Abstract:
The present invention relates to the discovery that IRGM, encoded by a uniquely human gene which confers risk for inflammatory diseases, affects autophagy through a hitherto unknown mechanism. The present invention shows that IRGM controls autophagy and that IRGM modulators, in particular, double-stranded RNA, including poly I:C, poly-U (polyU) and polyI:polyC and muramyl dipeptide and related analogs of same, including N-acetyl muramyl-L-alanyl-D-isoglutamine (DMP) and numerous other compounds as identified herein, which may be used alone, in combination, or in combination with alternative autophagy modulators and additional bioactive agents to provide effective therapies for a number of diseases, including cancer, bacterial infections and inflammatory diseases, especially including tuberculosis infections and Crohn's disease, among others. The present invention is also directed to compositions and methods for treating inflammatory or autophagy-related diseases including diseases which cause excessive inflammation in patients.
IRGM and Precision Autophagy Controls for Antimicrobial and Inflammatory Disease States and Methods of Detection of Autophagy

Related Applications and Grant Support

This application claims the benefit of priority of provisional applications US62/121,232, filed 26 February 2015, entitled "IRGM Controls the Core Autophagy Machinery to Conduct Antimicrobial Defense and Modulate Inflammatory Disease States" and US62/165,357, filed May 22, 2015, entitled "Methods for Regulating Inflammation By Precision Autophagy", the entire contents of each of these applications is incorporated by reference in its entirety herein.

This invention was made with government support under grant nos. AI04229 and AI11935, awarded by National Institutes of Health and grant no. ULTR000041, awarded by the National Center for Advancing Translation Sciences. The government has certain rights in the invention.

Field of the Invention

The present invention relates to the discovery that IRGM, encoded by a uniquely human gene which confers risk for inflammatory diseases, affects autophagy through a hitherto unknown mechanism. The present invention shows that IRGM controls autophagy and that IRGM modulators, in particular, double-stranded RNA, including poly I:C, poly-UG (polyUGUGU) and polyICLC and muramylidipeptide and related analogs of same, including N-acetyl muramyl-L-alanyl-D-isoglutamine (DMP) and numerous other compounds as identified herein, which may be used alone, in combination, or in combination with alternative autophagy modulators and/or additional bioactive agents to provide effective therapies for a number of diseases, including cancer, bacterial infections and inflammatory diseases, especially including tuberculosis infections and Crohn's disease, among others.

The present invention is also directed to compositions and methods for treating inflammatory or autophagy-related diseases including diseases which cause excessive inflammation in patients. The approach taken to the treatment of these disease states and conditions which cause excessive inflammation is referred to as precision autophagy. This method utilizes interferon, especially interferon-gammna (IFN-gamma), pegylated interferon (PEG-IFN) and related compounds and/or certain TRIM proteins or variants thereof having at
least 90% sequence identity as described herein, in particular, TRIM1 (SEQ ID NO:1), TRIM3 (SEQ ID NO:1 1), TRIM8 (SEQ ID NO:36), TRIM10 (SEQ ID NO:46), TRIM13 (SEQ ID NO:56), TRIM17 (SEQ ID NO:81), TRIM19 (SEQ ID NO:91), TRIM20 (SEQ ID NO:96), TRIM21 (SEQ ID NO:101), TRIM22 (SEQ ID NO:106), TRIM38 (SEQ ID NO:172), TRIM 41 (SEQ ID NO:187), TRIM43 (SEQ ID NO:197), TRIM44 (SEQ ID NO:202), TRIM45 (SEQ ID NO:207), TRIM46 (SEQ ID NO:212), TRIM54 (SEQ ID NO:247), TRIM55 (SEQ ID NO:252), TRIM56 (SEQ ID NO:257), TRIM58 (SEQ ID NO:262), TRIM59 (SEQ ID NO:267), TRIM60 (SEQ ID NO:272), TRIM65 (SEQ ID NO:297), TRIM66 (SEQ ID NO:302), TRIM75 (SEQ ID NO:338) and mixtures thereof, preferably TRIM 1 (SEQ ID NO:1), TRIM 8 (SEQ ID NO:36), TRIM 20 (SEQ ID NO:96), TRIM 21 (SEQ ID NO:101), TRIM 22 (SEQ ID NO:106), TRIM 56 (SEQ ID NO:257), TRIM 65 (SEQ ID NO:297), and mixtures thereof to treat extreme inflammation associated with disease states that cause excessive inflammation. Methods and pharmaceutical compositions are disclosed herein.

Background of the Invention

Autophagy is a cellular homeostatic mechanism with broad roles in human health and disease (Mizushima et al., 2008). Autophagy is at the intersection of metabolic (Rabinowitz and White, 2010; Settembre and Ballabio, 2014) and antimicrobial processes (Deretic et al., 2013; Ma et al., 2013). Thus, the system responds to a range of inputs such as starvation (Chauhan et al., 2013; Efeyan et al., 2013; Mihaylova and Shaw, 2011), lysosomal disruption (Settembre and Ballabio, 2014), endogenous danger associated molecular patterns and microbial products commonly referred to as pathogen-associated molecular patterns (PAMPs) (Deretic et al., 2013; Ma et al, 2013). Autophagic responses to PAMPs lead to direct antimicrobial action through a process termed xenophagy (Gomes and Dikic, 2014; Levine, 2005) and control of inflammation and other immune processes (Deretic et al., 2013).

Among the better-established links between autophagy and human diseases are the genetic polymorphisms in ATG16L1 and IRGM conferring risk for Crohn's disease (CD), an intestinal inflammatory disorder (Consortium, 2007; Craddock et al, 2010; Murthy et al., 2014). The human population polymorphisms in IRGM have been linked to autophagy (Consortium, 2007; Craddock et al., 2010) and to its effector outputs including antimicrobial defense (Brest et al., 2011; McCarroll et al., 2008). In keeping with its autophagy-mediated
antimicrobial role, IRGM is additionally a genetic risk factor for tuberculosis in different human populations (Bahari et al., 2012; Che et al., 2010; Intemann et al., 2009; King et al., 2011; Song et al., 2014) and may afford protection in leprosy (Yang et al., 2014). However, the molecular mechanism of IRGM's function in autophagy has remained a mystery.

IRGM has no homologs among the Atg genes in yeast, which makes it difficult to assign to it an autophagy-specific function; instead, IRGM has been considered to affect autophagy indirectly (Singh et al., 2006). A complicating factor in understanding the exact function of IRGM is that it is distinctly a human gene (Bekpen et al., 2010). Its orthologs are present only in African great apes and Homo sapiens but active alleles are absent in ancestral evolutionary lineages leading up to them (Bekpen et al., 2009). The mouse genome encodes a large family of immunity related GTPase (21 IRG genes) compared to a single gene (IRGM) in humans; furthermore, all murine IRGs encode ca. 40-kDa proteins that are much larger than the human IRGM (21 kDa). The prevailing view of the murine IRGs is that they have predominantly non-autophagy functions (Choi et al., 2014; Zhao et al., 2008). Thus the significant information gathered in the murine systems may have limited import on how the human IRGM works.

Given the significance of IRGM in human populations and the notoriously high prevalence of diseases such as CD and tuberculosis, it is surprising that IRGM's mechanism of action in autophagy remains unknown. Here we report that unexpectedly, IRGM physically interacts with key autophagy regulators, ULK1, Beclin 1, ATG14L and ATG16L1. We also show that, remarkably, IRGM links inputs from PAMP sensors by making molecular complexes with NOD2, another genetic risk factor in CD (Eckmann and Karin, 2005; Hugot et al., 2001; Ogura et al., 2001). The formation of NOD2-IRGM complex is stimulated in response to PAMPs, whereas increased association of NOD2 with IRGM promotes IRGM directed assembly of autophagy regulators. IRGM undergoes post-translational modifications that stabilize components of the core autophagic machinery, and mutant IRGM protein that cannot direct these modifications is disabled for its role in autophagic defense against invasive bacteria.

Therapies to modulate autophagy are entering clinical trials but methods of monitoring whether drugs modulate autophagy in patients during such treatment are currently unavailable, but badly needed. In one aspect, the present invention addresses that need.
Brief Description of the Invention

IRGM, encoded by a uniquely human gene conferring risk for inflammatory diseases, affects autophagy through a hitherto unknown mechanism. The present invention is directed to showing that IRGM controls autophagy. IRGM interacts with ULK1 and Beclin 1 and promotes their coassembly into molecular complexes. IRGM stabilizes ULK1 and affects the stability of Beclin 1-interactors thus governing the composition of autophagy initiation complexes. We further show that IRGM interacts with pattern recognition receptors including NOD2. IRGM, NOD2 and ATG16L1, all of which are Crohn's disease risk factors and form a molecular complex to modulate autophagic responses to microbial products. NOD2 enhances K63-linked polyubiquitination of IRGM, which is required for interactions of IRGM with the core autophagy factors and for bacterial clearance. Thus, IRGM plays a direct role in organizing the core autophagy machinery to endow it with antimicrobial functions.

In one embodiment, the present invention relates to the use of IRGM modulators for the treatment of disease, in particular, bacterial infections and inflammatory diseases, most notably tuberculosis and Crohn's disease amongst a number of others. The compounds which are useful as modulators of IRGM include the double stranded RNA compounds, including poly I:C, poly-UG (poly UGUGU) and poly ICLC, among others, and muramyl dipeptide and its analogs and derivates as otherwise disclosed herein.

In one embodiment, the present invention provides a method of modulating autophagy in a biological system, in particular a patient or subject. In this aspect of the invention, a compound identified herein as an IRGM modulator (which can be an inhibitor or agonist of IRGM and/or its pathway(s), is presented to the biological system, including administration to a patient or subject in need, in order to modulate autophagy and effect a favorable result in the biological system, often a patient or subject. The resulting modulation may be monitored or applied in the biological system to effect a favorable result, including the inhibition, treatment and/or prevention of cancer, including metastasis of cancer, or the inhibition, treatment (including the amelioration of symptoms) and/or prevention of one or more disease states or conditions in which the modulation, especially including upregulation or inhibition of autophagy provides a favorable result in numerous disease states and/or
conditions including neurodegeneration (including, for example, Alzheimer's disease, Parkinson's disease; other ataxias), chronic inflammatory diseases (including, for example, inflammatory bowel disease, including Crohn's disease, rheumatoid arthritis, lupus, multiple sclerosis, chronic obstructive pulmonary disease/COPD, pulmonary fibrosis, cystic fibrosis, Sjogren's disease), diabetes and metabolic syndrome, muscle degeneration and atrophy, frailty in aging, stroke and spinal cord injury, arteriosclerosis, infectious diseases, especially bacterial infections such as tuberculosis, viral infections (HIV I and II, HBV, HCV, including secondary disease states or conditions associated with infectious diseases, including AIDS) and tuberculosis, among others. The common principle of this embodiment of the invention is that compounds which modulate IRGM, are outstanding autophagy modulators (i.e., inhibitors or activators of autophagy), depending upon the disease state, condition or symptom to be treated, may cure, prevent (including reducing the likelihood of), improve prognosis, ameliorate symptoms and/or improve the quality of the patient's or subject's life. In addition, in the therapeutic aspects of the invention, the administration of an autophagy modulator (i.e., one or more IRGM modulators alone or in combination with an additional autophagy modulator and/or an additional bioactive agent) may prolong the life of the patient, as well as improve the quality of life in the aging patient or subject.

In one embodiment the method of treating an autophagy-mediated disease state or condition comprising administering at least one dsRNA or a muramyl dipeptide analog or derivative (collective referred to as "IRGM modulators"), optionally in combination with at least one additional autophagy modulator and/or bioactive agent to a patient in need. In this method at least one IRGM modulator as described above, alone or in combination with an additional autophagy modulator, such as an autophagy modulator selected from the group consisting of flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon and nortriptyline, tetrachlorisophthalonitrile and phenylmercuric acetate, pharmaceutically acceptable salts thereof and mixtures thereof, alone, optionally in further combination with at least one additional bioactive agent, optionally in combination with a pharmaceutically acceptable carrier, additive or excipient, may be administered to a patient or subject in need to treat an autophagy-mediated disease state and/or condition. It is noted that flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine,
memantine, bromhexine, norcyclobenzaprine, diperodon, nortriptyline and their pharmaceutically acceptable salts show activity as agonists or inducers of autophagy in the treatment of an autophagy-mediated disease, tetrachlorisophthalonitrile, phenylmercuric acetate and their pharmaceutically acceptable salts, find use as antagonists or inhibitors of autophagy. All of these compounds will find use as modulators of autophagy in the various autophagy-mediated disease states and conditions described herein, with the agonists being preferred in most disease states other than cancer and in the case of the treatment of cancer, the inhibitors described above are preferred, alone or in combination with an autophagy agonist as described above and/or an additional anticancer agent as otherwise described herein.

Pharmaceutical compositions according to the present invention comprise an effective amount of at least one IRGM modulator as described herein in combination with an autophagy modulator selected from the group consisting of flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon, nortriptyline, tetrachlorisophthalonitrile, phenylmercuric acetate and their pharmaceutically acceptable salts, optionally in combination with a pharmaceutically acceptable carrier, additive and/or excipient and further optionally, in combination with at least one additional bioactive agent (e.g., an anticancer agent, antibiotic, anti-tuberculosis agent, antiviral agent such as an anti-HIV agent, anti-HBV agent or anti-HCV agent, etc.), preferably at least one anticancer agent as otherwise disclosed herein or at least one additional autophagy modulator as otherwise described herein. In the present invention, an additional autophagy modulator (autostatin) may be selected from the group consisting of may be combined with an additional autophagy modulator selected from the group consisting of benzethonium, niclosamide, monensin, bromperidol, levobunolol, dehydroisoandrosterone 3-acetate, sertraline, tamoxifen, reserpine, hexachlorophene, dipyridamole, harmaline, prazosin, lidoflazine, thiethylperazine, dextromethorphan, desipramine, mebendazole, canrenone, chlorprothixine, maprotiline, homochlorycyclizine, loperamide, nicardipine, dextfenfluramine, nilvadipine, dosulepin, biperiden, denatonium, etomidate, toremifene, tomoxetine, clorgyline, zotepine, beta-escin, tridihexethyl, ceftazidime, methoxy-6-harmalan, melengestrol, albendazole, rimantadine, chlorpromazine, pergolide, cloperastine, prednicarbate, haloperidol, clotrimazol, nitrofural, iopanoic acid, naftopidil, methimazole, trimeprazine, ethoxyquin, clocortolone, doxycycline, pirlindole
mesylate, doxazosin, deptropine, nocodazole, scopolamine, oxybenzone, halcinonide, oxybutynin, miconazole, clomipramine, cyproheptadine, doxepin, dyclonine, salbutamol, flavoxate, amoxapine, fenofibrate, pimethixene and mixtures thereof.

In still another embodiment, the invention provides a method of treating a subject who has been infected with tuberculosis (e.g. *M. tuberculosis*) or who is at risk of such infection, the method comprising administering to the subject a pharmaceutically effective amount of a IRGM modulator as described hereinafter. In another embodiment, the invention provides a method of treating Crohn's disease comprising administering to a patient in need a pharmaceutically effective amount of a IRGM modulator as described hereinafter.

