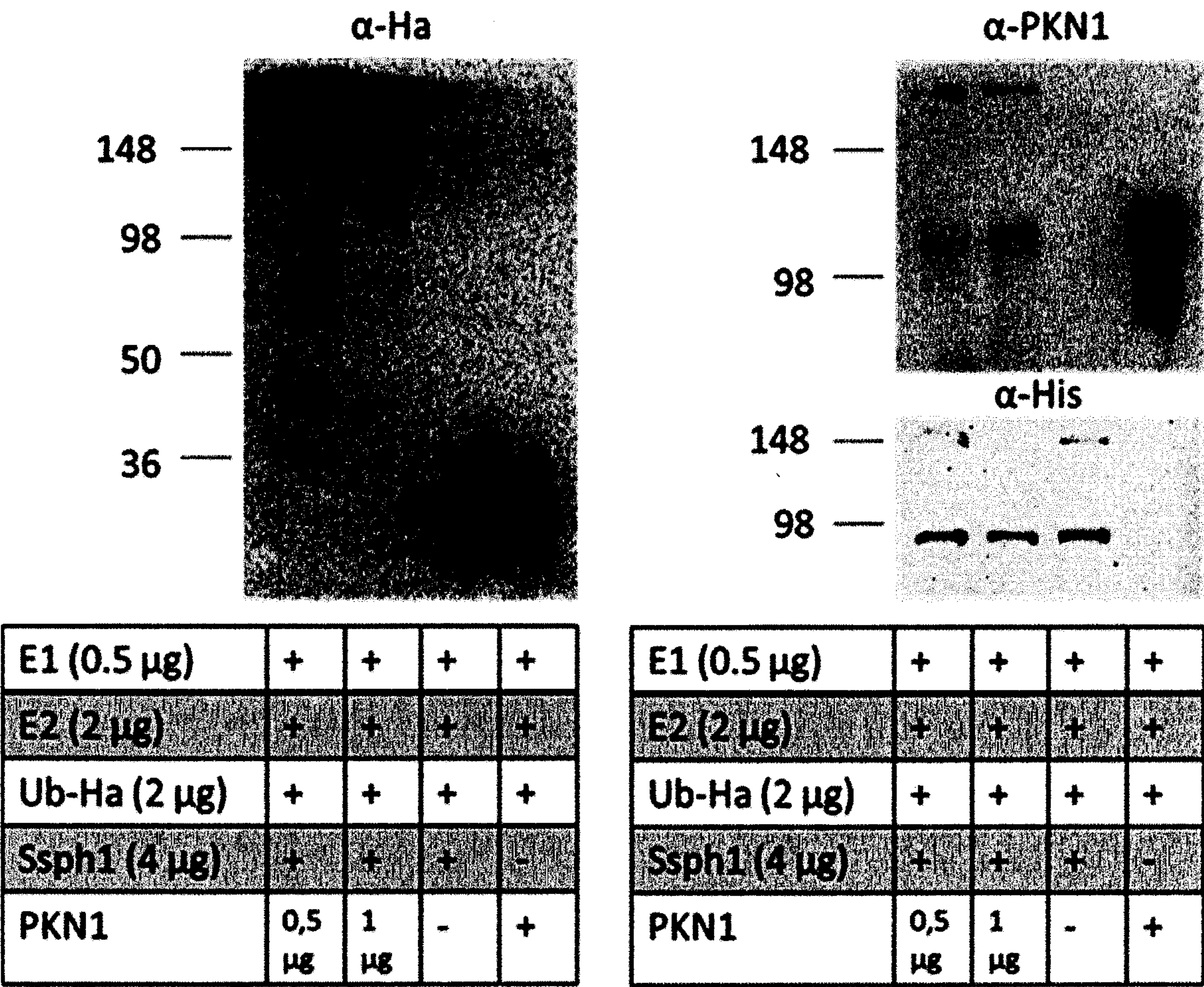


FIGURE 7



E1 (0.5 μ g)	-	+	+	-	+	+
E2 (2 μ g)	+	-	+	-	+	+
Ub (2 μ g)	+	+	+	+	+	+
Ssph1 (4 μ g)	+	+	-	+	+	+