The present invention provides methods of treating inflammatory or autophagy-related diseases. Autophagy is a eukaryotic intracellular pathway that carries out key aspects of cytoplasmic homeostasis. Autophagy has many biological effects that include immunological processes and inflammation, and one aspect is regulation of activation inflammasome activity. We disclose the methods to regulate disease-causing excessive inflammation by one form of selective autophagy named precision autophagy. This method provide therapeutic options for inflammatory or autophagy-related diseases by modulating precision autophagy. Several forms of precision autophagy could be induced by compounds, such as IFN-gamma, or related compounds. The present invention could be used to upregulate autophagy, for example in the case of disease states such as tuberculosis and other disease states where an upregulation of autophagy would be beneficial for disease treatment. This therapy could be effected by administering an effective amount of one or more TRIM proteins as otherwise described herein to a patient in need, the result being the upregulation of autophagy and the treatment of a disease state and/or condition which is mediated through autophagy (an autophagy-mediated disease). In other instances, the present invention could be used to regulate (i.e. down-regulate) some forms of precision autophagy, and precision autophagy in turn modulate several forms of inflammation, such as inflammasome or type I interferon response in order to bring the autophagy response back in to balance. The targeting disorders for precision autophagy down-regulation include autophagy-related diseases or inflammatory diseases, including autoimmune diseases, infectious diseases, cardiovascular diseases, and metabolic diseases including diabetes mellitus. For example, the inflammatory response is essential to human beings, however, excessive inflammatory response is a lethal condition seen in several diseases in different stages, including autoimmune diseases and
acute viral/bacterial infection. The inventors have found that the excessive inflammation associated with these disease states and/or conditions could be regulated by precision autophagy, including the administration of siRNAs as described herein which specifically inhibit one or more TRIM proteins as otherwise described herein. In addition, the inventors find that certain disease states could benefit from an initial upregulation of autophagy which could benefit the disease treatment, followed by down-regulation of autophagy during the course of therapy for the disease state and/or condition in order to reduce an excessive autophagy response.

Thus, the present invention utilizes certain preferred precision autophagy modulators to treat disease states and conditions which cause excessive inflammation and particularly seen in a number of disease states, especially including inflammatory diseases as otherwise described herein, autoimmune diseases, infectious diseases (generally, after an initial period of beneficial upregulation of autophagy), cardiovascular diseases and metabolic diseases, including diabetes mellitus. These precision autophagy modulators may include interferons such as interferon gamma (IFN-gamma) and pegylated interferon (PEG-IFN), as well as the preferred TRIM (tripartite motif containing) proteins or variants exhibiting 90% sequence identity to the TRIM proteins, preferably TRIM proteins selected from at least one TRIM protein selected from the group consisting of TRIM1, TRIM3, TRIM8, TRIMIO, TRIM13, TRIM17, TRIM19, TRIM20, TRIM21, TRIM22, TRIM38, TRIM 41, TRIM43, TRIM44, TRIM45, TRIM46, TRIM54, TRIM55, TRIM56, TRIM58, TRIM59, TRIM60, TRIM65, TRIM66 and TRIM75 with TRIM 1, TRIM 8, TRIM 20, TRIM 21, TRIM 22, TRIM 56 and TRIM 65 and mixtures thereof being preferred as autophagy upregulators.

The present invention relates to a method of treating excessive inflammation in inflammatory diseases, autoimmune diseases, infectious diseases, cardiovascular diseases and metabolic diseases in a patient in need thereof comprising administering to said patient an effective amount of a precision autophagy modulator selected from the group consisting of an interferon, including interferon gamma (IFN-gamma) and pegylated interferon (PEG-IFN) and at least one TRIM protein (including a TRIM protein variant), preferably a TRIM protein selected from the group consisting of TRIM1 (SEQ ID NO:1), TRIM3 (SEQ ID NO:1 1), TRIM8 (SEQ ID NO:36), TRIMIO (SEQ ID NO:46), TRIM13 (SEQ ID NO:56), TRIM17 (SEQ ID NO:81), TRIM19 (SEQ ID NO:91), TRIM20 (SEQ ID NO:96), TRIM21 (SEQ ID NO:101), TRIM22 (SEQ ID NO:106), TRIM38 (SEQ ID NO:172), TRIM 41 (SEQ ID NO:187), TRIM43 (SEQ ID NO:197), TRIM44 (SEQ ID NO:202), TRIM45 (SEQ ID
NO.207), TRIM46 (SEQ ID NO:212), TRIM54 (SEQ ID NO:247), TRIM55 (SEQ ID NO:252), TRIM56 (SEQ ID NO:257), TRIM58 (SEQ ID NO:262), TRIM59 (SEQ ID NO:267), TRIM60 (SEQ ID NO:272), TRIM65 (SEQ ID NO:297), TRIM66 (SEQ ID NO:302), TRIM75 (SEQ ID NO:338) and mixtures thereof, preferably TRIM 1 (SEQ ID NO:1), TRIM 8 (SEQ ID NO:36), TRIM 20 (SEQ ID NO:96), TRIM 21 (SEQ ID NO:101), TRIM 22 (SEQ ID NO:106), TRIM 56 (SEQ ID NO:257), TRIM 65 (SEQ ID NO:297). and mixtures thereof, optionally in combination with an additional autophagy modulator (including an alternative TRIM protein) and/or an additional bioactive agent. In certain instances, it may be beneficial to down-regulate autophagy and inhibit TRIM protein response in order to reduce an excessive autophagy response through the use of one or more siRNA as described herein which specifically inhibits one or more TRIM protein. Additional autophagy modulators for the present invention include, for example, flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon, nortriptyline, tetrachlorisopthalonitrile and phenylmercuric acetate, benzethonium, niclosamide, monensin, bromperidol, levobunolol, dehydroisoandrosterone 3-acetate, sertraline, tamoxifen, reserpine, hexachlorophene, dipyriramole, harmaline, prazosin, lidoflazine, thiethylperazine, dextromethorphan, desipramine, mebendazole, canrenone, chlorprothixene, maprotiline, homochlorcyclizine, loperamide, nicardipine, dexfenfluramine, nilvadipine, dosulepin, biperiden, denatonium, etomidate, toremifene, tomoxetine, clorgyline, zotepine, beta-escin, tridihexethyl, ceftazidime, methoxy-6-harmalan, melengestrol, albendazole, rimantadine, chlorpromazine, pergolide, cloperastine, prednicarbate, haloperidol, clotrimazole, nitrofural, iopanoic acid, naftopidil, methimazole, tremeprazine, ethoxyquin, clocortolone, doxycycline, pirlindole mesylate, doxazosin, deprotoxine, nocardazole, scopolamine, oxybenzone, halcinonide, oxybutynin, miconazole, clomipramine, cyproheptadine, doxepin, dyclonine, salbutamol, flavoxate, amoxapine, fenofibrate, pimethixene, pharmaceutically acceptable salts thereof and mixtures thereof, alternative TRIM proteins or variants exhibiting 90% sequence identity to the TRIM proteins, including, but are not limited to, TRIM2 (SEQ ID NO:6), TRIM 4 (SEQ ID NO:16), TRIM5 (TRIM5a) (SEQ ID NO:21), TRIM6 (SEQ ID NO:26), TRIM7 (SEQ ID NO:31), TRIM9 (SEQ ID NO:41), TRIM11 (SEQ ID NO:51), TRIM14 (SEQ ID NO:61), TRIM15 (SEQ ID NO:66), TRIM16 (SEQ ID NO:71), TRIM18 (SEQ ID NO:86), TRIM23 (SEQ ID NO:11), TRIM24 (SEQ ID NO:16), TRIM25 (SEQ ID NO:121), TRIM27 (SEQ ID NO:126), TRIM28 (SEQ ID NO:131), TRIM29 (SEQ ID
Neutral lipids such as lipids selected from the group consisting of triglycerides, diglycerides, monoglycerides, glycolated mono- or diacylglycerides, dolichol, polyprenol, polyprenal or very long chain fatty acids may also be administered in combination with the precision autophagy modulators according to the present invention to increase lipid storage and enhance the therapeutic effect of autophagy modulators used to treat excessive inflammation as otherwise disclosed herein. Additional bioactive agents as otherwise described herein may be administered in combination with the one or more of the above precision autophagy modulators and optionally, additional modulators and bioactive agents as otherwise described herein.

Pharmaceutical compositions according to the present invention comprise an effective amount of interferon, including interferon gamma (IFN-gamma) and pegylated interferon (PEG-IFN) in combination with at least one TRIM protein or a variant thereof, preferably a TRIM protein selected from the group consisting of TRIM1 (SEQ ID NO:1), TRIM3 (SEQ ID NO:1), TRIM8 (SEQ ID NO:36), TRIMIO (SEQ ID NO:46), TRIM13 (SEQ ID NO:56), TRIM17 (SEQ ID NO:81), TRIM19 (SEQ ID NO:91), TRIM20 (SEQ ID NO:96), TRIM21 (SEQ ID NO:101), TRIM22 (SEQ ID NO:106), TRIM38 (SEQ ID NO:172), TRIM 41 (SEQ ID NO:187), TRIM43 (SEQ ID NO:197), TRIM44 (SEQ ID NO:202), TRIM45 (SEQ ID NO:207), TRIM46 (SEQ ID NO:212), TRIM54 (SEQ ID NO:247), TRIM55 (SEQ ID NO:252), TRIM56 (SEQ ID NO:257), TRIM58 (SEQ ID NO:262), TRIM59 (SEQ ID NO:267), TRIM60 (SEQ ID NO:272), TRIM65 (SEQ ID NO:297), TRIM66 (SEQ ID NO:302), TRIM67 (SEQ ID NO:307), and TRIM73 (SEQ ID NO:328), TRIM74 (SEQ ID NO:333), and mixtures thereof, with TRIM2 (SEQ ID NO:6), TRIM5 (SEQ ID NO:21), TRIM6 (SEQ ID NO:26), TRIM11 (SEQ ID NO:51), TRIM23 (SEQ ID NO:11), TRIM27 (SEQ ID NO:126), TRIM28 (SEQ ID NO:131), TRIM31 (SEQ ID NO:141), TRIM 32 (SEQ ID NO:146), TRIM33 (SEQ ID NO:151), TRIM42 (SEQ ID NO:192), TRIM49 (SEQ ID NO:227), TRIM50 (SEQ ID NO:232), TRIM51 (SEQ ID NO:237), TRIM68 (SEQ ID NO:312), TRIM72 (SEQ ID NO:323), TRIM73 (SEQ ID NO:328), TRIM74 (SEQ ID NO:333) and TRIM (SEQ ID NO:343) being preferred. Neutral lipids such as lipids selected from the group consisting of triglycerides, diglycerides, monoglycerides, glycolated mono- or diacylglycerides, dolichol, polyprenol, polyprenal or very long chain fatty acids may also be administered in combination with the precision autophagy modulators according to the present invention to increase lipid storage and enhance the therapeutic effect of autophagy modulators used to treat excessive inflammation as otherwise disclosed herein. Additional bioactive agents as otherwise described herein may be administered in combination with the one or more of the above precision autophagy modulators and optionally, additional modulators and bioactive agents as otherwise described herein.
NO:302), TRIM75 (SEQ ID NO:338) and mixtures thereof, preferably TRIM 1 (SEQ ID NO:1), TRIM 8 (SEQ ID NO:36), TRIM 20 (SEQ ID NO:96), TRIM 21 (SEQ ID NO:101), TRIM 22 (SEQ ID NO:106), TRIM 56 (SEQ ID NO:257), TRIM 65 (SEQ ID NO:297) and mixtures thereof, optionally in combination with an additional autophagy modulator (including an alternative TRIM protein as otherwise described herein) and/or an additional bioactive agent as otherwise described herein in combination with a pharmaceutically acceptable carrier, additive or excipient. Compositions comprising an effective amount of at least one TRIM protein or a variant thereof, preferably a TRIM protein selected from the group consisting of TRIM1 (SEQ ID NO:1), TRIM3 (SEQ ID NO:1 1), TRIM8 (SEQ ID NO:36), TRIM10 (SEQ ID NO:46), TRIM13 (SEQ ID NO:56), TRIM17 (SEQ ID NO:81), TRIM19 (SEQ ID NO:91), TRIM20 (SEQ ID NO:96), TRIM21 (SEQ ID NO:101), TRIM22 (SEQ ID NO:106), TRIM38 (SEQ ID NO:172), TRIM 41 (SEQ ID NO:187), TRIM43 (SEQ ID NO:197), TRIM44 (SEQ ID NO:202), TRIM45 (SEQ ID NO:207), TRIM46 (SEQ ID NO:212), TRIM54 (SEQ ID NO:247), TRIM55 (SEQ ID NO:252), TRIM56 (SEQ ID NO:257), TRIM58 (SEQ ID NO:262), TRIM59 (SEQ ID NO:267), TRIM60 (SEQ ID NO:272), TRIM65 (SEQ ID NO:297), TRIM66 (SEQ ID NO:302), TRIM75 (SEQ ID NO:338) and mixtures thereof, with TRIM 1 (SEQ ID NO:1), TRIM 8 (SEQ ID NO:36), TRIM 20 (SEQ ID NO:96), TRIM 21 (SEQ ID NO:101), TRIM 22 (SEQ ID NO:106), TRIM 56 (SEQ ID NO:257), TRIM 65 (SEQ ID NO:297), and mixtures thereof being preferred in combination with at least one additional bioactive agent, including an autophagy modulator as otherwise described herein including an alternative TRIM protein. In addition, neutral lipids such as lipids selected from the group consisting of triglycerides, diglycerides, monoglycerides, glycolated mono- or diacylglycerides, dolichol, polyenol, polyenral or very long chain fatty acids may also be included in the pharmaceutical compositions according to the present invention in combination with the precision autophagy modulators according to the present invention to increase lipid storage and enhance the therapeutic effect of autophagy modulators used to treat excessive inflammation as otherwise disclosed herein.

Methods of treating a disease state and/or condition with precision autophagy in a patient or subject in need (where upregulation of autophagy is desirable) comprise administering to said patient an effective amount of at least one compound selected from the group consisting of interferon gamma (IFN-gamma), pegylated interferon (PEG-IFN) and at least one TRIM protein or a variant thereof, preferably a TRIM protein selected from the group consisting of TRIM1 (SEQ ID NO:1), TRIM3 (SEQ ID NO:1 1), TRIM8 (SEQ ID
NO:36), TRIM10 (SEQ ID NO:46), TRIM13 (SEQ ID NO:56), TRIM17 (SEQ ID NO:81), TRIM19 (SEQ ID NO:91), TRIM20 (SEQ ID NO:96), TRIM21 (SEQ ID NO:101), TRJ M22 (SEQ ID NO:106), TRIM38 (SEQ ID NO:172), TRIM 41 (SEQ ID NO:187), TRIM 43 (SEQ ID NO:197), TRIM44 (SEQ ID NO:202), TRIM45 (SEQ ID NO:207), TRIM46 (SEQ ID NO:212), TRIM54 (SEQ ID NO:247), TRIM55 (SEQ ID NO:252), TRIM56 (SEQ ID NO:257), TRIM58 (SEQ ID NO:262), TRIM59 (SEQ ID NO:267), TRIM60 (SEQ ID NO:272), TRIM65 (SEQ ID NO:297), TRIM66 (SEQ ID NO:302), TRIM75 (SEQ ID NO:338) and mixtures thereof, preferably TRIM 1 (SEQ ID NO:1), TRIM 8 (SEQ ID NO:36), TRIM 20 (SEQ ID NO:96), TRIM 21 (SEQ ID NO:101), TRIM 22 (SEQ ID NO:106), TRIM 56 (SEQ ID NO:257), TRIM 65 (SEQ ID NO:297) and mixtures thereof, optionally in combination with an additional autophagy modulator (including an alternative TRIM protein) and/or an additional bioactive agent. In these methods, neutral lipids such as lipids selected from the group consisting of triglycerides, diglycerides, monoglycerides, glycolated mono- or diacylglycerides, dolichol, polyrenol, polyrenal or very long chain fatty acids may also be administered in combination with the precision autophagy modulators according to the present invention to increase lipid storage and enhance the therapeutic effect of autophagy modulators used to treat excessive inflammation as otherwise disclosed herein. The present methods apply to a number of disease states and/or conditions which are mediated through autophagy and which often can result in an excessive autophagy response. In certain preferred aspects, the administration of TRIM20, alone or in combination with an additional autophagy modulator and/or bioactive agent as otherwise described herein is useful for upregulating autophagy and treating disease through modulation (up-regulation) of autophagy. This approach is especially useful in the case of certain disease states and/or conditions, especially microbial infections such as bacterial and viral infections where upregulation of TRIM proteins, especially TRJM20 is useful in inhibiting early stages of disease, especially viral and bacterial infections, including early stage tuberculosis (note that in later stage tuberculosis it may be preferable to down-regulate the autophagy response and inhibit the TRIM proteins by administering a TRIM protein inhibitor, especially including a siRNA). In this embodiment, a TRIM protein, especially including TRIM20 may be administered alone or in combination with interferon-gamma (IFN-gamma), pegylated interferon (PEG-IFN) and/or an additional autophagy modulator and/or an additional bioactive agent in order to treat a disease state and/or condition which is mediated through autophagy (an autophagy-mediated disease state and/or condition).
Methods according to the present invention also include down-regulating autophagy where an inflammatory response is elevated (in autoimmune disease, inflammatory diseases and in later stage disease states such as viral and/or bacterial infections, especially including tuberculosis, among others, the method comprising administering an inhibitor of a TRIM protein (including TRIM21) as otherwise set forth herein, especially siRNA which is an inhibitor of a TRIM protein. In preferred aspects, a siRNA inhibitor of TRIM21 is particularly useful in treating these disease states, especially including tuberculosis at any time during a tuberculosis infection. In other embodiments, a siRNA inhibitor of TRIM20 is administered at a later stage of tuberculosis in order to enhance the therapy of the disease state by reducing and/or the impact of autophagy.