FIGURE 8

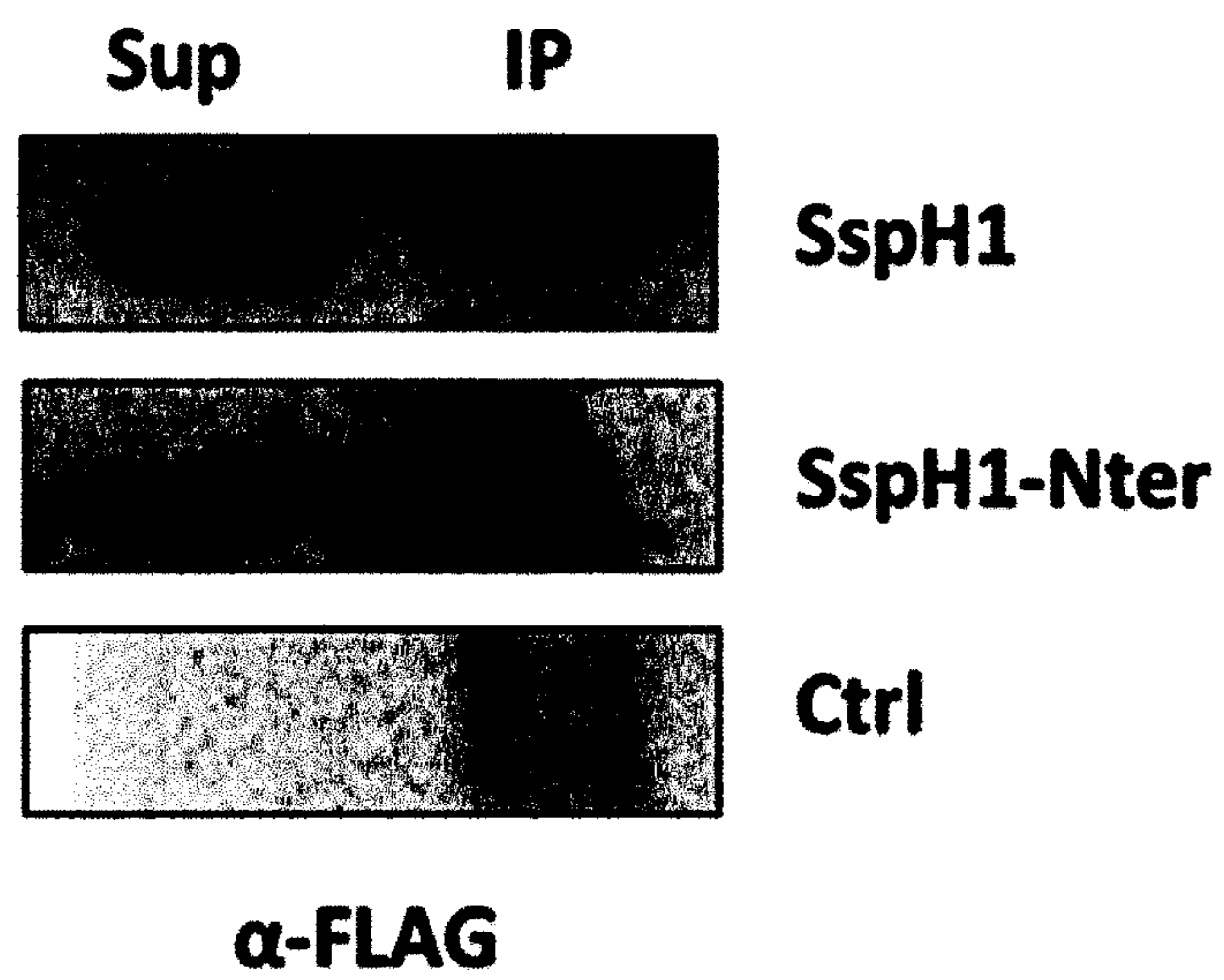


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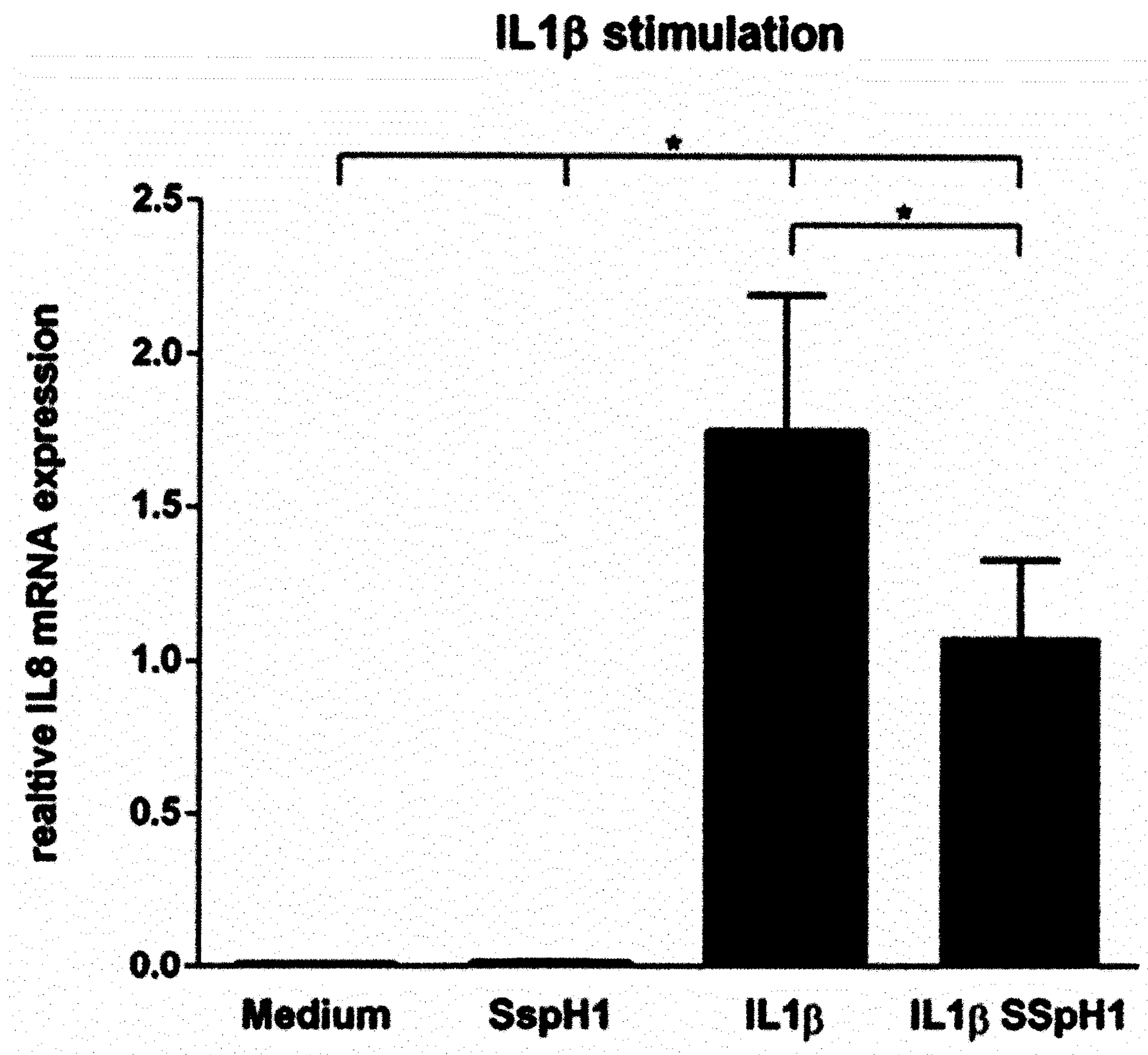
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FIGURE 9



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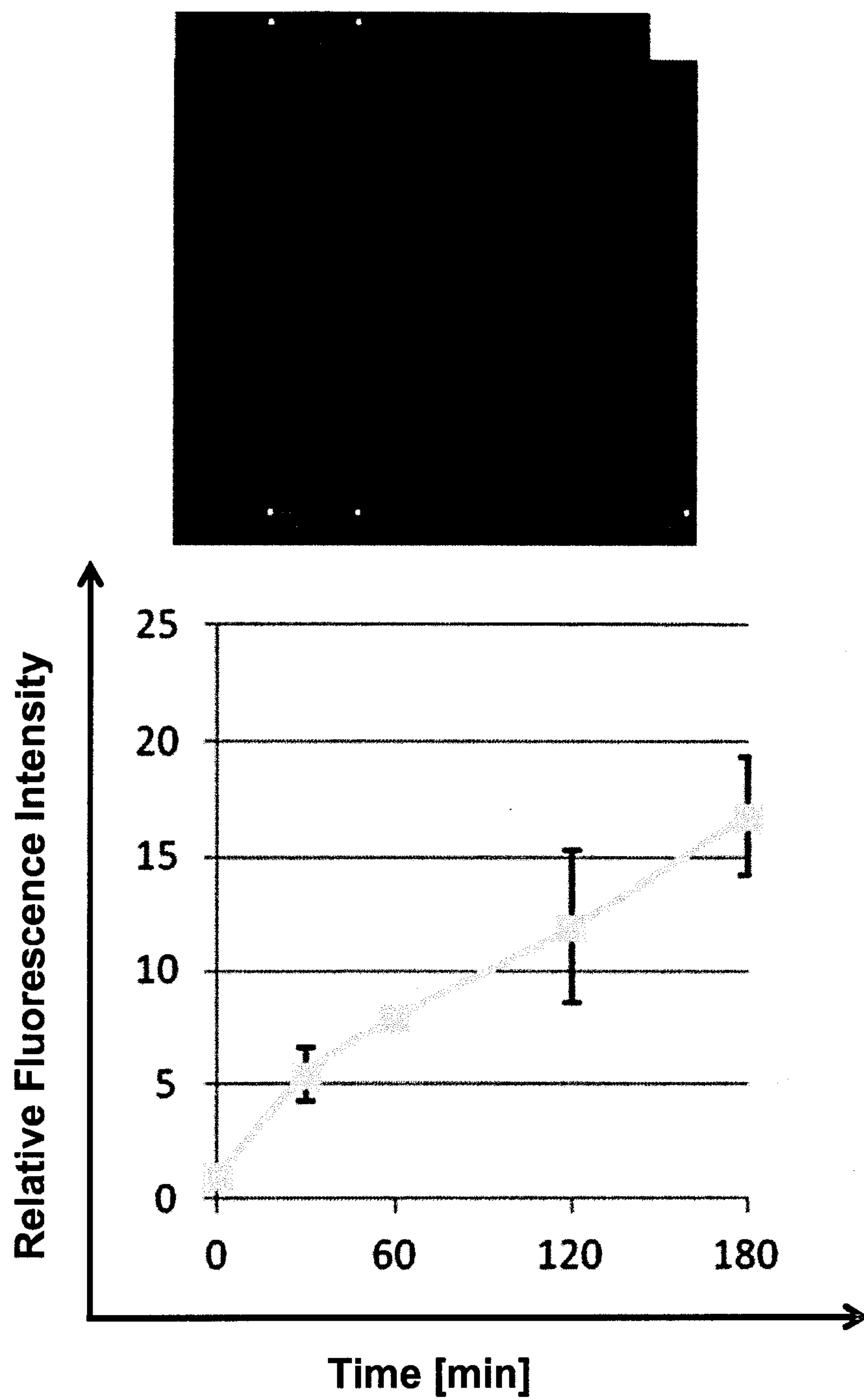
FIGURE 10