Brief Description of the Figures

FIGURE 1 IRGM shows that IRGM activates AMPK signaling and interacts with core autophagy machinery. (A) Lysates from HT-29 colon epithelial cells transfected with control and IRGM siRNA were subjected to Western blotting with antibodies to phospho-AMPK (Thr-172), AMPK, IRGM and actin. (B) Levels of phospho-AMPK (Thr-172) and phospho-Beclin 1 (Ser-93/96) in lysates from HEK293T cells co-expressing Flag-Beclin 1 and GFP or GFP-IRGM. (C) Levels of active phospho-ULK1 (Ser-555 and Ser-317) in lysates of HEK293T cells co-expressing Myc-ULK1 and either GFP or GFP-IRGM. Numbers beneath bands in B, C, quantification of phosphorylated proteins relative to the total abundance of the same protein. (D) Co-immunoprecipitation (Co-IP) analysis of interaction between IRGM and endogenous ULK1 and AMBRA1 in HEK293T lysates of cells expressing GFP or GFP-IRGM. (E) Top, confocal microscopy images of HEK293T cells expressing IRGM-V5 and Myc-ULK1 subjected to starvation for 2 h. Arrowheads, co-localization. Bottom, fluorescence intensity line tracing. (F) Co-IP analysis in lysates of HEK293T cells expressing indicated proteins. (G) Confocal microscopy images of HEK293T cells transiently expressing V5-IRGM and Flag-Beclin 1 subjected to starvation for 2 h. Details as for panel E. (H) Lysates of HEK293T cells expressing GFP or GFP-IRGM with Myc-ULK1 subjected to immunoprecipitation with anti-GFP and blots probed with phospho-ULK1 Ser-317 or Ser-757 antibodies. (I) Lysates of cells expressing Myc-ULK1, Flag-Beclin 1 and increasing concentrations of GFP-IRGM subjected to immunoprecipitation with anti-Flag; blots probed as indicated. (J) HEK293T cell lysates co-expressing GFP-IRGM and Flag-Beclin 1 subjected to Western blotting with antibody to phospho-Beclin 1 (Ser-15) and antibodies as
indicated. (K) Co-IP analysis of Flag-IRGM and endogenous ATG14. (L, M) Mapping of Beclin 1 regions interacting with IRGM. (L) Lysates of HEK293T cells co-expressing GFP-IRGM and Flag-Beclin 1 variants in panel M were subjected to immunoprecipitation with anti-Flag and blots probed as indicated. (M) Beclin 1 domain organization indicating its interacting proteins along with deletion constructs used in Co-IP analysis in panel L. (N) Co-IP analysis of the effects of IRGM overexpression on the interaction of Beclin 1 with its regulatory proteins. Lysates of HEK293T cells co-expressing GFP-IRGM and Flag-Beclin 1 were subjected to immunoprecipitation with anti-Flag and blots probed as indicated. (O) Model of IRGM-dependent autophagy induction based on the results obtained in FIGURE 1 IRGM and FIGURE S1 IRGM. See also FIGURE S1 IRGM.

FIGURE 2 IRGM shows that IRGM is required for stable levels of the autophagy initiation proteins. (A,C,E) U937 cells transfected with control or IRGM siRNAs, untreated or treated with LPS (500 ng/ml for 4 h) were lysed and subjected to Western blotting with antibody to (A) ULK1, (C) ATG14L, AMBRA1 and ATG5, and (E) ATG16L1. IRGM knock down efficiency and quantifications are shown in Supplementary FIGURE S2 IRGM A,B. (B,D,F) Left, confocal images of U937 cells transfected with control or IRGM siRNA treated with LPS (500 ng/ml for 4 h), Immunofluorescence analysis was performed with (B) phopho-ULK1 (Ser-317), (D) ATG5, and (F) ATG16L1. Graphs, means ± SD (corrected total cell fluorescence of cells; >30 cells from 5 fields measured using Image J). *; p < 0.05 (Student's unpaired t test). (G) Lysates from HEK293T cells expressing GFP or GFP-IRGM were subjected to immunoprecipitation with anti-GFP and blot probed with indicated antibodies. (H) Schematic of ATG16L1 domain structure indicating IRGM interacting regions mapped in panels I. (I) Lysates of HEK293T cells co-expressing GFP-IRGM and the indicated Flag-ATG16L1 variants in panel H were subjected to immunoprecipitation with anti-Flag and blots probed as indicated. Results, representative of three independent experiments. See also FIGURE S2 IRGM

FIGURE 3 IRGM shows that IRGM is required for PAMPs induced autophagy. (A) Abundance of IRGM mRNA (relative to GAPDH) in THP-1 cells (control or infected with invasive E. coli LF82) determined by quantitative real-time PCR (qRT-PCR). (B) Effect of LPS (30 min) or (C) MDP exposure (16 h) on IRGM mRNA levels in U937 cells. Gene expression (qRT-PCR) was normalized relative to GAPDH. Data, means ± SD (n>3); *; p < 0.05 (Student's unpaired t test). (D) Schematic summary of the physiological signals
activating \textit{IRGM} expression based on data in panels A-C and in FIGURE S3A-H. (E, F) Left, Western blot analysis of LC3-II abundance in U937 cells transfected with control or IRGM siRNA: (E) treated or not with LPS (500 ng/ml; 4 h); (F) treated or not with MDP (5 \(\mu\)g/ml; for 8 h). Right, densitometric analysis of Western blots using ImageJ software. (G, H) Left, confocal images of LC3 puncta in LPS treated (500 ng/ml; 4 h) (G) or MDP-treated (5 \(\mu\)g/ml; 8 h), (H) U937 cells transfected with control or IRGM siRNA. Graphs (right of panels G and H), represent mean corrected total cell fluorescence ± SE (25-35 cells from 10-15 fields measured using ImageJ. *p<0.05 (ANOVA). (I) Analysis of endogenous interactions (Co-IP) using THP-1 lysates infected with invasive E. coli LF82 (1 h) or stimulated with LPS (2 \(\mu\)g/ml; 2 h) or MDP (10 \(\mu\)g/ml; 2 h). Lysates were subjected to immunoprecipitation with IRGM antibody or control IgG and probed as indicated. (J) Schematic summary of the results obtained in FIGURE 3E-I. See also FIGURE S3 IRGM.

**FIGURE 4 IRGM** shows that IRGM interacts and co-localizes with ATG16L1 and NOD2. (A, B) Co-IP analysis of endogenous (A) or overexpressed (B) IRGM, with NOD2 and ATG16L1 in (A) starved HT29 cells and (B) HEK293T cells. (C) Top, confocal microscopy images of HEK293T cells transiently expressing GFP-IRGM and Flag-NOD2. Bottom, fluorescence intensity line tracing corresponding to dashed line. (D) Schematic of NOD2 domain organization along with deletion constructs used in Co-IP analysis in panel E. (E) Left panel, lysates of HEK293T cells co-expressing GFP-IRGM and the Flag-NOD2 variants shown in panel D subjected to immunoprecipitation with anti-Flag and blot probed with antibodies as indicated. Right panel, densitometric analysis of Western blots (IP blot/Input blot). (F) Flag tag pull-down assays performed with affinity purified NOD2 variants from 293T cell lysates and purified recombinant GST-IRGM shown in the schematic (left panel). (G) Top, confocal microscopy images showing co-localization of GFP-IRGM and Flag-NOD2 and Rhodamine-MDP in HEK293T cells. Bottom, fluorescence intensity line tracing corresponding to red line. (H) Effect of MDP (10 \(\mu\)g/ml; 8 h) on GFP-IRGM and Flag-NOD2 interactions in HCT1 16 cells. (I) Model of IRGM-NOD2 interactions. See also FIGURE S4 IRGM.

**FIGURE 5 IRGM** shows that NOD2 promotes K63-linked polyubiquitination of IRGM, enhancing its interactions with autophagy initiation factors. (A-C) Effects of NOD2 expression on IRGM self-association (A), and IRGM's interaction with Beclin 1 (B) or with ULK1 (C) in HEK293T cells. (D, E) Analysis of IRGM ubiquitination in HEK293T cells.
Cells co-expressing GFP or GFP-IRGM and (D) HA-tagged Ubiquitin C or (E) HA-tagged Ubiquitin C mutated for all lysines except lysine 48 (HA-K48) or Lysine 63 (HA-K63) and Flag-NOD2 were subjected to immunoprecipitation with GFP antibody and blots probed with indicated antibodies. Blot in (E) was processed to remove irrelevant lanes (dashed vertical line). (F) Cells co-expressing GFP-IRGM, HA-K63 and Flag-NOD2 deletion variants as in Figure 4D were subjected to immunoprecipitation analysis with anti-GFP and blot probed with indicated antibodies. (G) Cells co-expressing GFP or GFP-IRGM or GFP-IRGM-K\textsuperscript{mut} (IRGM variant with all lysine residues mutated to alanine) and HA-K63 were subjected to immunoprecipitation analysis with anti-GFP and blot was probed with indicated antibodies. Blot was processed (dashed vertical line) to remove irrelevant lanes. (H) Lysates of cells co-expressing GFP or GFP-IRGM or GFP-IRGM-K\textsuperscript{mut} and Flag-IRGM were subjected to immunoprecipitation with anti-GFP and blot probed with indicated antibodies. (I) Lysates of cells expressing GFP or GFP-IRGM or GFP-IRGM-K\textsuperscript{mut} were subjected to immunoprecipitation with anti-GFP and blots probed with indicated antibodies. Results representative of three independent experiments. See also FIGURE S5 IRGM.

**FIGURE 6 IRGM** shows that ubiquitination of IRGM is required for NOD2 degradation and ULK1 stability. (A) Effects of IRGM expression on NOD2 levels in transfected HEK293T cells. Data, means ± SE; *, p < 0.05 (Student's unpaired t test). (B) Lysates of FIEK293T cell co-expressing GFP or GFP-IRGM and Flag-NOD2, untreated/treated with Bafilomycin A1 (100 nM for 8 h) were subjected to Western blotting. (C) Lysates of cells co-expressing Flag-NOD2 and GFP, GFP-IRGM, or GFP-IRGM-K\textsuperscript{mut} were subjected to Western blotting. (D, E) Lysates from HEK293T cells co-expressing Myc-ULK1 and either GFP or increasing amounts of GFP-IRGM were subjected to Western blotting as in (D) with the relative abundance of Myc-ULK1 shown in (E). Blot was processed (dashed vertical line) to remove irrelevant lanes. (F) HEK293T cells transfected with plasmids encoding GFP, GFP-IRGM, or GFP-IRGM-K\textsuperscript{mut} and either Myc-ULK1 or Flag-Beclin 1 were lysed and subjected to Western blotting. Data from densitometric analyses of Western blots (B, C, E), means ± SE, n=3 *, p < 0.05 (ANOVA). (G) Depiction of the role of IRGM ubiquitination in NOD2 degradation and ULK1 stabilization. See also FIGURE S6 IRGM.

**FIGURE 7 IRGM** shows that ubiquitination of IRGM is important for preventing inflammation. (A) Effect of IRGM (WT and K\textsuperscript{mut}) expression with and without NOD2 on the
nuclear localization of NF-kB-p65 in HeLa cells upon E. coli LF82 infection. (B) Graph, mean % cells with NFkB-p65 nuclear localization (from 10 microscopic fields) ± SD; *, p < 0.05 (ANOVA). (C) Effect of E. coli infection on IL-1β mRNA expression in THP-1 cells subjected to IRGM knockdown (qRT-PCR normalized to GAPDH). Data, means ± SD (n>3); *, p < 0.05 (ANOVA). (D, E, F) Lysates of cells co-expressing either GFP or GFP-IRGM and (D) Flag-NOD1, (E) Flag-Rig-I, or (F) Flag-TLR3, subjected to immunoprecipitation with anti-GFP (D, E) or anti-Flag (F); blots were probed with indicated antibodies. (G) Effect of FLAG-tagged NOD1, RIG-I, or TLR3 expression on IRGM ubiquitination (K63-linked) in HEK293T cells. (H) Model of IRGM-mediated xenophagy. IRGM expression is induced by physiological cues including starvation, microbes, or microbial products (PAMPs). IRGM protein increases the abundance of active AMPK, which subsequently promotes autophagy by activating ULK1 and Beclin 1. Not only does IRGM amplify this fundamental autophagy signaling but it also assembles the core autophagy machinery. Association of IRGM with NOD2, which is enhanced in the presence of MDP, promotes IRGM ubiquitination and the assembly of autophagy initiation factors. Together, these molecular events promote antimicrobial autophagy and suppress excessive inflammatory responses. See also FIGURE S7 IRGM.

FIGURE S1 IRGM, related to FIGURE 1 IRGM shows that IRGM interacts with core autophagy machinery. (A) Left panel, Western blotting with lysates of bafilomycin (100 nM, 2 hr) treated or untreated HCT116 cells expressing GFP or GFP-IRGM. Right panel, densitometric analysis of Western blots. (B) Co-IP analysis with HEK293T cell co-expressing either GFP or GFP-IRGM and Myc-ULK1. (C-F) Co-IP experiment with HEK293T cell expressing GFP or GFP-IRGM (C, D, F) and Myc-AMBRA1 (E) were subjected to Western blotting with indicated antibodies.

FIGURE S2 IRGM, related to FIGURE 2 IRGM shows that IRGM stabilizes core autophagy machinery. (A) Graph showing the knockdown efficiency of IRGM in U937 monocytic cells. (B) Graph showing the densitometric analysis of Western blots in FIGURE 2 IRGM A, C, E. Result shown are mean ± S.D of three independent experiments. *, p > 0.05. (C) U937 monocyte cells transfected with control or IRGM siRNA, untreated or treated with LPS (500 ng/ml for 4 h) were lysed and subjected to Western blotting with indicated antibodies.
**FIGURE S3 IRGM**, related to **FIGURE 3 IRGM** shows that starvation induces IRGM expression through AMPK. (A, B) Analysis of IRGM expression in several cell lines by quantitative real-time PCR (qRT-PCR). PBMC-Peripheral blood mononuclear cell (C, D) Starvation induces IRGM expression in several cell lines and notably in (D) HT-29 cells (-20 fold). RNA isolated from fed and starved cells were subjected to qRT-PCR. (E) Western blot from fed and starved HT-29 cells lysates showing induction of IRGM and LC3B. (F, G) AMPK is required for starvation induced IRGM expression in HT-29 cells. (F) qRT-PCR from RNA isolated from fed or starved HT-29 cells, treated with increasing concentration of compoundC (20, 40, 80, 160 µM). CompoundC is potent inhibitor of AMPK. (G) Knocking down AMPKa2 blunted starvation induced IRGM expression. Inset, Western blotting showing AMPK-sx2 knock down efficiency. (H) RNA isolated from U937 cells treated with IFNγ were subjected to qRT-PCR. (I) Graph showing knockdown efficiency of IRGM in U937 monocyic cells.