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FIGURE 11a

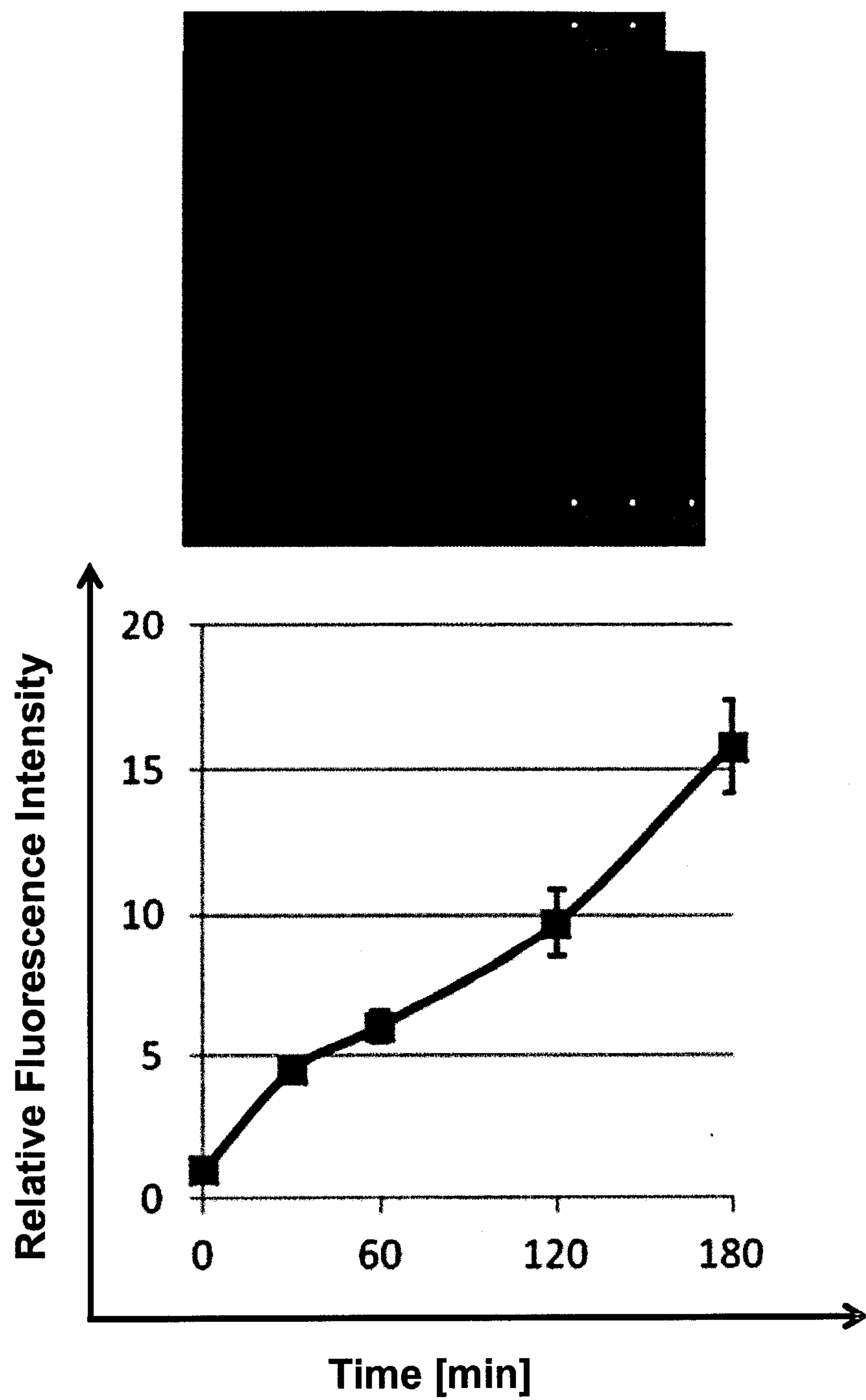
IpaH1.4



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FIGURE 11b

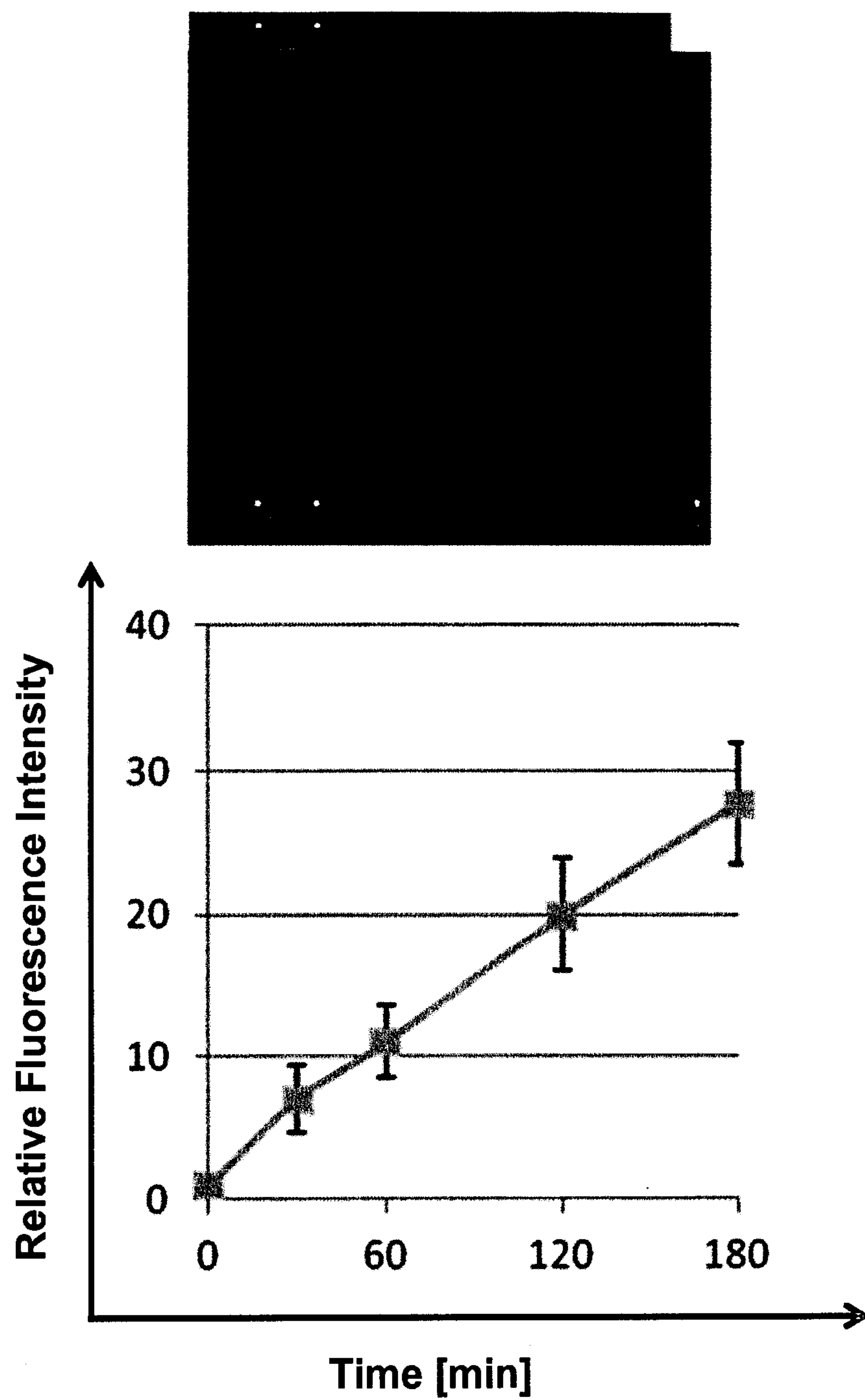
IpaH4.5



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FIGURE 11c