**FIGURE S4 IRGM**, related to **FIGURE 4 IRGM** shows that IRGM interacts and co-localizes with ATG16L1 and NOD2. (A) Endogenous IRGM interact with NOD2 and ATG16L1 in starved HT-29 cells. Co-IP analysis using IRGM antibody and Western blotting with indicated antibodies . (B) Lysates from cells expressing GFP-IRGM and Flag-NOD2 were subjected to immunoprecipitation with anti-GFP and blots were probed with antibodies as indicated. (C) Representative confocal images of HEK293T cells expressing GFP-IRGM alone or with NOD2 (D, E) HEK293T cells expressing GFP-IRGM (D) or GFP-IRGM and Flag-NOD2 (E) were subjected to immunofluorescence with Tom 20 (mitochondrial marker) antibody. Bottom, co-localization profile measurement along straight line using LSM 510 software.

**FIGURE S5 IRGM**, related to **FIGURE 5 IRGM** shows that NOD2 enhances ubiquitination of IRGM. (A) HEK293T cell lysates expressing the indicated set of proteins were subjected to immunoprecipitation with Flag antibody and Western blotted with antibodies as indicated. (B) HEK293T cell lysates co-expressing GFP-IRGM alone or along with NOD2 were subjected to immunoprecipitation with GFP antibody and Western blotted with antibody to GFP. (C) HEK293T cell lysates co-expressing IRGM-V5 and HA-K63 were subjected to immunoprecipitation with V5 antibody and Western blotted with antibody to HA. (D) Analysis of effect of NOD2 on IRGM/IRGMkmut- Beclin 1 interaction by Co-IP experiment.
FIGURE S6 IRGM, related to FIGURE 7 IRGM shows that ubiquitination of IRGM is important for its anti-inflammatory function. (A) Starvation reduces intracellular replication of invasive E. coli LF82 in HEK 293T cells. Results are expressed as mean ± standard error of colony-forming units (cfu) per ml per 104 live cells. *, p<0.05. (B) Representative confocal images of GFP-IRGM transfected HEK293T cells infected with invasive E. coli LF82 (red, LPS antibody). (C) Analysis of NFicB-p65 nuclear translocation following LF82 infection in HeLa cells expressing GFP or GFP-IRGM or GFP-IRGM-Kmut and/or Flag-NOD2. (D) Graph showing the knock down efficiency of IRGM in LF82 infected THP-1 cells (E) Lysates of cells co-expressing control vector or Flag-IRGM and GFP-TLR4 were subjected to immunoprecipitation with anti-Flag and blots were probed with indicated antibodies.

FIGURE 1 PRECISION shows that TRIM proteins regulate IFN-γ-induced autophagy. (A) THP-1 cells were subjected to TRIM knockdown, treated with 1,000 U/mL IFN-γ for 4 h, and high content (HC) analysis was performed using a Cellomics HCS scanner (epifluorescence) and iDEV software. HC (magenta, endogenous LC3B immunofluorescence [IF]; blue, nuclei stained with Hoechst). Mask overlay, software-defined objects (primary objects, cell outlines; internal secondary objects, LC3 puncta). (B) Average count of LC3 puncta per cell from cells treated as in (A) (Data from two 96-well plates with identical siRNA arrangements; the corresponding data are shown in Supplementary figure 1C). Encircled are INF-γ-treated wells (right) and wells with vehicle controls (bottom left). TRIM knockdowns that reduced LC3 puncta readout in both two experiments by 3 SDs (horizontal dot lines) from the average of IFN-γ-treated controls (horizontal solid line) are indicated by corresponding TRIM numbers (open circle). TRIMs that were chosen in follow-up experiments in Figure 1C are also indicated with number. (C) Similar to (B), except that THP-1 cells were subjected to specific TRIM or scrambled (Scr; control) knockdown, and were analyzed in more than quadruplicate manner. (D) Model of TRIMs-mediated IFN-γ-induced autophagy based on the results obtained in Figure 1 and Figure SI thus far. (E) THP-1 cells were treated with TRIM20 or Scr siRNAs, treated with or without IFN-γ for 4h in the presence of bafilomycin A1, and LC3-II conversion was determined by immunoblots. (F) HeLa cells were transfected with GFP or GFP-TRIM20, and HC analysis performed. Data, means ± SE, n ≥ 3, *P < 0.05; †P ≥ 0.05 (ANOVA). Scale bars, 5 µη.
FIGURE 2 PRECISION shows that TRIM20 interacts with ULK1 and Beclin 1. (A,B) Co-immunoprecipitation analysis of GFP-TRIM20 (T20) with (A) Myc-ULK1 or (B) Flag-Beclin 1 in HEK293 cells extracts. IP, immunoprecipitation; WB, western blot. (C) Confocal microscopy of HeLa cells co-expressing mCherry-TRIM20 with GFP-ULK1. Line tracing corresponds to arrow. White outline, cell boundary defined by background fluorescence. Scale bars, 10 μm. (D) Co-immunoprecipitation analysis of TRIM20 complexes with p-ULK1 (Ser-317) in HEK293 cells. (E) TRIM20 domains and deletion constructs used. Dotted lines, deleted regions. (F) Co-immunoprecipitation analysis of interactions between deletion variants of TRIM20 (as GFP fusions) with Myc-ULK1 in HEK293 cells. (G) GST pull-down analysis of radiolabeled Myc-ULK1 with GST-tagged deletion variants of TRIM20. Top, autoradiogram of pull-down products. Bottom, Coomassie Brilliant Blue (CBB)-stained SDS-polyacrylamide gel with GST-deletion variants of TRIM20. Data representative of three or more experiments.

FIGURE 3 PRECISION shows that TRIM20 assembles ULK1 and Beclin 1 in a complex and interacts with ATG16L1. (A) TRIM20 domains and deletion constructs used. (B) Co-immunoprecipitation analysis of interaction between deletion variants of TRIM20 (as GFP fusions; asterisks denote fusion products on the bottom blot) with Flag-Beclin 1 in HEK293 cells. (C) Co-immunoprecipitation analysis of ULK1 in Beclin 1 complexes in the presence and absence of TRIM20 from HEK293T cell lysates. (D) Co-immunoprecipitation analysis of GFP-TRIM20 with endogenous ATG16L1. (E) TRIM20 domains and deletion constructs used. (F) Co-immunoprecipitation analysis of interaction between deletion variants of TRIM20 with Flag-ATG16L1 in HEK293 cells. (G) ATG16L1 domains and deletion constructs used. (H) Co-immunoprecipitation analysis of interactions between deletion variants of Flag-ATG16L1 and GFP-TRIM20 in HEK293 cells. (I) Model of TRIM20-dependent autophagy induction based on Figure 2, 3, and Supplementary Figure 2. Data representative of three or more experiments.

FIGURE 4 PRECISION shows that TRIM20 interacts with mammalian Atg8 paralogs (mAtg8s). (A) GST pull-down analysis of interactions between radiolabeled Myc-TRIM20 and GST-tagged mAtg8s. Top, autoradiogram of pull-down products. Bottom, CBB-stained SDS-polyacrylamide gel with GST-mAtg8s. (B) TPJM20 domains and deletion constructs used. (C) GST pull-down analysis of binding between radiolabeled Myc-TRIM20 deletion
variants and GST-GABARAP and GST-LC3A. (D) Identification of GABARAP interacting regions on TRIM20 by peptide array. Three series of TRIM20 peptides (regions of primary sequence staggered by 3 amino acid residues), with either three or four positive consecutive binding signals, were identified. The peptide sequences corresponding to the positive binding signals (encompassed spots; defined as Region I, II, III) were mutated as described, and were subjected to the GST pull-down experiments in (E) and Figure S2F. (E) GST pull-down analysis of interaction between radiolabeled Myc-TRIM20 triple mutants and GST-GABARAP. Data representative of three or more experiments.

FIGURE 5 PRECISION shows that TRIM20 degrades inflammasome components through autophagy. (A) Levels of NLRP3 were determine in lysates from THP-1 cells subjected to TRIM20 or Scr siRNA were activated with 1,000 U/mL IFN-γ for 3 h, and 2.5 µg/mL LPS for 2 h (for optimal TRIM20 expression; Supplementary Figure S3B). RI, relative intensity. (B) Levels of NLRP3 were determined from THP-1 subjected to TRIM20 or control knockdown and treated or not with bafilomycin A1 (Baf Aj). (C) The abundance of NLRP3 protein was determined from THP-1 cells subjected to TRIM20 or control knockdown and exposed to Escherichia coli strain LF82 and IFN-γ in the presence or absence of bafilomycin. (D) The abundance of NLRP3 protein was determined from primary human MDMs subjected to TRIM20 or control knockdown and exposed to LPS and IFN-γ in the presence or absence of bafilomycin. (E) Levels of NLRP3 were determined from THP-1 cells subjected to ULK1, Beclin 1, or Scr siRNA were treated with IFN-γ and LPS. (F-H) Levels of NLRP3 (F), NLRP1 (G), or pro-capsase 1 (H) were determined in cells expressing GFP or GFP-TRIM20 following autophagy induction (EBSS, 3 h) in the presence or absence of bafilomycin A1. Data, means ± SE, n ≥ 3, *P < 0.05, †P ≥ 0.05 (ANOVA).

FIGURE 6 PRECISION shows that ULK1 is recruited to NLRP3 complexes by wild type TRIM20 but not by FMF disease-associate TRIM20 mutants. (A) Co-immunoprecipitation analysis of ULK1 in NLRP3 complexes in HEK293T cells expressing GFP-TRIM20 or GFP alone. (B) The effect of NLRP3 expression on the presence of phospho-ULK1 in TRIM20 complexes. Lysates from HEK293 cells transiently expressing Myc-ULK1, GFP-TRIM20 (or GFP alone), and Flag-NLRP3 (or not) were immunoprecipitated with anti-GFP and immunoblots were probed as indicated. (C) Model of TRIM20's function in autophagy as a regulator-receptor: TRJM20 assembles autophagy machinery (ULK1, Beclin 1, ATG16L1,
mAtg8s) and recognizes substrates (NLRP3, pro-caspase 1 and NLRP1) delivering them for autophagic degradation. The recognition of substrate enriches active p-ULK1 on the TRIM20 platform. (D) FLICA-positive cells were quantified using THP-1 cells that had been subjected to knockdown of TRIM20, treated with IFN-γ, and then treated with or without LPS (2 h) and nigericin (10 min), and stained for active caspase-1 (with FLICA); >150 cells per experiment were analyzed for quantification. (E) The levels of IL-1β were determined from supernatants of THP-1 cells that had been subjected to knockdown of ULK1 or TRIM20, treated with IFN-γ and LPS, and stimulated with nigericin for 30 min. (F) Predominant FMF-associated point mutations of TRIM20 reside in the PRY/SPRY domain. (G) Levels of NLRP3 were determined from lysates of HEK293 cell expressing GFP-TRIM20 (wild type or FMF-associated variants) or GFP and induced for autophagy by starvation in EBSS for 3 h. (H) Effects of FMF-associated variants on ULK1 presence in TRIM20 complexes. HEK293 cells were transiently transfected with Myc-ULK1, and either GFP-TRIM20 (wild type or FMF-associated variants) or GFP alone. Lysates were immunoprecipitated with anti-GFP, and immunoblots were probed as indicated. Numbers indicate relative intensity of the indicated band. (I) Model of FMF-associated mutation in NLRP3 degradation. The presence of NLRP3 promote phosphorylation of ULK1 in TRIM20 complex, leading to autophagic degradation of NLRP3. TRIM20 mutants harbored less ULK1 and phospho-ULK1, which results in less autophagic activity and less degradation of inflammasome components. Asterisks denote common FMF-associated point mutations in TRIM20. Data, means ± SE, n ≥ 3, *P < 0.05, (ANOVA).

FIGURE 7 PRECISION shows that TRIM21 interacts with autophagy regulators and effectors. (A and B) Co-immunoprecipitation analyses of GFP-TRIM21 (T21) with (A) Myc-ULK1 and (B) Flag-Beclin 1 in HEK293 cells extracts. (C) GST pull-down analysis of binding between radiolabeled Myc-TRIM21 and GST-mAtg8s. Top, autoradiogram of pull-down products. Bottom, CBB-stained SDS-polyacrylamide gel with GST-mAtg8s. (D) TRIM21 domains and deletion constructs used. (E) GST pull-down analysis of binding between radiolabeled Myc-TRIM21 deletion mutants and GST-GABARAP and GST-p62. Asterisks and squares denote presence or absence of Myc-TRIM21, respectively. (F) p62 domains and deletion constructs used. (G) GST pull-down analysis of interaction between radiolabeled Myc-TRIM21 and GST-tagged p62. Data representative of three or more experiments.
**FIGURE 8 PRECISION** shows that TRIM21 promotes autophagic degradation of IRF3 dimers and attenuates type I interferon production. (A) Confocal microscopy of HeLa cells co-expressing mCherry-TRIM21, Flag-IRF3, and GFP-LC3B in the presence of bafilomycin A1. White outline, cell boundary. Arrows indicate the colocalization. (B) Confocal microscopy of HEK293 cells co-expressing mCherry-TRIM21, Flag-IRF3, and GFP-ULK1. (C) Co-immunoprecipitation analysis of IRF3-ULK1 complexes in the presence and absence of TRIM21. Lysates from HEK293 cells transiently expressing Myc-ULK1, Flag-IRF3, and either GFP-TRIM20 or GFP were immunoprecipitated with anti-Myc, and immunoblots were probed as indicated. (D) Levels of dimerized IRF3 were assessed by native PAGE from THP-1 cells subjected to TRIM21 or control knockdown, and stimulated for 12h by herring testis DNA (HT-DNA) transfected into the cells in the presence of 200 U/mL IFN-γ. (E) The effect of autophagy inhibition with bafilomycin on TRIM21-dependent IRF3 dimer degradation in THP-1 cells. (F) The effect of TRIM21 knockdown on IFN-β mRNA levels following stimulation of THP-1 cells with IFN-γ and HT-DNA. (G) Model of TRIMs' roles in regulation of inflammation by precision autophagy. TRIM20 targets the inflammasome components for autophagic degradation, whereas TRIM21 targets IRF3, to suppress inflammasome activity and type I IFN response, respectively. TRJM20 and TRIM21, both of whose expression response to IFN-γ, directly bind their respective cargo, cooperate in IFN-γ induction of autophagy (dashed line), and recruit autophagic machinery to execute degradation. Scale bars, 10 μm. Data, means ± SE, n ≥ 3, *P < 0.05, †P ≥ 0.05 (ANOVA).

**FIGURE S1 PRECISION** shows that TRIM proteins regulate IFN-γ-induced autophagy. (A and B) High content image analysis of LC3 puncta in (A) THP-1 cells or (B) human MDM cells treated with IFN-γ for 4 h. HC and mask overlays are as in Fig 1. (C) Screen data from Figure 1B showing average ± range. (D) Knockdown efficacy of TRIMs were determined by RT-PCR. (E and F) THP-1 cells were treated with (E) escalating doses of IFN-γ for 4 h or (F) 1,000 U/ml of IFN-γ for indicated times, and TRIM20 mRNA levels were determined by quantitative RT-PCR. Values are standardized to (E) no IFN-γ control or (F) 0 h time point. (G) THP-1 cells were subjected to TRIM20 or scrambled siRNA, treated with IFN-γ for 4 h, and HC analysis performed. (H) Knockdown of TRIM20 mRNA levels was examined by quantitative RT-PCR. Values are standardized to control (Scr, scrambled; no IFN-γ). (I) LC3-II conversion in HEK293 cells transfected with GFP-TRIM20 (T20) or GFP. Data, means ±
**FIGURE S2 PRECISION** shows that TRIM20 interacts with ULK1, Beclin 1, and mAtg8s. (A,B) Co-immunoprecipitation analysis of GFP-TRIM20 with endogenous (A) ULK1 or (B) Beclin 1 in HEK293 cells extracts. (C) Beclin 1 domains and deletion constructs used. (D) Co-immunoprecipitation analysis of interactions between deletion variants of Flag-Beclin 1 (asterisks and squares in the top blot denote presence or absence of Flag-Beclin 1, respectively) and GFP-TRIM20 in HEK293 cells. (E) Confocal microscopy of HEK293 cells co-expressing mCherry-TRIM20 with GFP-GABARAP. Line tracings correspond to arrows. (F) Confocal microscopy of HeLa cells co-expressing mCherry-TRIM20 with GFP-LC3B in the presence of bafilomycin A1. Line tracing corresponds to arrows. (G) GST pull-down analysis of interaction between radiolabeled Myc-TRIM20 harboring single or double mutants (corresponding to Figure 4D) and GST-GABARAP. Data representative of three independent experiments. Scale bars, (E) 5 μη (2 μη for inset) and (F) 10 μη.

**FIGURE S3 PRECISION** shows that TRIM20 degrades NLRP3 through autophagy. (A) Co-immunoprecipitation analysis of deletion variants of TRIM20 (as GFP fusions; asterisks denote fusion products on the bottom blot) with NLRP3 in HEK293 cells. (B) THP-1 cells were treated with IFN-γ for 3 h, additionally treated with 2.5 μg/ml of LPS for indicated periods, and TPJM20 mRNA levels were determined by quantitative PCR. Values are standardized to IFN-γ-untreated control. (C and D) Levels of NLRP3 were determined in lysates from THP-1 cells subjected to TRTM20 or Scr siRNA were (C) untreated either IFN-γ or LPS, or (D) LPS alone for 2h. (E) THP-1 cells were treated with 1.0 μg/ml of LPS for 3h, and levels of NLRP3 in lysate were determined by immunoblots. (F) Knockdown efficacies of ULK1 and Beclin 1 by siRNA were examined by quantitative RT-PCR. (G and H) Levels of GFP-TRIM20 were determined in cells co-expressing (G) with or (H) without NLRP3 following autophagy induction (EBSS, 3 h) in the presence or absence of bafilomycin A1. (I) Co-immunoprecipitation analysis of ULK1 in NLRP3 protein complexes in the presence and absence of TRIM20 knockdown. Lysates from THP-1 cell subjected to each knockdown and treatment of 200 U/mL IFN-γ for 3h and additional LPS (1.0 μg/ml) treatment 2h, were immunoprecipitated with anti-NLRP3, and immunoblots were probed as indicated. (J) Co-immunoprecipitation analysis of AMPK in GFP-TRIM20 complexes in HEK293T cells.
expressing Myc-ULK1 and Flag-NLRP3 (or not). Data, means ± SE, n ≥ 3 experiments, *P < 0.05, †P > 0.05 (C, t-test; D, ANOVA).

**FIGURE S4 PRECISION** shows the effects of TRIM20 on inflammasome activity and FMF-associated variants of TRIM20 decrease number of TRIM20 and LC3 puncta. (A) LDH release of supernatants in Figure 6D. (B and C) Supernatants were harvested from THP-1 cells that had been subjected to double knockdown as indicated, treated with IFN-γ and LPS, additionally stimulated with nigericin (20 μM) for 30 min, and levels of IL-1β and LDH release were measured. (D) Knockdown efficacy of NLRP3 by siRNA was examined by immunoblotting. (E) Confocal microscopy of THP-1 cells that had been subjected to knockdown of TRIM20, treated with IFN-γ, and then treated with or without LPS (2h) and nigericin (10min), and stained for active caspase-1 (with FLICA) and nucleus (TO-PRO-3). Arrowheads, FLICA-positive puncta; asterisk, cell; white outline, cell boundary. (F) Confocal microscopy of GFP-TRIM20 (wild type or FMF-associated variants) or GFP in HEK293 cells. (G) HC image (epifluorescence) analysis of TRIM20 puncta in HeLa cells expressing GFP-TRIM20 (wild type or FMF-associated variants) or GFP. (H) HC image analysis of LC3 puncta in HeLa cells expressing GFP-TRIM20 (wild type or triple mutant TRIM20). Data, means ± SE, n ≥ 3 experiments, *P < 0.05, †P > 0.05 (t test or ANOVA). Scale bar, 5 μm.

**FIGURE S5 PRECISION** shows that TRIM21 affects the level of dimerized IRF3 in HIV1 infection. (A) THP-1 cells were treated with 1,000 U/ml of IFN-γ for indicated times, and TRIM21 mRNA levels were determined by quantitative RT-PCR. (B) Co-immunoprecipitation analysis of GFP-TRIM20 with Flag-TRIM21 in HEK293 cells extracts. (C) Knockdown efficacy of TRIM21 level was examined by immunoblotting. (D) Levels of dimerized IRF3 were assessed by native PAGE from THP-1 cells subjected to TRIM21 or control knockdown, untreated with IFN-γ, and transfected with herring testis DNA (HT-DNA). (E and F) THP-1 cells subjected to TRIM21 or control knockdown were infected with a single-round infection HIV1 virus in the presence of 200 U/mL IFN-γ for 20h, and (E) the levels of dimerized IRF3 or (F) mRNA levels of IFN-β were determined. (G) Model of TRIM21's dual function in autophagy as a regulator-receptor: TRIM21 assembles autophagy machinery (ULK1, Beclin 1, and mAtg8s) and recognizes substrates (dimerized IRF3) delivering them for autophagic degradation to suppress type I IFN response and inflammation. Dashed outlines (ULK1 and Beclin 1), domain binding location not mapped;
solid outline for mAtg8 (GABARAP) reflects mapping data. (H) The effect of TRIM21 knockdown on IFN-β mRNA levels following stimulation of THP-1 cells with 1,000 U/ml IFN-γ for 3h and then with 2^g/mL LPS for 2h. Data, means ± SE, n ≥ 3 experiments, *P < 0.05 (ANOVA).

Detailed Description of the Invention

It is noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the," include plural referents unless expressly and unequivocally limited to one referent. Thus, for example, reference to "a compound" includes two or more different compound. As used herein, the term "include" and its grammatical variants are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that can be substituted or other items that can be added to the listed items.

The term "compound" or "agent", as used herein, unless otherwise indicated, refers to any specific chemical compound disclosed herein and includes tautomers, regioisomers, geometric isomers as applicable , and also where applicable, optical isomers (e.g. enantiomers) thereof, as well as pharmaceutically acceptable salts thereof. Within its use in context, the term compound generally refers to a single compound, but also may include other compounds such as stereoisomers, regioisomers and/or optical isomers (including racemic mixtures) as well as specific enantiomers or enantiomerically enriched mixtures of disclosed compounds as well as diastereomers and epimers, where applicable in context. The term also refers, in context to prodrug forms of compounds which have been modified to facilitate the administration and delivery of compounds to a site of activity.

The term "patient" or "subject" is used throughout the specification within context to describe an animal, generally a mammal, including a domesticated mammal including a farm animal (dog, cat, horse, cow, pig, sheep, goat, etc.) and preferably a human, to whom treatment, including prophylactic treatment (prophylaxis), with the methods and compositions according to the present invention is provided. For treatment of those conditions or disease states which are specific for a specific animal such as a human patient, the term patient refers to that specific animal, often a human.
The terms "effective" or "pharmaceutically effective" are used herein, unless otherwise indicated, to describe an amount of a compound or composition which, in context, is used to produce or affect an intended result, for example the modulation of autophagy within the context of a particular treatment or alternatively, the effect of a bioactive agent which is coadministered with the autophagy modulator (autotoxin) in the treatment of disease.

The terms "treat", "treating", and "treatment", etc., as used herein, refer to any action providing a benefit to a patient at risk for or afflicted by an autophagy mediated disease state or condition as otherwise described herein. The benefit may be in curing the disease state or condition, inhibition its progression, or ameliorating, lessening or suppressing one or more symptom of an autophagy mediated disease state or condition, as well as inhibiting or reducing excessive autophagy. Treatment, as used herein, encompasses both prophylactic and therapeutic treatment.

As used herein, the term "autophagy mediated disease state or condition" (which term may include the term "IRGM modulated disease" as a subset) refers to a disease state or condition that results from disruption in autophagy or cellular self-digestion and wherein IRGM or its pathway and/or the TRIM proteins and their pathways are involved in the disease state or condition. Autophagy is a cellular pathway involved in protein and organelle degradation, and has a large number of connections to human disease. Autophagic dysfunction is associated with cancer, neurodegeneration, microbial infection and ageing, among numerous other disease states and/or conditions. Although autophagy plays a principal role as a protective process for the cell, it also plays a role in cell death. Disease states and/or conditions which are mediated through autophagy (which refers to the fact that the disease state or condition may manifest itself as a function of the increase or decrease in autophagy in the patient or subject to be treated and treatment requires administration of an inhibitor or agonist of autophagy in the patient or subject) include, for example, cancer, including metastasis of cancer, lysosomal storage diseases (discussed hereinbelow), neurodegeneration (including, for example, Alzheimer's disease, Parkinson's disease, Huntington's disease; other ataxias), immune response (T cell maturation, B cell and T cell homeostasis, counters damaging inflammation) and chronic inflammatory diseases (may promote excessive cytokines when autophagy is defective), including, for example, inflammatory bowel disease, including Crohn's disease, rheumatoid arthritis, lupus, multiple
sclerosis, chronic obstructive pulmony disease/COPD, pulmonary fibrosis, cystic fibrosis, Sjogren's disease; hyperglycemic disorders, diabetes (I and II), affecting lipid metabolism islet function and/or structure, excessive autophagy may lead to pancreatic β-cell death and related hyperglycemic disorders, including severe insulin resistance, hyperinsulinemia, insulin-resistant diabetes (e.g. Mendenhall's Syndrome, Werner Syndrome, leprechaunism, and lipoatrophic diabetes) and dyslipidemia (e.g. hyperlipidemia as expressed by obese subjects, elevated low-density lipoprotein (LDL), depressed high-density lipoprotein (HDL), and elevated triglycerides) and metabolic syndrome, liver disease (excessive autophagic removal of cellular entities—endoplasmic reticulum), renal disease (apoptosis in plaques, glomerular disease), cardiovascular disease (especially including ischemia, stroke, pressure overload and complications during reperfusion), muscle degeneration and atrophy, symptoms of aging (including amelioration or the delay in onset or severity or frequency of aging-related symptoms and chronic conditions including muscle atrophy, frailty, metabolic disorders, low grade inflammation, atherosclerosis and associated conditions such as cardiac and neurological both central and peripheral manifestations including stroke, age-associated dementia and sporadic form of Alzheimer's disease, pre-cancerous states, and psychiatric conditions including depression), stroke and spinal cord injury, arteriosclerosis, infectious diseases (microbial infections, removes microbes, provides a protective inflammatory response to microbial products, limits adaption of autophagy of host by microbe for enhancement of microbial growth, regulation of innate immunity) including bacterial, especially including *M. tuberculosis*, fungal, cellular and viral (including secondary disease states or conditions associated with infectious diseases), including HIV I and II, hepatitis B and C, AIDS and tuberculosis, among others, development (including erythrocyte differentiation), embryogenesis/fertility/infertility (embryo implantation and neonate survival after termination of transplacental supply of nutrients, removal of dead cells during programmed cell death) and ageing (increased autophagy leads to the removal of damaged organelles or aggregated macromolecules to increase health and prolong life, but increased levels of autophagy in children/young adults may lead to muscle and organ wasting resulting in ageing/progeria).

The term "lysosomal storage disorder" refers to a disease state or condition that results from a defect in lysosomal storage. These disease states or conditions generally occur when the lysosome malfunctions. Lysosomal storage disorders are caused by lysosomal dysfunction usually as a consequence of deficiency of a single enzyme required for
the metabolism of lipids, glycoproteins or mucopolysaccharides. The incidence of lysosomal storage disorder (collectively) occurs at an incidence of about about 1:5,000 - 1:10,000. The lysosome is commonly referred to as the cell's recycling center because it processes unwanted material into substances that the cell can utilize. Lysosomes break down this unwanted matter via high specialized enzymes. Lysosomal disorders generally are triggered when a particular enzyme exists in too small an amount or is missing altogether. When this happens, substances accumulate in the cell. In other words, when the lysosome doesn't function normally, excess products destined for breakdown and recycling are stored in the cell. Lysosomal storage disorders are genetic diseases, but these may be treated using autophagy modulators (autostatin) as described herein. All of these diseases share a common biochemical characteristic, i.e., that all lysosomal disorders originate from an abnormal accumulation of substances inside the lysosome. Lysosomal storage diseases mostly affect children who often die as a consequence at an early stage of life, many within a few months or years of birth. Many other children die of this disease following years of suffering from various symptoms of their particular disorder.

Examples of lysosomal storage diseases include, for example, activator deficiency/GM2 gangliosidosis, alpha-mannosidosis, aspartylglucoaminuria, cholesteryl ester storage disease, chronic hexosaminidase A deficiency, cystinosis, Danon disease, Fabry disease, Farber disease, fucosidosis, galactosialidosis, Gaucher Disease (Types I, II and III), GM! Ganliosidosis, including infantile, late infantile/juvenile and adult/chronic), Hunter syndrome (MPS II), I-Cell disease/Mucolipidosis II, Infantile Free Sialic Acid Storage Disease (ISSD), Juvenile Hexosaminidase A Deficiency, Krabbe disease, Lysosomal acid lipase deficiency, Metachromatic Leukodystrophy, Hurler syndrome, Scheie syndrome, Hurler-Scheie syndrome, Sanfilippo syndrome, Morquio Type A and B, Maroteaux-Lamy, Sly syndrome, mucolipidosis, multiple sulfate deficiency, Niemann-Pick disease, Neuronal ceroid lipofuscinoses, CLN6 disease, Jansky-Bielschowsky disease, Pompe disease, pycnodysostosis, Sandhoff disease, Schindler disease, Tay-Sachs and Wolman disease, among others.

The term "modulator of autophagy", "regulator of autophagy" or "autophagy modulator" is used to refer to a compound or composition which modulates IRGM "ARGM modulator") or its pathway or TRIM proteins and their pathways ("precision authophagy modulators" or "TRIM protein modulators") and has an influence on treating diseases which
are modulated through those mechanisms. IRGM modulators pursuant to the present invention include double stranded RNA (dsRNA), in particular poly I:C, poly U-G (UGUGU) and modified dsRNA such as poly ICLC (poly I:C modified with lysine and carboxymethyl cellulose) and muramyl peptides or muramyl dipeptides as disclosed herein. TRIM protein modulators include interferon gamma, pegylated interferon or preferably, any one or more of the TRIM proteins otherwise disclosed herein or an inhibitor of a TRIM protein such as a siRNA which specifically inhibits one or more TRIM proteins.

The term "muramyl peptide" or "muramyl dipeptide" include compounds according to the chemical structure:

```
O
\|_R
C - CH
\|_CH2OR1
R3 - CH
\|_O
R2O
\|_OR5
NHCOR4
```

wherein:
- $R^1$ represents a hydrogen atom or a $C_2$ acyl group;
- $R^2$ represents a hydrogen atom or a $C_2$ acyl group;
- $R^3$ represents a hydrogen atom or a $C_6$ alkyl group;
- $R^4$ represents a $C_1$ alkyl group or a $C_5$ or $C_{10}$ aryl group;
- $R^5$ represents a hydrogen atom; and
- $R$ represents the residue of an amino acid or a linear peptide built up of from 2 to 6 amino acid residues, at least one of the residues being optionally substituted with a lipophilic group including muramyl dipeptide and desmethylmuramyl dipeptide.

Preferred acyl groups for $R^1$ and $R^2$ are $C_1$-$C_5$ acyl groups such as acetyl; it will be appreciated that the carbon count in the acyl group does not include the carbonyl moiety. Preferred alkyl groups for $R^3$ are $C_1$-$C_4$ alkyl groups such as methyl and ethyl. Preferred
alkyl groups for R4 and CI-C6 alkyl groups, particularly CI-C4 alkyl groups, such as methyl or ethyl; phenyl is a preferred aryl group. R preferably represents a mono-, di- or tripeptide, more often a dipeptide. The proximal peptide residue (or the only peptide residue, if there is only one) is preferably that of an L-amino acid.