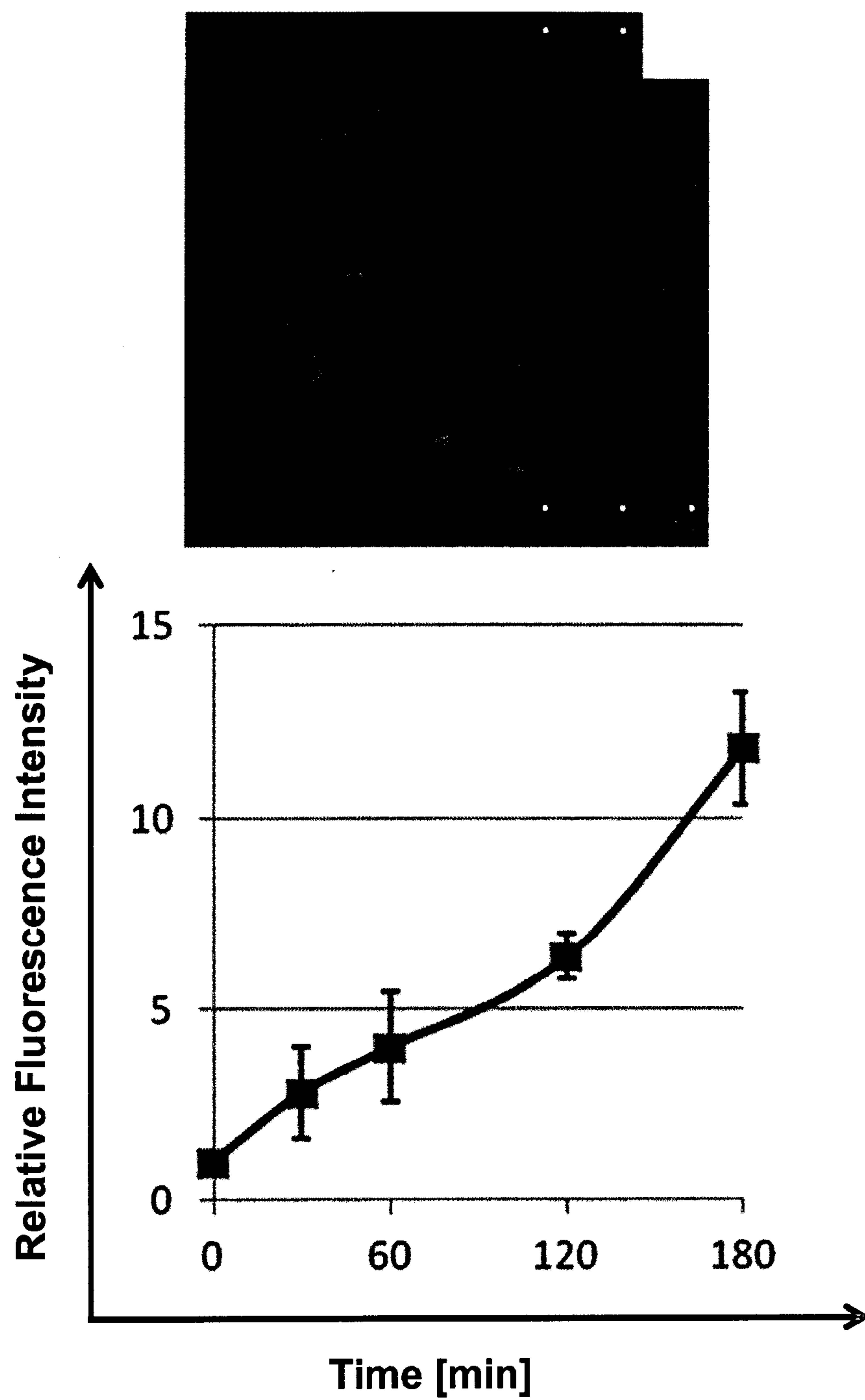
IpaH7.8



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FIGURE 11d

IpaH9.8



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FIGURE 11e

SlrP

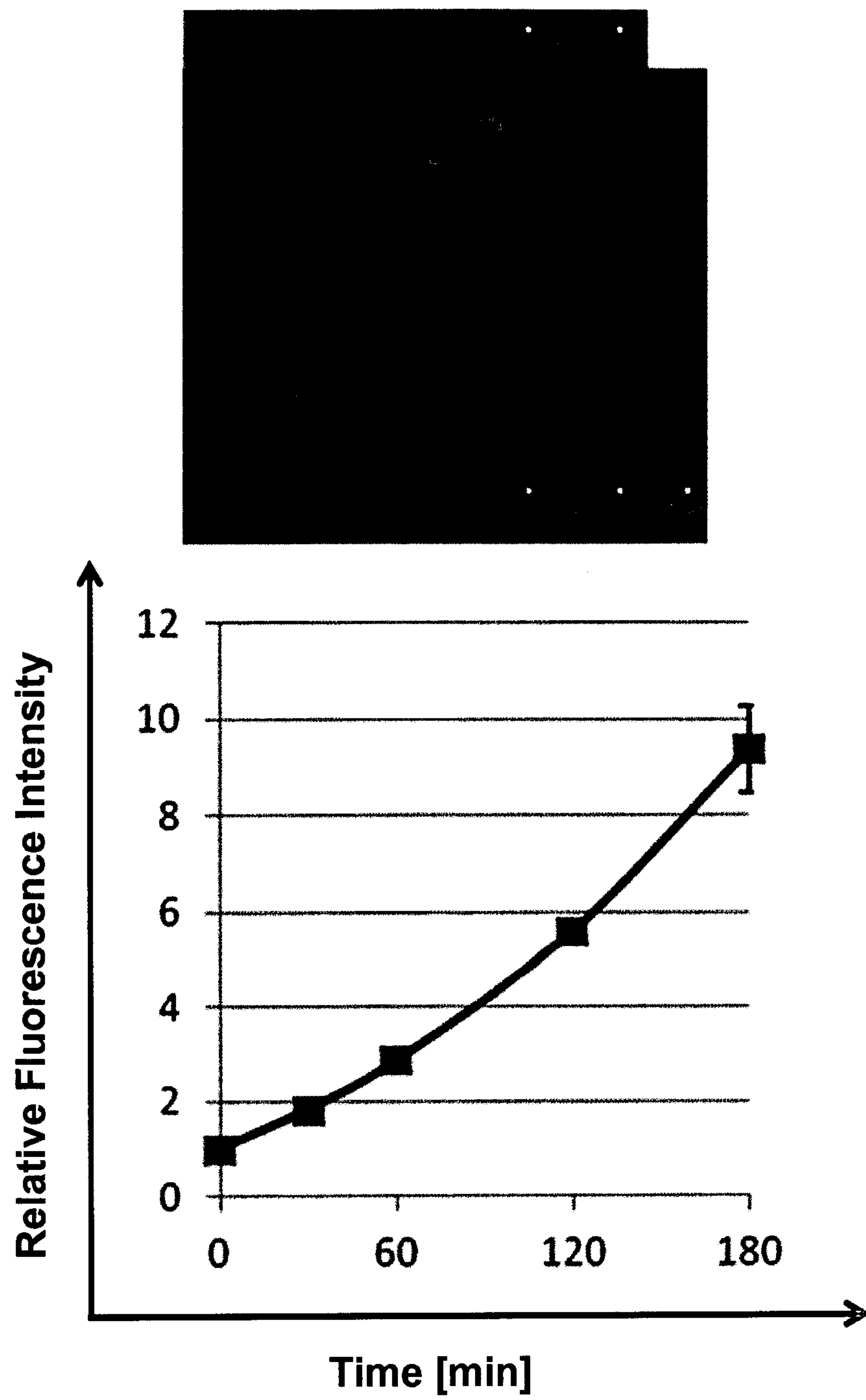


FIGURE 12

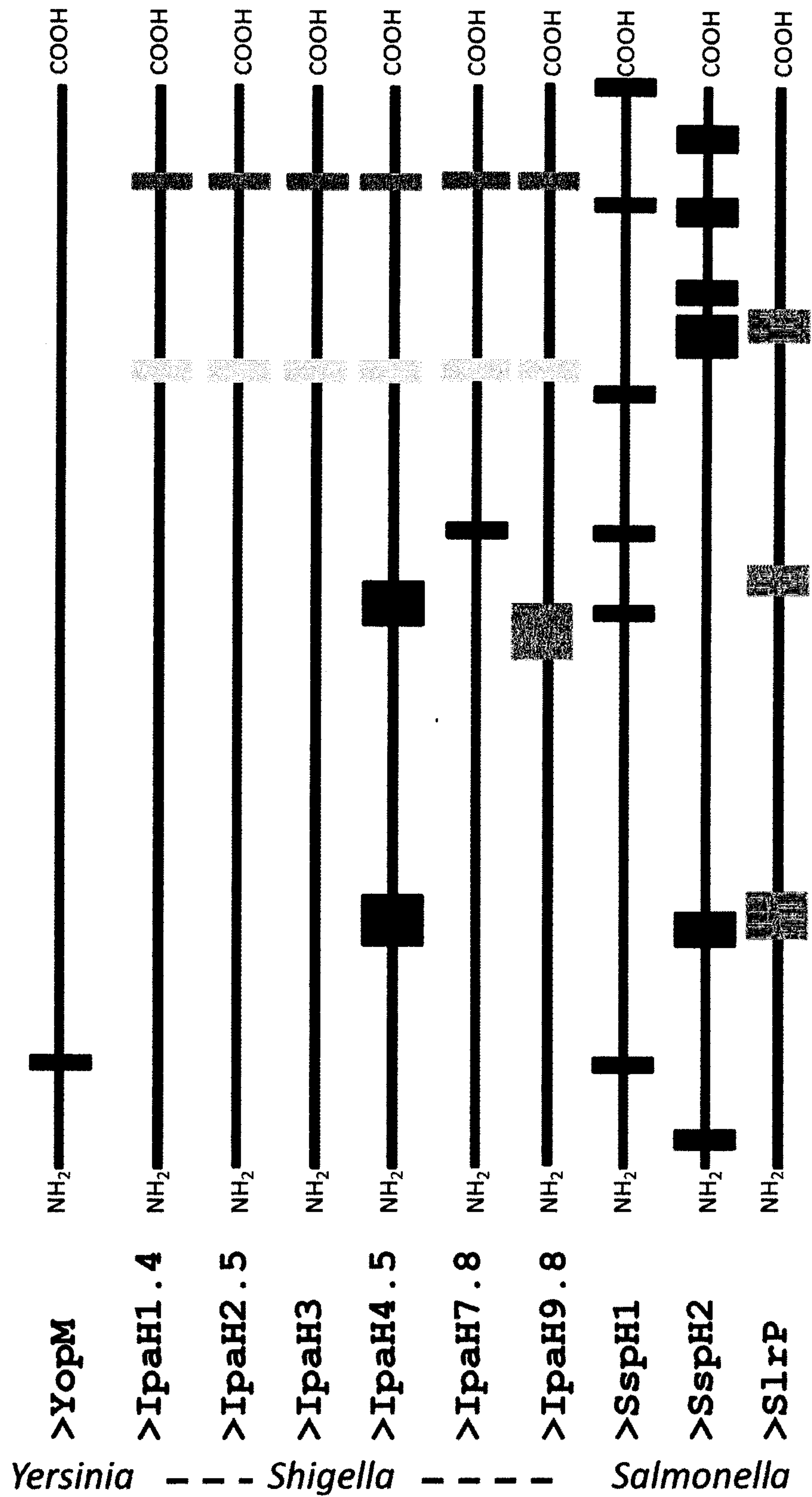
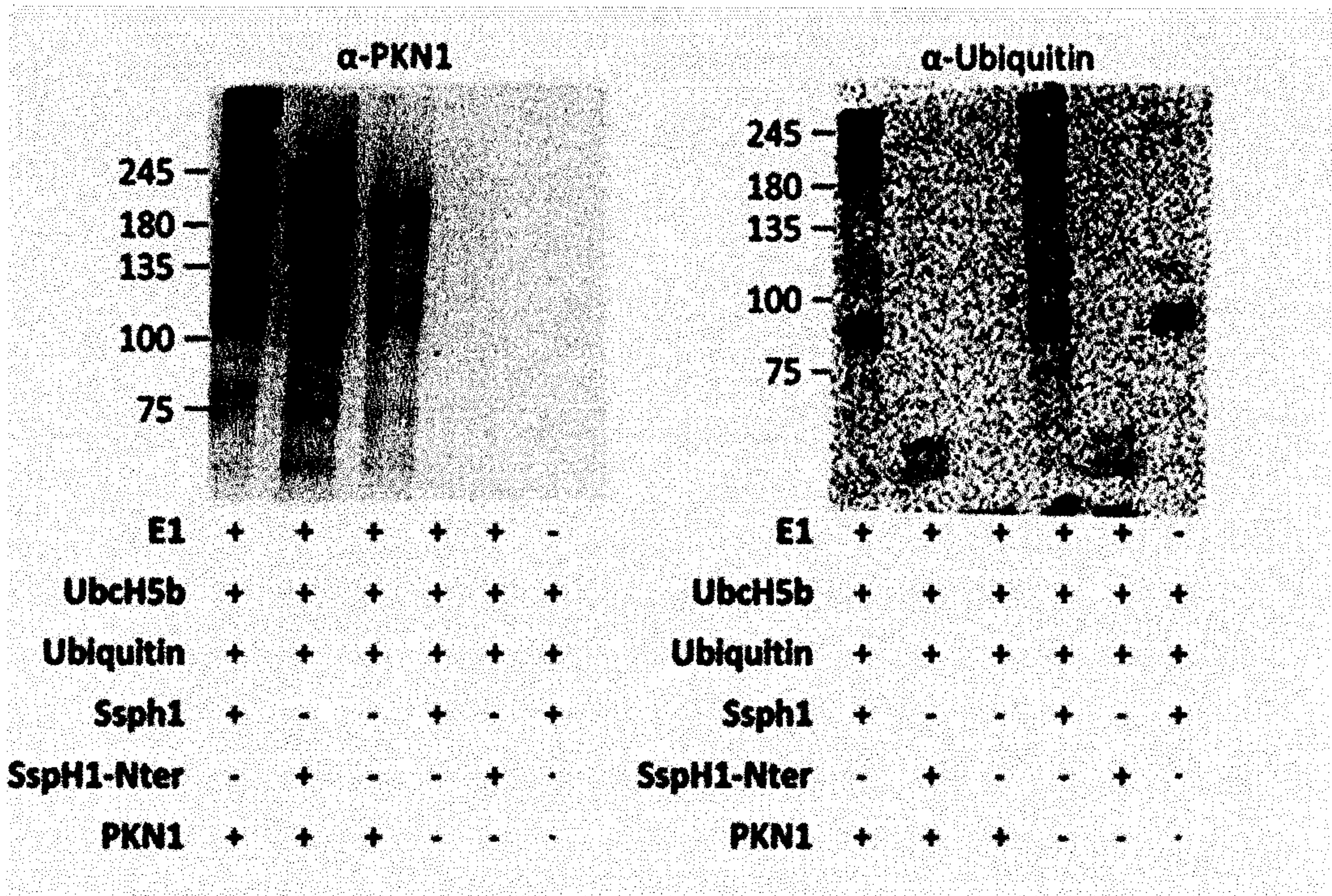