Examples include:
L-alanyl
L-valyl
L-leucyl
L-isoleucyl
L-a-aminobutyryl
L-seryl
L-threonyl
L-tryptophanyl
L-lysyl
L-ornithyl
L-arginy1
L-histidyl
L-glutamyl
L-glutaminyl
L-methionyl
L-cysteinyl
L-phenylalan1
L-tyrosyl
L-aspartyl
L-asparaginyl
L-prolyl
L-hydroxyprolyl
L-alanyl is preferred, as is L-threonyl.

The next amino acid from the proximal end of the peptide is preferably of the D-configuration. It is preferably acidic and may be D-glutamic or D-aspartic acid or a mono-, di- or mixed CI-C22 (preferably C1-C5) alkyl ester, amide or CI-C4 alkyl amide thereof. (The expression "mixed" is illustrated when one carboxyl group is amidated and the other
esterified. D-isoglutamine and D-glutamate are preferred. A third amino acid residue from the proximal end of the chain, if there is one, is preferably of the L-configuration, as indicated above in relation to the proximal amino acid residue. L-alanyl and L-lysyl are preferred.

The amino acid residue or linear peptide is optionally substituted with at least one lipophilic group. The lipophilic group may be a C_{10}-C_{22} acyl group such as stearoyl or a di-(C_{10}-C_{22} acyl)-sn-glycero-3'-hydroxyphosphoryloxy group wherein for example each of the C_{10}-C_{22} acyl groups can be a palmitoyl group. The lipophilic group may alternatively (or in addition, as more than one substitution may be present) be a C_{1}-C_{6} ester group, such as an acetyl group or a butyl ester are examples.

Examples of muramyl dipeptides within the scope of general formula I include:

- muroctasin, otherwise known as MDP-Lys (L18) (N^2-(N-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^6-stearoyl-L-lysine);
- MTP-PE (N-acetylmuramyl-L-alanyl-D-isoglutaminyl-Lalanyl-2-(l',2'-dipalmitoyl-sn-glycero-3'-hydroxyphosphoryloxy)ethylamide, monosodium);
- murabutide (N-acetylmuramyl-L-alanyl-D-glutamine-aN-butyl ester); and
- t-MDP (N-acetylmuramyl-L-threonyl-D-isoglutamine).


Other useful compounds are disclosed in WO96/01645 (the structures of these compounds may be found in the published PCT application and include the following compounds, among others:
GMDP;
GMDP-LL;
GMDP-Obu;
GMDO-Lys;
GMDB-Lys(St);
GMDB-Lys(St);
GMDB A-Lys(St);
GMDPA(OBzl)₂;
MeGMDP;
(GMDP)₂;
(GMDPA)₂;
(GMDPLys)₂;
[GMDP-Lys(St)]₂;
GMDP-Ad;
GMDP-tuftsin E;
GMDP-tuftsin A;
GMDP-tuftsin lipophilic;
GMDP-bursin;
GMDP-thymogen I;
GMDP-thymogen II;
GMDP-thymogen III;
Thr-MDP

The term "TRIM protein" or "tripartite motif containing protein" is used to describe a
TRIM protein or variant thereof as otherwise disclosed herein which is integral to an
autophagy response and may be integral as part of an upregulation of autophagy (TRIM20,
etc.) or down-regulation of autophagy (TRIM21, etc.). Many TRIM proteins are induced by
interferons, which are important components of resistance to pathogens and a number of
TRIM proteins are known to be required for the restriction of infection by lentiviruses. In
instances where a patient or subject is interferon deficient, the administration of TRIM
proteins alone or in combination with interferon gamma and/or pegylated interferon may
assist in treating disease, especially infections such as viral infections or bacterial infections,
especially *Mycobacterium* infections such as *M. tuberculosis* infections. TRIM proteins are
involved in pathogen-recognition and by regulation of transcriptional pathways in host
defence. Numerous TRIM proteins may be used in the present invention as otherwise
described herein. Sequences of these proteins are included as are the accession numbers for identifying these proteins. TRIM proteins are known in the art. TRIM proteins which are useful and preferred in the present invention include the human full length TRIM proteins (TRIM 1-75) as otherwise described herein. The sequences of TRIM proteins 1-75 as shown as well as polypeptide variants which have at least about a 90% sequence identity, and preferably at least about 95% sequence identify (about 96%, about 97%, about 98% and about 99% sequence identity) to the wild type polypeptide sequences of homo sapien TRIM proteins 1-75 are useful in the present invention. These sequences are set forth in the attached table on pages 92-116 just before the presentation of the claims. Note that TRIM proteins or polypeptide variants thereof or a pharmaceutically acceptable salt thereof may be used in the present invention. All 75 TRIM proteins as identified herein may be used in the present invention, although the preferred TRIM proteins have been identified and are more often used to modulate autophagy (either up-regulation or down-regulation) in order to favorably effect an intended outcome. SEQ ID NOs for TRIM proteins 1-75 and siRNA TRIM protein inhibitors are set forth in the table on pages 92-116 of the present application just before the claims.

In addition to TRIM proteins which find use in the present invention (pharmaceutical compositions comprising these proteins may be administered to patients in order to regulate (up- or down-regulate autophagy), inhibitors of these proteins, especially including small inhibitory RNAs or small interfering RNAs (siRNAs) may also be used to impact autophagy and treat disease states and/or conditions which are mediated through autophagy. A number of siRNAs can be used to inhibit any one or more of the TRIM proteins pursuant to the present invention. Exemplary siRNAs are presented herein in the table just before the claims. Thus, siRNAs which can be used in the present invention include the siRNAs according to the specific sequences indicated in the attached table, as well as oligos which are plus/minus up to five nucleotide units upstream or downstream of the identified siRNAs. Additional variants of these variants include those with 90% sequence identity to the siRNAs set forth in the table on pages 92-116 or variants that exhibit polymorphism to the disclosed siRNAs. These siRNAs range in size from about 9-10 nucleotide units up to about 29-30 nucleotide units, with 19-23 nucleotide units being preferred. Preferably, these siRNAs are the specific siRNAs which are disclosed in the table on pages 92-116 hereof or siRNAs which contain up to five nucleotide units more upstream and/or downstream to the disclosed siRNAs.
The term "modulator of autophagy", "regulator of autophagy" or "autostatin" is used to refer to a compound which functions as an agonist (inducer or up-regulator) or antagonist (inhibitor or down-regulator) of autophagy and are unrelated to the IRGM modulators, interferons, TRIM proteins or TRIM protein inhibitors (e.g. siRNAs as disclosed herein). These modulators may be used in combination with an IRGM modulator and/or a TRIM protein, interferon or siRNA inhibitor in methods and compositions pursuant to the present invention. Depending upon the disease state or condition, autophagy may be upregulated (and require inhibition of autophagy for therapeutic intervention) or down-regulated (and require upregulation of autophagy for therapeutic intervention). In most instances, in the case of cancer treatment with a modulator of autophagy as otherwise described herein, the autophagy modulator is often an antagonist of autophagy. In the case of cancer, the antagonist (inhibitor) of autophagy may be used alone or combined with an agonist of autophagy.

The following compounds have been identified as autophagy modulators according to the present invention and can be used in combination with an IRGM modulator or Trim protein as disclosed herein in the treatment of an autophagy mediated disease state or condition as otherwise described herein. It is noted that an inhibitor of autophagy is utilized where the disease state or condition is mediated through upregulation or an increase in autophagy which causes the disease state or condition and an agonist of autophagy is utilized where the disease state or condition is mediated through downregulation or a decrease in autophagy. The following compounds have been identified as autophagy modulators (autotaxins) in autophagy assays according to the present invention: flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon and nortriptyline, tetrachlorisophthalonitrile, phenylmercuric acetate and pharmaceutically acceptable salts thereof. It is noted that flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon, nortriptyline and their pharmaceutically acceptable salts show activity as agonists or inducers of autophagy in the treatment of an autophagy-mediated disease, whereas tetrachlorisophthalonitrile, phenylmercuric acetate and their pharmaceutically acceptable salts, find use as antagonists or inhibitors of autophagy. All of these compounds will find use
as modulators of autophagy in the various autophagy-mediated disease states and conditions described herein, with the agonists being preferred in most disease states other than cancer (although inhibitors may also be used alone, or preferably in combination with the agonists) and in the case of the treatment of cancer, the inhibitors described above are preferred, alone or in combination with an autophagy agonist as described above and/or an additional anticancer agent as otherwise described herein.

Other compounds which may be used in combination with the IRGM modulators and/or TRIM proteins and/or siRNAs as otherwise described herein either alone or in combination with the autophagy modulators which are described above, include for example, other "additional autophagy modulators" or "additional autostatins" which are known in the art. These can be combined with one or more of the autophagy modulators which are disclosed above to provide novel pharmaceutical compositions and/or methods of treating autophagy mediated disease states and conditions which are otherwise described herein. These additional autophagy modulators including benzethonium, niclosamide, monensin, bromperidol, levobunolol, dehydroisoandosterone 3-acetate, sertraline, tamoxifen, reserpine, hexachlorophene, dipyriramole, harmaline, prazosin, lidoflazine, thiethylperazine, dextromethorphan, desipramine, mebendazole, canrenone, chlorprothixene, maprotiline, homochlorcyclizine, loperamide, nicardipine, dexfenfluramine, nilvadipine, dosulepin, biperiden, denatonium, etomidate, toremifene, tomoxetine, clorgyline, zotepine, beta-escin, tridihexethyl, ceftazidime, methoxy-6-harmalan, melengestrol, albendazole, rimantadine, chlorpromazine, pergolide, cloperastine, prednicarbate, haloperidol, clotrimazole, nitrofural, ipanoic acid, naftopidil, Methimazole, Trimeprazine, Ethoxyquin, Cloctolone, Doxycycline, Pirlindole mesylate, Doxazosin, Deppropine, Nocodazole, Scopolamine, Oxybenzone, Halcinonide, Oxybutynin, Miconazole, Clomipramine, Cyproheptadine, Doxepin, Dyclonine, Salbutamol, Flavoxate, Amoxapine, Fenofibrate, Pimethixene and mixtures thereof.

The following compounds have been identified as autophagy modulators according to the present invention and can be used in the treatment of an autophagy mediated disease state or condition as otherwise described herein. These include interferon, especially interferon-gamma (IFN-gamma), pegylated interferon (PEG-IFN) and related compounds and certain TRIM proteins and variants thereof, including TRIM1, TRIM3, TRIM8, TRIM10, TRIM13, TRIM17, TRIM19, TRIM20, TRIM21, TRIM22, TRIM38, TRIM 41, TRIM43, TRIM44,
TRIM45, TRJM46, TRIM54, TRIM55, TRIM56, TRIM58, TRIM59, TRIM60, TRIM65, TRIM66 and TRIM75 with TRIM 1, TRIM 8, TRIM 20, TRIM 21, TRIM 22, TRIM 56 and TRIM 65 and mixtures thereof and preferably, TRIM 1, TRIM 8, TRIM 20, TRIM 21, TRIM 22, TRIM 56, TRIM 65 and mixtures thereof. The following compounds have been identified as autophagy modulators which may be used in combination with the above-identified autophagy agents. These agents include, for example flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon and nortriptyline, tetrachlorisophthalonitrile, phenylmercuric acetate and pharmaceutically acceptable salts thereof. It is noted that flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon, nortriptyline, benzethonium, niclosamide, monensin, bromperidol, levobunolol, dehydroisoandrosterone 3-acetate, sertraline, tamoxifen, reserpine, hexachlorophene, dipyridamole, harmalone, prazosin, lidoflazine, thiethylperazine, dextromethorphan, desipramine, mebendazole, canrenone, chlorprothixene, maprotiline, homochlorcyclizine, loperamide, nicardipine, dexfenfluramine, nilvadipine, dosulepin, biperiden, denatonium, etomidate, toremifene, tomoxetine, clorgyline, zotepine, beta-escin, tridihexethyl, ceftazidime, methoxy-6-harmalan, melengestrol, albendazole, rimantadine, chlorpromazine, pergolide, cloperastine, prednicarbate, haloperidol, clotrimazole, nitrofural, iopanoic acid, naftopidil, Methimazole, Trimeprazine, Ethoxyquin, Clocortolone, Doxycycline, Pirlindole mesylate, Doxazosin, Deftropine, Nocodazole, Scopolamine, Oxybenzone, Halcinonide, Oxybutynin, Miconazole, Clomipramine, Cyproheptadine, Doxepin, Dyclonine, Salbutamol, Flavoxate, Amoxapine, Fenofibrate, Pimethixene, and mixtures thereof. Additional autophagy agents include alternative TRIM proteins or variants thereof, such as, but not limited to, TRIM5a, TRIM6, TRIM10, TRIM17, TRIM41, TRIM55, TRIM72, TRIM76, TRIM2, TRIM23, TRIM26, TRIM28, TRIM31, TRIM 32, TRIM33, TRIM38, TRIM42, TRIM44, TRIM45, TRIM49, TRIM50, TRIM51, TRIM58, TRIM59, TRIM68, TRIM73, TRIM74 and TRIM76 and mixtures thereof. Neutral lipids such as lipids selected from the group consisting of triglycerides, diglycerides, monoglycerides, glycolated mono- or diacylglycerides, dolichol, polyrenol, polyrenal or very long chain fatty acids and mixtures thereof and their pharmaceutically acceptable salts may also be included for use in the present invention either alone or preferably in combination with one or more TRIM protein. All of
these compounds will find use as modulators of autophagy in the various autophagy-mediated
disease states and conditions described herein.

The term "co-administration" or "combination therapy" is used to describe a therapy
in which at least two active compounds in effective amounts are used to treat an autophagy
mediated disease state or condition as otherwise described herein, either at the same time or
within dosing or administration schedules defined further herein or ascertainable by those of
ordinary skill in the art. Although the term co-administration preferably includes the
administration of two active compounds to the patient at the same time, it is not necessary
that the compounds be administered to the patient at the same time, although effective
amounts of the individual compounds will be present in the patient at the same time. In
addition, in certain embodiments, co-administration will refer to the fact that two compounds
are administered at significantly different times, but the effects of the two compounds are
present at the same time. Thus, the term co-administration includes an administration in
which one active agent (especially an autophagy modulator) is administered at approximately
the same time (contemporaneously), or from about one to several minutes to about 24 hours
or more than the other bioactive agent coadministered with the autophagy modulator. The
additional bioactive agent may be any bioactive agent, but is generally selected from an
additional autophagy mediated compound as described herein, an additional anticancer agent,
or another agent, such as a mTOR inhibitor such as pp242, rapamycin, enirolimus,
everolimus or cidaforollimus, among others including epigallocatechin gallate (EGCG),
caffeine, curcumin or reseveratrol (which mTOR inhibitors find particular use as enhancers
of autophagy using the compounds disclosed herein and in addition, in the treatment of
cancer with an autophagy modulator (inhibitor) as described herein, including in combination
with tetrachlorisophthalonitrile, phenylmercuric acetate and their pharmaceutically
acceptable salts, which are inhibitors of autophagy. It is noted that in the case of the
treatment of cancer, the use of an autophagy inhibitor is preferred, alone or in combination
with an autophagy inducer (agonist) as otherwise described herein and/or a mTOR inhibitor
as described above. In certain embodiments, an mTOR inhibitor selected from the group
consisting of pp242, rapamycin, enirolimus, everolimus, cidaforollimus, epigallocatechin
gallate (EGCG), caffeine, curcumin, reseveratrol and mixtures thereof may be combined with
at least one agent selected from the group consisting of digoxin, xylazine, hexetidine and
sertindole, the combination of such agents being effective as autophagy modulators in
combination.
The term "cancer" is used throughout the specification to refer to the pathological process that results in the formation and growth of a cancerous or malignant neoplasm, i.e., abnormal tissue that grows by cellular proliferation, often more rapidly than normal and continues to grow after the stimuli that initiated the new growth cease. Malignant neoplasms show partial or complete lack of structural organization and functional coordination with the normal tissue and most invade surrounding tissues, metastasize to several sites, and are likely to recur after attempted removal and to cause the death of the patient unless adequately treated.