FIGURE 13a



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FIGURE 13b

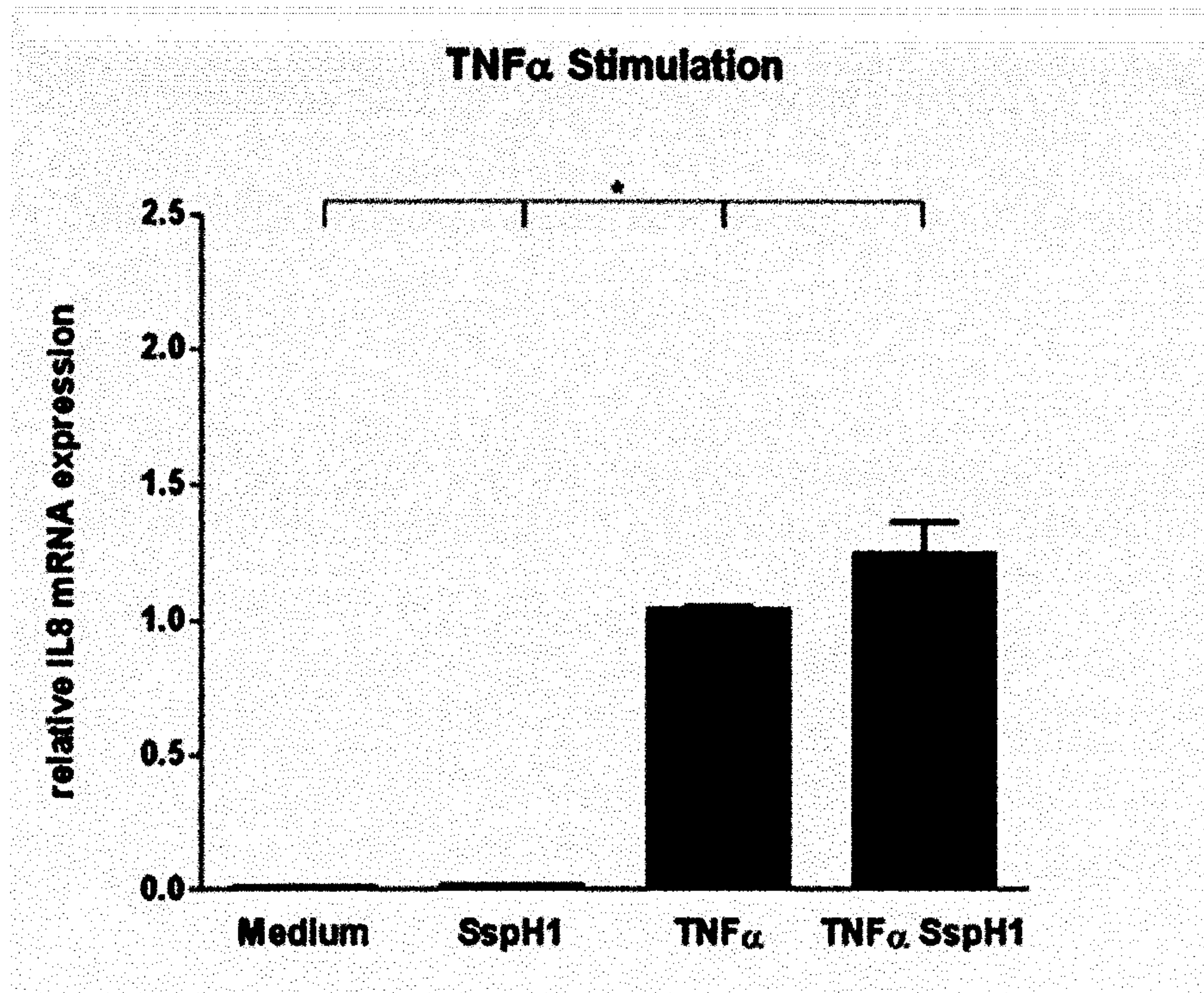


FIGURE 14

SlrP

>gi|267992540|gb|ACY87425.1| leucine-rich repeat-containing protein [Salmonella enterica subsp. enterica serovar Typhimurium str. 14028S]

(SEQ ID NO: 1)

MFNITNIQSTARHQSSISNEASTEVLKKEIWNKISAFFSSEHQVEAQNCIAYLCHPPETASPEEIKSK
FECLRMLAFPAYADNIQYSRGGADQYCILSENSQEILSIVFNTEGYTVEGGGKSVTYTRVTESEQA
SSASGSKDAVNYELIWSEWVKE**APAKEAANREEAVQRMRDCLKNNKTELRLKILGLTTIPAYIPE**
QITTLILDNNELKSLPENLQGNIKTLYANSNQLTSIFATLPDTIQEMELSINRITELPERLPSALQSLDL
FHNKISCLPENLPEELRYLSVYDNSIRTLPAHLPSETHLVQSNSTALPETLPPGLKTL EAGENAL
TSLPASLPPELQVLDVSKNQITVLPETLPPTITL DVSRNALTNLPENLPAALQIMQASRNNLVRLP
ESLPHFRGEGPQPTRIIVEYNPFSERTIQNMQRMLMSSVDYQGPRVLFAMGDFSIVRV

Area of LRR stretches: LRR 1-10 (AA 200-410 = SEQ ID NO: 28)

(AA 462-765 = SEQ ID NO: 19)

Maximal protein-transduction domains:

APAKEAANREEAVQRMRDCLKNNKTELRLKILGLTTIPA = SEQ ID NO: 37

LPAALQIMQASRNNLVRL = SEQ ID NO: 38

= SEQ ID NO: 39

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FIGURE 15**SspH1:**

>gi|267993082|gb|ACY87967.1| SspH1 [Salmonella enterica subsp. enterica serovar Typhimurium str. 14028S]

(SEQ ID NO: 2)

MFNIRNTQPSVSMQAIAGAAPEASPEEIVWEKIQVFFPQENYEEAQQCLAELCHPARGMLPDHIS
SQF**ARLKALTFPA**WEENIQCNRDGINQFCILDAGSKEILSITLDDAGNYTVNCQGYSEAHDFIMDT
EPGEECTEFAEGASGTSLRPATTVSQKAAEYDAVWSKWERDAPAGESPGRAAVVQEMRDCLNN
GNPVLNVGASGLTTLPDRLPPHITTLVIPDNNLTSLPELPEGLRELEVSGNLQLTSLPSLPQGLQKL
WAYNNWLASLPTLPPGLGDLAVSNNQLTSLPEMPPALREL RVSGNNLTSLPALPSGLQKLWAYN
NRLTSLPEMSPGLQELDVSHNQLTRLPOSITGLSSAARVYLDGNPLSVRTLQALRDIIGHSGIRIHF
DMAGPSVPRE

LRR stretch: LRR 1-8 (AA 217-381 = SEQ ID NO: 29)

(AA 404-700 = SEQ ID NO: 20)