As used herein, the term neoplasia is used to describe all cancerous disease states and embraces or encompasses the pathological process associated with malignant hematogenous, ascitic and solid tumors. Representative cancers include, for example, stomach, colon, rectal, liver, pancreatic, lung, breast, cervix uteri, corpus uteri, ovary, prostate, testis, bladder, renal, brain/CNS, head and neck, throat, Hodgkin’s disease, non-Hodgkin’s lymphoma, multiple myeloma, leukemia, melanoma, non-melanoma skin cancer (especially basal cell carcinoma or squamous cell carcinoma), acute lymphocytic leukemia, acute myelogenous leukemia, Ewing’s sarcoma, small cell lung cancer, choriocarcinoma, rhabdomyosarcoma, Wilms’ tumor, neuroblastoma, hairy cell leukemia, mouth/pharynx, oesophagus, larynx, kidney cancer and lymphoma, among others, which may be treated by one or more compounds according to the present invention. In certain aspects, the cancer which is treated is lung cancer, breast cancer, ovarian cancer and/or prostate cancer.

The term "tumor" is used to describe a malignant or benign growth or tumefacient.

The term "additional anti-cancer compound", "additional anti-cancer drug" or "additional anti-cancer agent" is used to describe any compound (including its derivatives) which may be used to treat cancer. The "additional anti-cancer compound", "additional anti-cancer drag" or "additional anti-cancer agent" can be an anticancer agent which is distinguishable from a CIAE-inducing anticancer ingredient such as a taxane, vinca alkaloid and/or radiation sensitizing agent otherwise used as chemotherapy/cancer therapy agents herein. In many instances, the co-administration of another anti-cancer compound according to the present invention results in a synergistic anti-cancer effect. Exemplary anti-cancer compounds for co-administration with formulations according to the present invention
include anti-metabolites agents which are broadly characterized as antimitabolites, inhibitors of topoisomerase I and II, alkylating agents and microtubule inhibitors (e.g., taxol), as well as tyrosine kinase inhibitors (e.g., surafenib), EGF kinase inhibitors (e.g., tarceva or erlotinib) and tyrosine kinase inhibitors or ABL kinase inhibitors (e.g. imatinib).

Anti-cancer compounds for co-administration include, for example, Aldesleukin; Alemtuzumab; alitretinoin; allopurinol; altretamine; amifostine; anastrozole; arsenic trioxide; Asparaginase; BCG Live; bexarotene capsules; bexarotene gel; bleomycin; busulfan intravenous; busulfan oral; calusterone; capecitabine; carboplatin; carmustine; carmustine with Polifeprosan 20 Implant; celecoxib; chlorambucil; cisplatin; cladribine; cyclophosphamide; cytarabine; cytarabine liposomal; dacarbazine; dactinomycin; actinomycin D; Darbepoetin alfa; daunorubicin liposomal; daunorubicin, daunomycin; Denileukin difitox, dexamethasone; docetaxel; doxorubicin; Dromostanolone propionate; Elliott's B Solution; epirubicin; Epoetin alfa estramustine; etoposide phosphae; etoposide (VP-16); exemestane; Filgrastim; floxuridine (intraarterial); fludarabine; fluorouracil (5-FU); fulvestrant; gemtuzumab ozogamicin; gleevec (imatinib); goserepl acetate; hydroxyurea; Ifritumomab Tiuxetan; idarubicin; ifosfamide; imatinib mesylate; Interferon alfa-2a; Interferon alfa-2b; irinotecan; letrozole; leucovorin; levamisole; lomustine (CCNU); meclorethamine (nitrogen mustard); megestrol acetate; melphalan (L-PAM); mercaptopurine (6-MP); mesna; methotrexate; methoxsalen; mitomycin C; mitotane; mitoxantrone; nandrolone phenpropionate; Nofetumomab; LOddC; Oprelevkin; oxaliplatin; paclitaxel; pamidronate; pepagademase; Pegaspargase; Pegfilgrastim; pentostatin; pipobroman; plicamycin; mithramycin; porfimer sodium; procarbazine; quinacrine; Rasburicase; Rituximab; Sargramostim; streptozocin; surafenib; talbuvidine (LDT); tefo; tamoxifen; tarceva (erlotinib); temozolomide; teniposide (VM-26); testolactone; thiouguanine (6-TG); thiotepa; topotecan; toremifene; Tositumomab; Trastuzumab; tretinoin (ATRA); Uracil Mustard; valrubicin; valtorcitabine (monoval LDC); vinblastine; vinorelbine; zoledronate; and mixtures thereof, among others.

Co-administration of one of the formulations of the invention with another anticancer agent will often result in a synergistic enhancement of the anticancer activity of the other anticancer agent, an unexpected result. One or more of the present formulations comprising an IRGM modulator optionally in combination with an autophagy modulator (autostatin) as described herein may also be co-administered with another bioactive agent (e.g., antiviral
agent, antihyperproliferative disease agent, agents which treat chronic inflammatory disease, among others as otherwise described herein).

The term "antiviral agent" refers to an agent which may be used in combination with autophagy modulators (autostatins) as otherwise described herein to treat viral infections, especially including HIV infections, HBV infections and/or HCV infections. Exemplary anti-HIV agents include, for example, nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors, fusion inhibitors, among others, exemplary compounds of which may include, for example, 3TC (Lamivudine), AZT (Zidovudine), (-)-FTC, ddl (Didanosine), ddC (zalcitabine), abacavir (ABC), tenofovir (PMPA), D-D4FC (Reverset), D4T (Stavudine), Racivir, L-FddC, L-FD4C, NVP (Nevirapine), DLV (Delavirdine), EFV (Efavirenz), SQVM (Saquinavir mesylate), RTV (Ritonavir), IDV (Indinavir), SQV (Saquinavir), NFV (Nelfinavir), APV (Amprenavir), LPV (Lopinavir), fusion inhibitors such as T20, among others, fuseon and mixtures thereof, including anti-HIV compounds presently in clinical trials or in development. Exemplary anti-HBV agents include, for example, hepsera (adefovir dipivoxil), lamivudine, entecavir, telbivudine, tenofovir, emtricitabine, clevudine, valtomicitabine, amdoxovir, pradefovir, racivir, BAM 205, nitazoxanide, UT 231-B, Bay 41-4109, EHT899, zadaxin (thymosin alpha-1) and mixtures thereof. Anti-HCV agents include, for example, interferon, pegylated interletron, ribavirin, NM 283, VX-950 (telaprevir), SCH 50304, TMC435, VX-500, BX-813, SCH503034, R1626, ITMN-191 (R7227), R7128, PF-868554, TT033, CGH-759, GI 5005, MK-7009, SIRNA-034, MK-0608, A-837093, GS 9190, ACH-1095, GSK625433, TG4040 (MVA-HCV), A-831, F351, NS5A, NS4B, ANA598, A-689, GNI-104, IDX102, ADX184, GL59728, GL60667, PSI-7851, TLR9 Agonist, PHX1766, SP-30 and mixtures thereof.

An "inflammation-associated metabolic disorder" includes, but is not limited to, lung diseases, hyperglycemic disorders including diabetes and disorders resulting from insulin resistance, such as Type I and Type II diabetes, as well as severe insulin resistance, hyperinsulinemia, and dyslipidemia or a lipid-related metabolic disorder (e.g. hyperlipidemia (e.g., as expressed by obese subjects), elevated low-density lipoprotein (LDL), depressed high-density lipoprotein (HDL), and elevated triglycerides) and insulin-resistant diabetes, such as Mendenhall's Syndrome, Werner Syndrome, leprechaunism, and lipoatrophic diabetes, renal disorders, such as acute and chronic renal insufficiency, end-stage chronic
renal failure, glomerulonephritis, interstitial nephritis, pyelonephritis, glomerulosclerosis, e.g., Kimmelstiel-Wilson in diabetic patients and kidney failure after kidney transplantation, obesity, GH-deficiency, GH resistance, Turner's syndrome, Laron's syndrome, short stature, increased fat mass-to-lean ratios, immunodeficiencies including decreased CD4+ T cell counts and decreased immune tolerance or chemotherapy-induced tissue damage, bone marrow transplantation, diseases or insufficiencies of cardiac structure or function such as heart dysfunctions and congestive heart failure, neuronal, neurological, or neuromuscular disorders, e.g., diseases of the central nervous system including Alzheimer's disease, or Parkinson's disease or multiple sclerosis, and diseases of the peripheral nervous system and musculature including peripheral neuropathy, muscular dystrophy, or myotonic dystrophy, and catabolic states, including those associated with wasting caused by any condition, including, e.g., mental health condition (e.g., anorexia nervosa), trauma or wounding or infection such as with a bacterium or human virus such as HIV, wounds, skin disorders, gut structure and function that need restoration, and so forth.

An "inflammation-associated metabolic disorder" also includes a cancer and an "infectious disease" as defined herein, as well as disorders of bone or cartilage growth in children, including short stature, and in children and adults disorders of cartilage and bone in children and adults, including arthritis and osteoporosis. An "inflammation-associated metabolic disorder" includes a combination of two or more of the above disorders (e.g., osteoporosis that is a sequela of a catabolic state). Specific disorders of particular interest targeted for treatment herein are diabetes and obesity, heart dysfunctions, kidney disorders, neurological disorders, bone disorders, whole body growth disorders, and immunological disorders.

In one embodiment, "inflammation-associated metabolic disorder" includes: central obesity, dyslipidemia including particularly hypertriglyceridemia, low HDL cholesterol, small dense LDL particles and postpranial lipemia; glucose intolerance such as impaired fasting glucose; insulin resistance and hypertension, and diabetes. The term "diabetes" is used to describe diabetes mellitus type I or type II. The present invention relates to a method for improving renal function and symptoms, conditions and disease states which occur secondary to impaired renal function in patients or subjects with diabetes as otherwise described herein. It is noted that in diabetes mellitus type I and II, renal function is impaired from collagen deposits, and not from cysts in the other disease states treated by the present invention.
Mycobacterial infections often manifest as diseases such as tuberculosis. Human infections caused by mycobacteria have been widespread since ancient times, and tuberculosis remains a leading cause of death today. Although the incidence of the disease declined, in parallel with advancing standards of living, since the mid-nineteenth century, mycobacterial diseases still constitute a leading cause of morbidity and mortality in countries with limited medical resources. Additionally, mycobacterial diseases can cause overwhelming, disseminated disease in immunocompromised patients. In spite of the efforts of numerous health organizations worldwide, the eradication of mycobacterial diseases has never been achieved, nor is eradication imminent. Nearly one third of the world's population is infected with mycobacterium tuberculosis complex, commonly referred to as tuberculosis (TB), with approximately 8 million new cases, and two to three million deaths attributable to TB yearly. Tuberculosis (TB) is the cause of the largest number of human deaths attributable to a single etiologic agent (see Dye et al., J. Am. Med. Association, 282, 677-686, (1999); and 2000 WHO/OMS Press Release).

Mycobacteria other than M. tuberculosis are increasingly found in opportunistic infections that plague the AIDS patient. Organisms from the M. avium-intracellulare complex (MAC), especially serotypes four and eight, account for 68% of the mycobacterial isolates from AIDS patients. Enormous numbers of MAC are found (up to $10^{10}$ acid-fast bacilli per gram of tissue), and consequently, the prognosis for the infected AIDS patient is poor.

In many countries the only measure for TB control has been vaccination with M. bovis bacille Calmette-Guerin (BCG). The overall vaccine efficacy of BCG against TB, however, is about 50% with extreme variations ranging from 0% to 80% between different field trials. The widespread emergence of multiple drug-resistant M. tuberculosis strains is also a concern.

M. tuberculosis belongs to the group of intracellular bacteria that replicate within the phagosomal vacuoles of resting macrophages, thus protection against TB depends on T cell-mediated immunity. Several studies in mice and humans, however, have shown that Mycobacteria stimulate antigen-specific, major histocompatibility complex (MHC) class II- or class I-restricted CD4 and CD8 T cells, respectively. The important role of MHC class I-restricted CD8 T cells was convincingly demonstrated by the failure of p2-microglobulin deficient mice to control experimental M. tuberculosis infection.
As used herein, the term "tuberculosis" comprises disease states usually associated with infections caused by mycobacteria species comprising \textit{M. tuberculosis} complex. The term "tuberculosis" is also often associated with mycobacterial infections caused by mycobacteria other than \textit{M. tuberculosis}. Other mycobacterial species include \textit{M. avium-intracellulare}, \textit{M. kansarii}, \textit{M. fortuitum}, \textit{M. chelonae}, \textit{M. leprae}, \textit{M. africanum}, and \textit{M. microti}. \textit{M. avium paratuberculosis}, \textit{M. intracellulare}, \textit{M. scrofulaceum}, \textit{M. xenopi}, \textit{M. marinum}, \textit{M. ulcerans}.


In certain embodiments, an "infectious disease" is selected from the group consisting of tuberculosis, leprosy, \textit{Crohn's Disease}, acquired immunodeficiency syndrome, \textit{Lyme disease}, cat-scratch disease, \textit{Rocky Mountain spotted fever} and \textit{influenza} or a \textit{viral infection selected from HIV} (I and/or II), \textit{hepatitis B virus} (HBV) or \textit{hepatitis C virus} (HCV).

While not being limited by way of theory, it is believed that autophagy-mediated disease states which evidence upregulated autophagy and upregulated TRIM proteins include inflammatory disease states and autoimmune disease states as otherwise described herein. These disease states and/or conditions may benefit from the inhibition of TRIM proteins where there is evidence that autophagy is up-regulated and needs to be brought back into balance in order to facilitate healing of the disease state and/or condition. In these disease states, the inhibition of TRIM proteins, including inhibiting TRIM proteins by administration
of small interfering RNAs (siRNAs) which inhibit the synthesis of the TRIM protein to be reduced in order to down regulate autophagy may be useful. This approach may provide beneficial treatment in a large number of disease states and conditions where upregulation of autophagy is responsible for the disease state and/or exacerbating the disease state. In other disease states, in particular, bacterial and viral infections, especially tuberculosis and in some instances of cancer, autophagy is often down-regulated and may benefit from the upregulation of autophagy through the administration of one or more TRIM proteins (especially TRIM20) alone or in combination with interferon-gamma, pegylated interferon and/or one more more additional autophagy agents including alternative TRIM proteins as otherwise disclosed herein.

According to various embodiments, the compounds according to the present invention may be used for treatment or prevention purposes in the form of a pharmaceutical composition. This pharmaceutical composition may comprise one or more of an active ingredient as described herein.

As indicated, the pharmaceutical composition may also comprise a pharmaceutically acceptable excipient, additive or inert carrier. The pharmaceutically acceptable excipient, additive or inert carrier may be in a form chosen from a solid, semi-solid, and liquid. The pharmaceutically acceptable excipient or additive may be chosen from a starch, crystalline cellulose, sodium starch glycolate, polyvinylpyrididone, polyvinylpolypyrrolidone, sodium acetate, magnesium stearate, sodium laurylsulfate, sucrose, gelatin, silicic acid, polyethylene glycol, water, alcohol, propylene glycol, vegetable oil, corn oil, peanut oil, olive oil, surfactants, lubricants, disintegrating agents, preservative agents, flavoring agents, pigments, and other conventional additives. The pharmaceutical composition may be formulated by admixing the active with a pharmaceutically acceptable excipient or additive.