Maximal protein-transduction domains:

ARLKALTFPA = SEQ ID NO: 40

LQKLWAYNNRL = SEQ ID NO: 41

LSVRTLQALR = SEQ ID NO: 42

= SEQ ID NO: 43

= SEQ ID NO: 44

= SEQ ID NO: 45

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FIGURE 16**SspH2:**

>gi|267994325|gb|ACY89210.1| leucine-rich repeat-containing protein [Salmonella enterica subsp. enterica serovar Typhimurium str. 14028S]

(SEQ ID NO: 3)

MPFHIGSGCLP**ATISNRRIYRIA**WSDTPPEMSSWEKMKEFFCSTHQTEALECIWTICHPPAGTTRE
DVINRFELLRTLAYAGWEESIHSGQHGENYFCILDEDSQEILSVTLDDAGNYTVNCQGYSETHRLT
LDTAQGEEGTGHAEGASGTFRTSFLPATTAPQTPAEYD**AVWSAWRRAAPAEESRGRAAVVQK**
MRACLNNGNNAVLNVGESGLTTLPDCLPAHITLVIPDNNLTSLPALPPELRTLEVSGNQLTSLPVLP
PGLLELSIFSNPLTHLPALPSGLCKLWIFGNQLTSLPVLP PGLQELSVSDNQLASLPALPSELCKLW
AYNNQLTSLPMLPSGLQELSVSDNQLASLPTLPSELYKLWAYNNRLTSLPALPSGLKELIVSGNRL
TSLPVLPSELKELMVSGNRLTSLPMLPSGLLSLSVYRNQLTRLPESLIHLSSETTVNLEGNPLSERT
LQALREITSAPGYSGPIIRFDMAGASAPRE

LRR stretch: LRR 1-12 (AA 223-466 = SEQ ID NO: 30)

(AA 492-788 = SEQ ID NO: 21)

Maximal protein-transduction domains:

ATISNRRIYRIA = SEQ ID NO: 46

AVWSAWRRAAPAEESRGRAAVVQKMRACLNN = SEQ ID NO: 47

= SEQ ID NO: 48

= SEQ ID NO: 49

= SEQ ID NO: 50

= SEQ ID NO: 51

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FIGURE 17

IpaH1.4

>tr|Q9AJU5|Q9AJU5_SHIFL Putative uncharacterized protein ipaH1.4 OS=Shigella flexneri
GN=ipaH1.4 PE=4 SV=1

(SEQ ID NO: 4)

MIKSTNIQAIGSGIMHQINNVYSLTPLSLPMELTPSCNEFYLKTWSEWEKNGTPGEQRNIAFNRLKI
CLQNQEAELNLSELDLKTLPDLPPQITTLERKNLLTHLPDLPPMLKVIHAQFNQLES LPALPETLEE
LNAGDNKIKELFELPENLTHLRVHNNRLHILPLLPELKLWVSGNRLDSIPFPDKLEGLALANNFIE
QLPELPFSMNRAVLMNNNLTTLPESVLRRLAQNAFVN
NHIA

(AA 241- 571 = SEQ ID NO: 22)

Area of LRR stretches: (AA 92-213 = SEQ ID NO: 31)

Maximal protein-transduction domains:

= SEQ ID NO: 52

= SEQ ID NO: 53

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FIGURE 18**IpaH2.5**

>gi|12329051|emb|CAC05782.1| IpaH2.5, member of the IpaH family, probably secreted by the Mxi-Spa machinery, function unknown [Shigella flexneri 5a str. M90T]

(SEQ ID NO: 5)

MIKSTNIQVIGSGIMHQINNIHSLTLFSLPVSLSPSCNEYYLKVVSEWEKNGTPGEQRNIAFNRLKIC
LQNQEAELNLSELDLKTLPDLPPQITTLERKNLLTHLPDLPPMLKVIHAQFNQLES LPALPETLEEL
NAGDNKIKELPFLPENLTHLRVHNNRLHILPLLPPELKLLVSGNR LDSIPPFPDKLEGLALANNFIE
QLPELPFSMNRAVLMNNNLTTLPESVLR LAQNAFVN

(AA 241-563 = SEQ ID NO: 23)

Area of LRR stretches: (AA 92-213 = SEQ ID NO: 32)

Maximal protein-transduction domains:

= SEQ ID NO: 54

= SEQ ID NO: 550

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FIGURE 19**IpaH3**

Shigella flexneri 5a str. M90T

>tr|I0VDT7|I0VDT7_SHIFL Invasion plasmid antigen OS=Shigella flexneri 5a str. M90T

GN=ipaH_3 PE=4 SV=1

(SEQ ID NO: 6)

MLPTNNNHRLISNSFSTYSIDTSRAYENYLTHWTEWKNNRIQEEQRDIAFQRLVSC LQNQETNLDL
SELGLTTLPEIPP **GIKSINISKNNLSLISPLPASLTQLNVSYNRLJELPALPQGLKLLNASHNQLITLPTL**
PISLKELVHSNNQLQSLPVLPELLETLDVSONGLAVLPPLPFSLQEISAIGNLLSELPPLPHNIHSIWA
DNMLTDIPYLPENLRNGYFDINQISHIPESILNLRNECSIDISDNPLSSHALQSLQRLTSSPDYHGPQI
YFSMSDGQQNTLHR [REDACTED]
[REDACTED]
[REDACTED]
[REDACTED]
[REDACTED]

[REDACTED] (AA 291- 583 = SEQ ID NO: 24)

Area of **LRR stretches**: (AA 92-213 = SEQ ID NO: 33)**Maximal protein-transduction domains:**

[REDACTED] = SEQ ID NO: 55

[REDACTED] = SEQ ID NO: 56

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FIGURE 20

IpaH4.5

>gi|12329057|emb|CAC05788.1| IpaH4.5, member of the IpaH family, probably secreted by the Mxi-Spa secretion machinery, function unknown [Shigella flexneri 5a str. M90T]

(SEQ ID NO: 7)

MKPINNHSSFFRSLCGLSCISRLSVEEQCTRDYHRIWDD **WARECTITTENRIQAVRLLKICLDTREPV**
LNLSLLKLRSLPPLPLHIRELNISNNELISLPENSPLLTTELHVNGNNLNILPTLPSQLIKLNISFNRLS
CLPSLPPYLQSLSARFNSLETLPSTLTILRIEGRRLTVLPPELPHRLQELFVSGNRLQELPEFPQS
LKYLKVGGENQLRRLSRLPQELLALDVSNNLLTSLPENITLPICTNVNIISGNPLSTHVLSLQRLTSS
PDYHGPQIYFSMSDGQQNTL [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

[REDACTED]

LRR stretch: LRR 1-10 (AA 63-270 = SEQ ID NO: 34)

[REDACTED] (AA 293 – 574 = SEQ ID NO: 25)

Maximal protein-transduction domains:

WARECTITTENRIQAVRLLKICLDTREPVLNLSLLKLRSL = SEQ ID NO: 57

QSLKYLKVGGENQLRRLSRLPQELLALD = SEQ ID NO: 58

[REDACTED] = SEQ ID NO: 59

[REDACTED] = SEQ ID NO: 551

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FIGURE 21

IpaH7.8

>gi|12329056|emb|CAC05787.1| IpaH7.8, member of the IpaH family, secreted by the Mxi-Spa secretion machinery, function unknown [Shigella flexneri 5a str. M90T]

(SEQ ID NO: 8)

MFSVNNTHSSVSCSPSINSNSTSNEHYLRILTEWEKNSSPGEERGIAFNRLSQCFONQEAVLNLS
DLNLTSLPELPKHISALIVENNKLTSLPKLPAFLKELNADNNRLSVIPELPESLTTL SVRSNQL ENLPV
LPNHLTSLFVENNRLYNLPALPEKLKFLHVYYNRLTTL PDL PDKLEILCAQRNNLVTFPQFSDRNNI
RQKEYYFHFNQITTLPEFSQQLDSSYRINISGNPLSTRVLOSLOQLTSSPDYHGPQIYFSMSDGQQ
NTL

LRR stretch: LRR 1-9 (AA 58-248 = SEQ ID NO: 35)

(AA 271-565 = SEQ ID NO: 26)

Maximal protein-transduction domains

TRVLOSLOQLT = SEQ ID NO: 60

= SEQ ID NO: 61

= SEQ ID NO: 62

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FIGURE 22**IpaH9.8**

>gi|12329122|emb|CAC05853.1| IpaH9.8, secreted by the Mxi-Spa secretion machinery, function unknown [Shigella flexneri 5a str. M90T]

(SEQ ID NO: 9)

MLPINNNFSLPQNSFYNTISGTYADYFSAWDKWEKQALPGEERDEAVSRLKECLINNSDELRLDRL
 NLSSLPDNLPAQITLLNVSYNQLTNLPPLPVTLLKKLYSASNKLSELPVLPPALESLOVQHNELENLP
 ALPDSLITMNI SYNEIVSLPSLPOALKNL RATRNFELTELP AFSEGNNPVVREYFFDRNQISHIPESIL
 NLRNECSIHISDNPLSSHALQALQRLTSSPDYHGPRIYFSMSDGQQNTL

[REDACTED]
 [REDACTED]
 [REDACTED]
 [REDACTED]
 [REDACTED]

[REDACTED] (AA 251-545 = SEQ ID NO: 27)

Area of LRR stretches: LRR 1-8 (AA 57-228 = SEQ ID NO: 36)

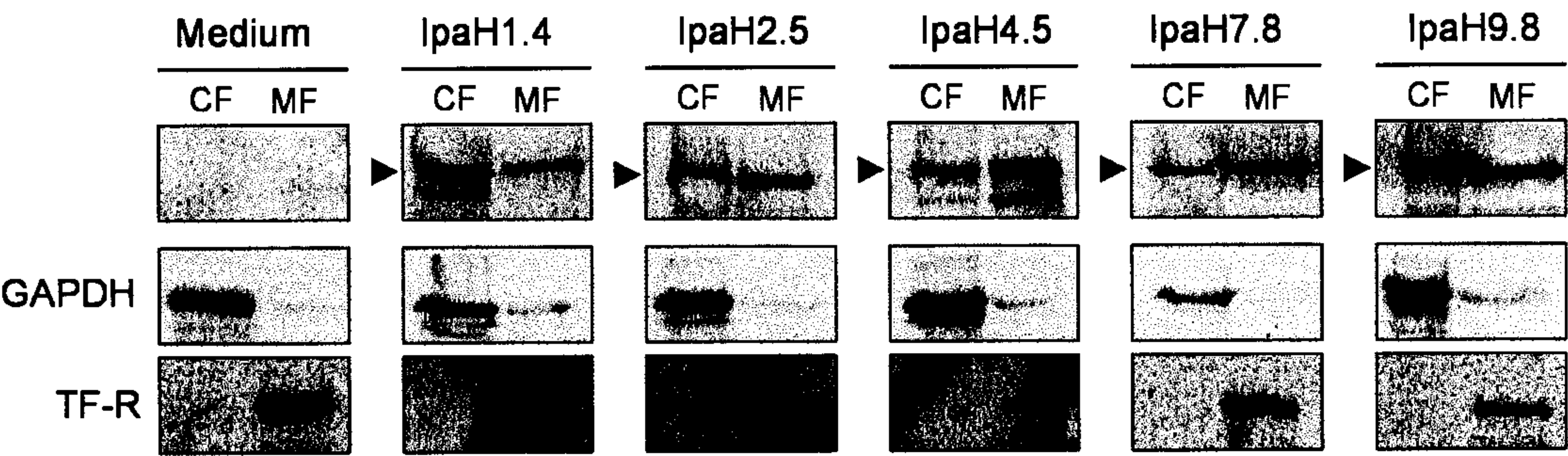
Maximal protein-transduction domains:

SLPSLPOALKNL RATRNFELT = SEQ ID NO: 63

[REDACTED] = SEQ ID NO: 64

[REDACTED] = SEQ ID NO: 65

FIGURE 23

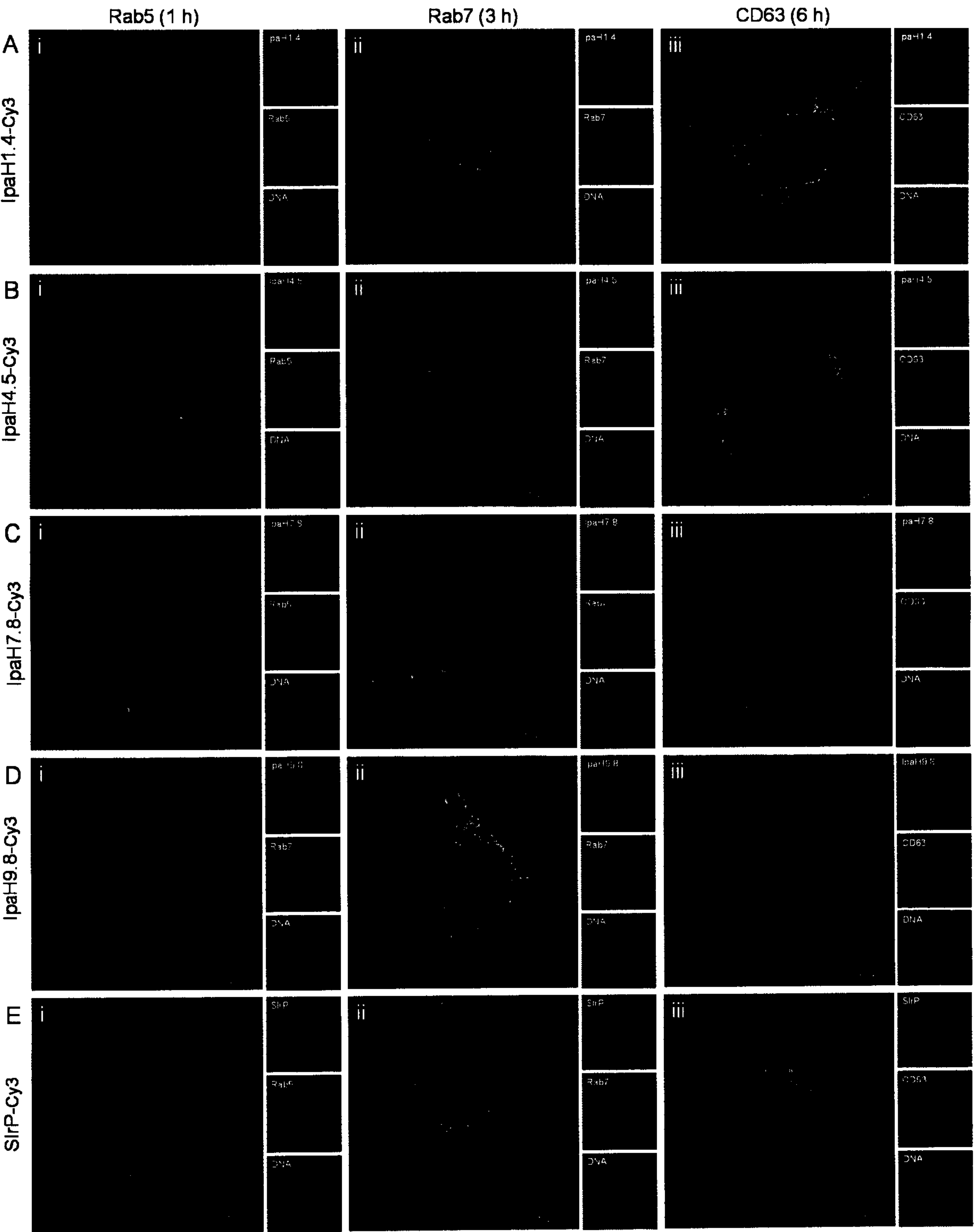


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FIGURE 24



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FIGURE 25

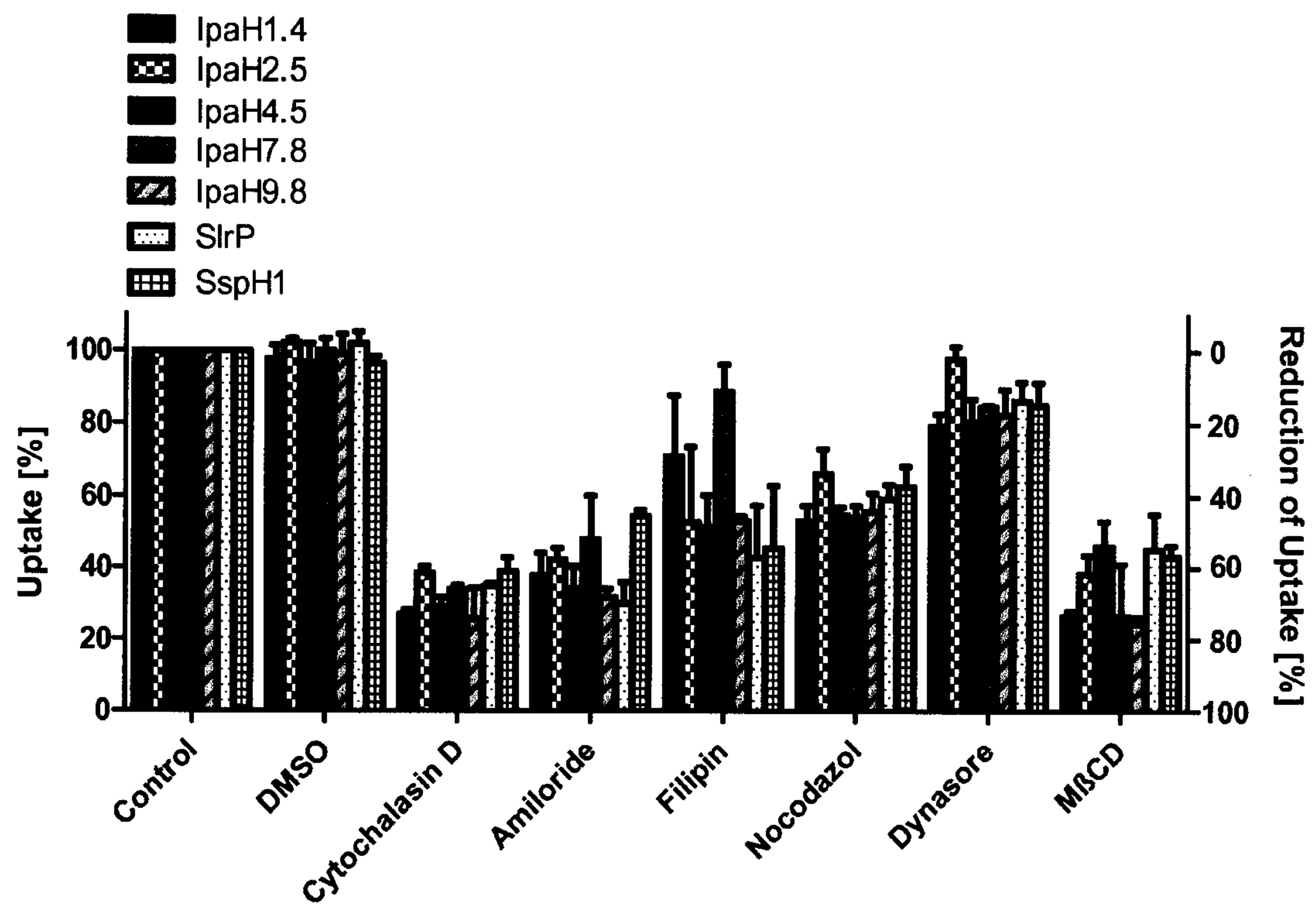


FIGURE 26

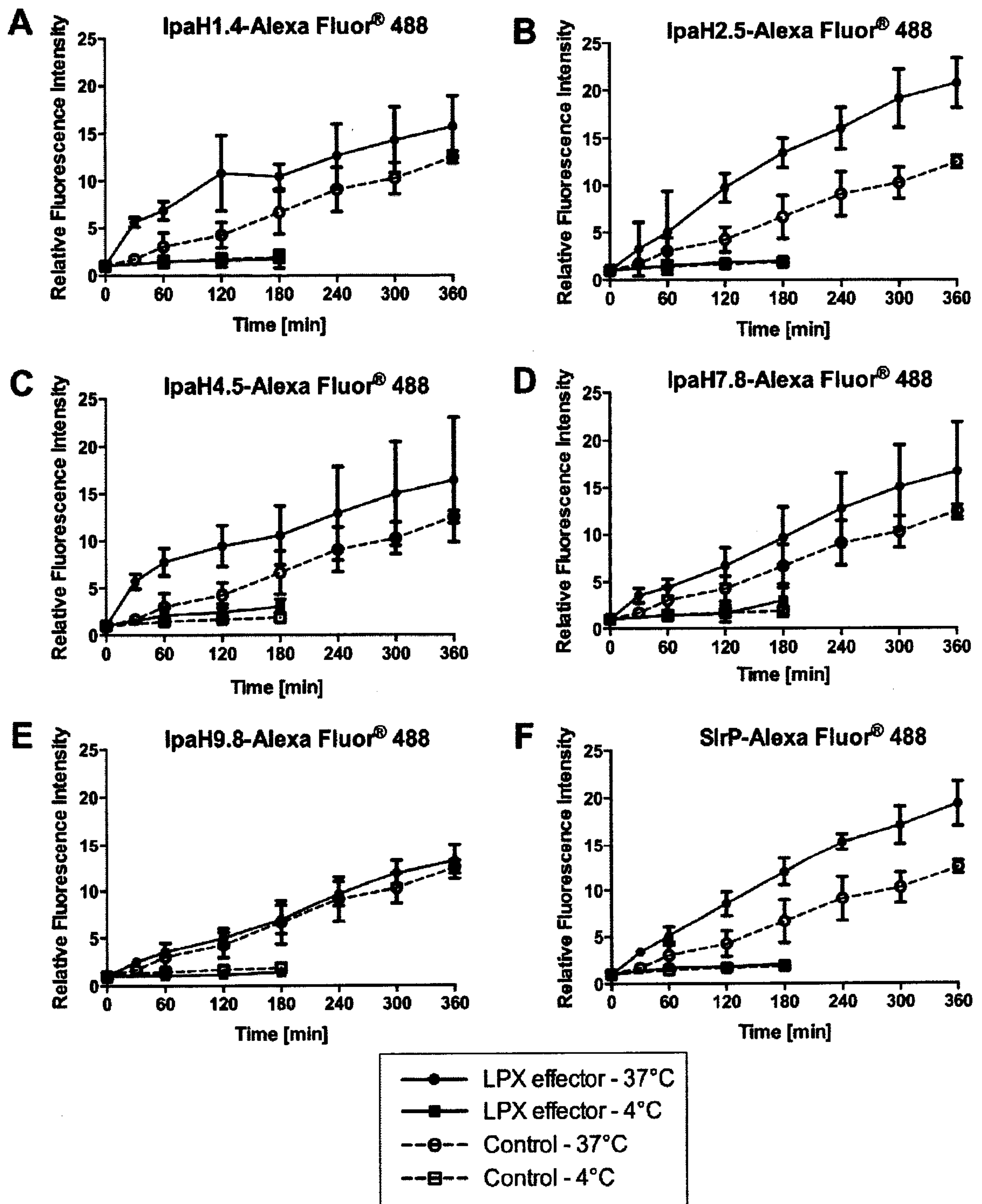


FIGURE 27

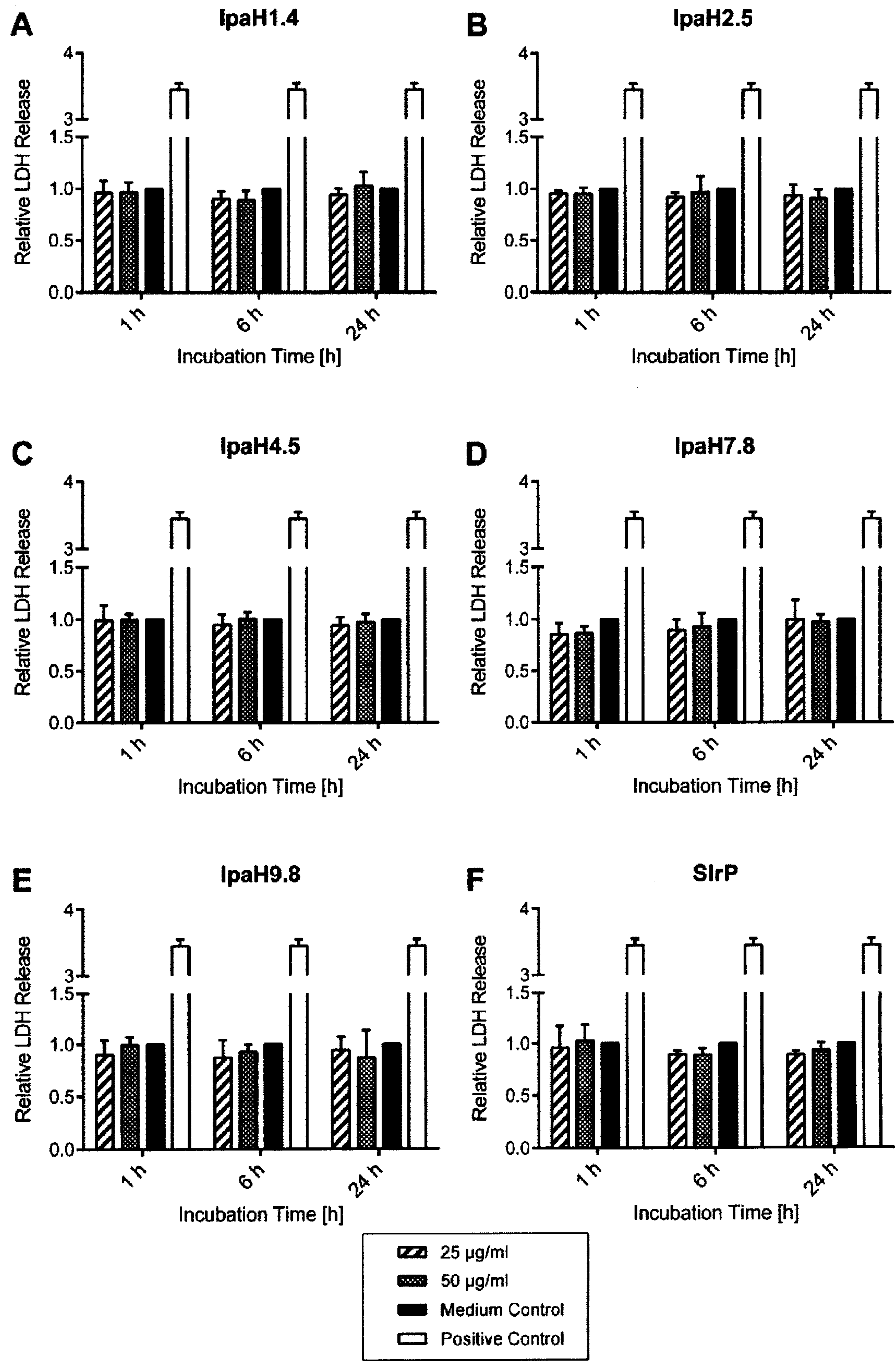


FIGURE 28