The pharmaceutical composition may be in a form chosen from sterile isotonic aqueous solutions, pills, drops, pastes, cream, spray (including aerosols), capsules, tablets, sugar coating tablets, granules, suppositories, liquid, lotion, suspension, emulsion, ointment, gel, and the like. Administration route may be chosen from subcutaneous, intravenous, intestinal, parenteral, oral, buccal, sublingual, nasal, intramuscular, transcutaneous, transdermal, intranasal, intratracheal, intrathecal, pulmonary, intraperitoneal, and topical, among others. The pharmaceutical compositions may be immediate release,
sustained/controlled release, or a combination of immediate release and sustained/controlled release depending upon the compound(s) to be delivered, the compound(s), if any, to be coadministered, as well as the disease state and/or condition to be treated with the pharmaceutical composition. A pharmaceutical composition may be formulated with differing compartments or layers in order to facilitate effective administration of any variety consistent with good pharmaceutical practice.

The subject or patient may be chosen from, for example, a human, a mammal such as domesticated animal, or other animal. The subject may have one or more of the disease states, conditions or symptoms associated with autophagy as otherwise described herein.

The compounds according to the present invention may be administered in an effective amount to treat or reduce the likelihood of an autophagy-mediated disease and/or condition as well one or more symptoms associated with the disease state or condition. One of ordinary skill in the art would be readily able to determine an effective amount of active ingredient by taking into consideration several variables including, but not limited to, the animal subject, age, sex, weight, site of the disease state or condition in the patient, previous medical history, other medications, etc.

For example, the dose of an active ingredient which is useful in the treatment of an autophagy mediated disease state, condition and/or symptom for a human patient is that which is an effective amount and may range from as little as 100 µg or even less to at least about 500 mg up to a gram or more, which may be administered in a manner consistent with the delivery of the drug and the disease state or condition to be treated. In the case of oral administration, active is generally administered from one to four times or more daily. Transdermal patches or other topical administration may administer drugs continuously, one or more times a day or less frequently than daily, depending upon the absorptivity of the active and delivery to the patient's skin. Of course, in certain instances where parenteral administration represents a favorable treatment option, intramuscular administration or slow IV drip may be used to administer active. The amount of active ingredient which is administered to a human patient preferably ranges from about 0.05 mg/kg to about 10 mg/kg, about 0.1 mg/kg to about 7.5 mg/kg, about 0.25 mg/kg to about 6 mg/kg, about 1.25 to about 5.7 mg/kg.
The dose of a compound according to the present invention may be administered at the first signs of the onset of an autophagy mediated disease state, condition or symptom. For example, the dose may be administered for the purpose of lung or heart function and/or treating or reducing the likelihood of any one or more of the disease states or conditions which become manifest during an inflammation-associated metabolic disorder or tuberculosis or associated disease states or conditions, including pain, high blood pressure, renal failure, or lung failure. The dose of active ingredient may be administered at the first sign of relevant symptoms prior to diagnosis, but in anticipation of the disease or disorder or in anticipation of decreased bodily function or any one or more of the other symptoms or secondary disease states or conditions associated with an autophagy mediated disorder to condition.

Synthesis of TRIM proteins according to the present invention may be performed by the routineer skilled in the art and may be provided by engineering polynucleotide sequences corresponding to the amino acid sequences of the TRIM proteins into plasmids for expression, transfecting the plasmids into both eukaryotic and/or prokaryotic cells and accumulating protein from the growth of the cells containing the plasmids. Alternatively, the proteins may be readily synthesized by standard, well-known peptide synthesis methods, including solid phase synthesis.

The following examples are provided to further describe the present invention. The examples, while descriptive of the present invention, are not to be construed as limiting the present invention.

EXAMPLES
First Set- IRGM Examples

Antibodies, plasmids, and siRNA
Antibodies were from Cell Signaling (AMPK, AMPK Thr-172, ULK1, ULK1 p-Ser317, p-Ser 757, p-Ser555, NOD2, Beclin 1 p-Ser-93/96 and ATG5), MBL international corp. (ATG16L1, ATG14L, Rubicon and UVRAG), Abeam (GFP, IRGM, LPS, TRAF6 and BCL2), Sigma (LC3B, Flag), Millipore (V5 tag and HA tag), Abbiotec (Beclin 1 p-Ser15) and Novus biological (AMBRA1). GFP-tagged IRGM expression plasmid (GFP-IRGMd) was described previously (Singh et al., 2010). GFP-IRGM-Kmut was generated from GFP-IRGMd plasmid by replacing wild type IRGMd gene with synthetic mutated IRGMd gene.
(GeneScript) with all lysine residues mutated to arginine. Flag-IRGM and IRGM-V5 were generated by Gateway cloning (Life technologies). HA-UbiquitinC, HA-UbiquitinC-K63 (all lysine mutated except K63, Plasmid 17606), HA-UbiquitinC-K48 (all lysine mutated except K48, Plasmid 17605), Flag-TLR3 (Plasmid 13084) and YFP-TLR4 (Plasmid 13018) were from Addgene. Flag-NOD2 and variants were from Dr. Thomas Kufer (University of Cologne, Germany). Flag-ATG16L1 and variants were from Dr. Ramnik Xavier (Massachusetts General Hospital, Boston). Flag-TRAF6 was from Dr. Edward Harhaj (Johns Hopkins School of Medicine, US). IRGM siRNA, TRAF6 siRNA, AMPKa2 siRNA were from Dharmacon (siGENOME SMART pool).

**Autophagy induction**

U937 cells were treated with LPS (500 ng/ml) for 4 h or by transfecting MDP (5 μg/ml) with calcium phosphate for 8 h. For induction of autophagy by starvation, cells were cultured in EBSS.

**Protein interactions analyses**

For co-immunoprecipitation assays, the cells were lysed using NP-40 buffer containing protease inhibitor cocktail and PMSF. Lysates were incubated with antibody for 2 h followed by incubation with proteinG Dynabeads (Life technologies) for 2 h. Beads were washed for four times with 1XPBS and then boiled with SDS-PAGE buffer for analysis of interacting protein by Immunoblotting. Immunoblots were quantified using Image J software.

**Microscopy analyses and quantification**

Immunofluorescence was performed as described earlier (Kyei et al., 2009). For quantification of puncta, images from different fields were captured and analyzed. For quantification of total cell fluorescence, image J was used as described previously (Chauhan et al., 2013).

**Gene expression analysis**

Total RNA was isolated from cell culture using Trizol as per the manufacturer’s instruction (Invitrogen). For quantitative real-time PCR: TURBO DNA-free kit (Ambion) was used to remove contaminating residual DNA; cDNA was prepared using the high
capacity cDNA reverse transcription kit as per the manufacturer's instruction (Applied Biosystem). Taqman probes (Applied Biosystem) and realtime PCR master mixes (Applied Biosystem) were used for real-time PCR as per the manufacturer's instruction. Data were normalized using GAPDH.

**Bacterial survival analyses**

AIEC LF82 survival assay was performed as described previously (Lapaquette et al., 2009). HEK293T cells were infected with AIEC LF82 of MOI of 1:20 for 3 h. Cells were treated with gentamycin (100 µg/ml) for 1 h followed by incubation in fresh media for 2 h. Cells were lysed and surviving bacteria quantified by plating and determining colony forming units.

**Cytokine and NF-kB responses**

For NFkB-p65 nuclear localization assay, HeLa cells were plated on cover slips a day before infection. Cells were infected with AIEC LF82 strain at MOI of 1:20 for 2 h followed by washings with PBS and fixing the cells with 4% paraformaldehyde. Immunofluorescence imaging was performed as described earlier (Kyei et al., 2009). Cells were visualized using a laser confocal microscope and images were captured using LSM510 software. For IL-Ιβ measurement, IL-Ιβ transcription was determined using qRT-PCR in THP-1 cells.

**Results (IRGM Examples) All Figures for this Section are Labeled As Figures IRGM for the attached figures**

**IRGM activates the core regulators of autophagy**

Prior work has indicated that IRGM affects autophagy through processes influencing mitochondrial function, including mitochondrial fission and membrane potential collapse (Singh et al., 2010). Similar changes in mitochondrial function often lead to AMPK activation (Romanello et al., 2010; Turkieh et al., 2014). Thus, we tested the activation status of AMPK. A knockdown of IRGM reduced the total amounts of AMPK in both control or starved cells (Figure 1A) and decreased the levels of the activated form of AMPK.
phosphorylated at Thr-172 (Figure 1A). Overexpression of IRGM increased levels of Thr-172 phosphorylated AMPK (Figure 1B).

AMPK has been previously shown to induce autophagy by directly phosphorylating ULK1 (Egan et al., 2011; Kim et al., 2011) and Beclin 1 (Kim et al., 2013). When we tested the phosphorylation status of ULK1 and Beclin 1, we observed that the expression of IRGM, which caused induction of autophagy (Figure S1A), enhanced phosphorylation at activating sites of Beclin 1 at Ser93/96 (Kim et al., 2013), and ULK1 at Ser-555 (Egan et al., 2011) and at Ser-317 (Kim et al., 2011) (Figure 1B,C).

**IRGM assembles the core regulatory machinery for autophagy**

The entire signaling cascade described above could explain how IRGM induces autophagy, e.g. by its effects on AMPK and activation of downstream autophagy regulators. However, IRGM showed a further, more direct role by interacting with the key regulators of autophagy. We found that IRGM co-immunoprecipitated and co-localized with both endogenous and overexpressed ULK1 and Beclin 1 (Figure 1D-G and S1B-C) but not with AMPK (Figure S1D). IRGM complexes with ULK1 were enriched for the activated, AMPK-dependent Ser-317, form of ULK1 relative to the inhibitory, mTOR-dependent, Ser-757 form (Figure 1H). Furthermore, expression of IRGM enriched ULK1 in the immunoprecipitated Beclin 1 complexes (Figure II and S1G). In keeping with this, cells overexpressing IRGM also showed increased Beclin 1 Ser-15 phosphorylation, the phosphorylated form of Beclin 1 dependent on ULK1 activity (Kim et al., 2013) (Figure 1J).

**IRGM determines the composition of the Beclin 1 complex**

We found that IRGM complexes also included autophagy-enhancing Beclin 1 interactors, AMBRA1 (Figure 1D and S1E), ATG14L (Figure 1K) and UVRAG (Figure SIF) but not the autophagy inhibitory factor Rubicon (Figure SIF) (Fimia et al., 2007; Itakura et al., 2008; Matsunaga et al., 2009). Next, we mapped Beclin 1 regions required for interaction with IRGM (Figure 1M). IRGM interacted with two Beclin 1 regions: (i) BH3-containing 1-125 N-terminal portion, and (ii) a segment encompassing CCD and ECD, whereas it did not bind to the intervening CCD domain alone (Figure 1L,M).

Incidentally, two Beclin1 negative regulators Bcl-2 and Rubicon bind respectively to the regions spanning Beclin 1's BH3 domain and Beclin 1's CCD and ECD domains,
whereas ATG14L, a factor enabling Beclin 1 to activate the initiation complex (Kim et al., 2013), binds to the CCD domain of Beclin 1 (Sun et al., 2008). This domain occupancy on Beclin 1 is compatible with simultaneous binding of IRGM and ATG14L and exclusion of autophagy negative regulators. When IRGM was overexpressed, it dis-enriched Rubicon and Bcl-2 from Beclin 1 and enriched ATG14L in Beclin 1 complexes (Figure IN). The above data indicate that IRGM forms protein complexes with the central regulators of autophagy and activates Beclin 1 by displacing its negative regulators (Figure 10, Right). This, taken together with IRGM's ability to sponsor the phosphorylation cascade that activates ULK1 and Beclin 1, shows how IRGM promotes autophagy (Figure 10, Left).

**IRGM affects levels of autophagy regulators**

As observed with AMPK (Figure 1A), IRGM affected the levels of a number of other autophagy regulators. IRGM knockdown in U937 monocytic cells (Figure S2A) reduced total amount of ULK1 (Figure 2A,B, Figure S2B), ATG14L (Figure 2C, Figure S2B), and AMBRA1 (Figure 2C, Figure S2B). In contrast to the above suite of autophagy regulators, Beclin 1 was not affected (Figure S2C). In addition to Beclin 1, IRGM did not alter cytoplasmic levels of ATG5-ATG12 conjugates (Figure 2C). However, the physical organization of ATG5-ATG12 was affected, since the numbers of its puncta, revealed by ATG5 immunofluorescence, were reduced upon IRGM knockdown (Figure 2D).

ATG5 puncta formation is governed by ATG16L1 (Mizushima, 2003). We thus looked at the effects of IRGM on ATG16L1 levels and observed that they were reduced in IRGM knockdown cells (Figure 2E,F). This prompted us to test whether IRGM might interact with ATG16L1. IRGM was in complexes with endogenous Atg16L1 (Figure 2G). Further domain mapping showed that IRGM primarily interacted with the WD repeats region of ATG16L1 (Figure 2H,I). The residual weak interaction between IRGM and ATG16L1 outside of the WD repeats (construct ATG16L1(1-341)) was not due to FIP200, previously shown to bridge ATG16L1 with ULK1 (Gammoh et al., 2013) since interaction was not reduced upon FIP200 knockdown, and if anything was slightly increased (Figure S2D). In summary, in addition to directing the assembly of key autophagy-specific regulators, IRGM also stabilizes them. Furthermore IRGM interacts with and stabilizes ATG16L1, a component of the ATG5-Atg12/ATG16L1 E3 complex, which governs LC3 conjugation and autophagosome formation (Mizushima, 2003).
Expression of IRGM and its assembly with autophagy factors responds to microbial signals

Infection with CD-associated adhesive invasive *Escherichia coli* (AIEC) LF82 (Lapaquette et al., 2010) or treatment with LPS or muramyl dipeptide (MDP) induced *IRGM* expression in U937 cells (Figure 3A-C). The induction of *IRGM* was similar to other physiological inducers of autophagy: starvation and IFN-γ (Gutierrez et al., 2004) which acted in a cell type-dependent manner, and, in the case of starvation, showed AMPK dependence (Figure 3D, Figure S3A-I). When autophagy was induced by LPS (Figure S3J) or MDP (Figure S3K) (Cooney et al., 2010), a knockdown of IRGM (Figure S3L) precluded LC3B-II conversion and LC3B puncta formation in response to these stimuli (Figure 3E-H). Thus, IRGM is required for autophagy elicited by microbial products.

In experiments with endogenous proteins, we could not detect interactions of IRGM with ULK1 and ATG16L1 under basal conditions (Figure 3I, untreated lane). However, when a monocytic cell line (THP-1) was infected with *E. coli* LF82, immunoprecipitates of endogenous IRGM contained ULK1 and ATG16L1 (Figure 3I, AIEC lane). Similar effects were observed with MDP and LPS (Figure 3I). Of note, MDP (a NOD2-cogante ligand) was a stronger promoter of these effects than LPS. In contrast to ULK1 and ATG16L1, which showed interactions with endogenous IRGM only in samples from cells infected or treated with MDP or LPS, AMBRA1 showed association with endogenous IRGM even under basal conditions (Figure 3I). Thus, exposure of cells to microbes or their products affects IRGM expression and also influences interactions with the autophagic apparatus (Figure 3I).

Three Crohn’s disease risk factors, NOD2, IRGM, and ATG16L1 interact

A known receptor for MDP is NOD2, a risk factor for familial CD (Ogura et al., 2001). Furthermore, ATG16L1, harboring an important CD-associated polymorphism (Consortium, 2007), interacts with NOD2 (Cooney et al., 2010; Travassos et al., 2010). Hence, we wondered whether IRGM, a third genetic CD risk factor (incidentally co-discovered with ATG16L1) (Consortium, 2007), is apart of this complex. Endogenous and overexpressed IRGM immunoprecipitates contained both NOD2 and ATG16L1 (Figure 4A, B). IRGM increased interactions between NOD2 and ATG16L1 (Figure S4A). In contrast, co-expression of NOD2 did not affect IRGM-ATG16L1 interactions (Figure S4B), suggesting that IRGM is important for promoting the assembly of the tri-partite complex.
Morphologically, NOD2 co-expression changed IRGM intracellular distribution from diffuse cytosolic to punctate (Figure S4C). A subset of these profiles colocalized with mitochondrial markers (Tom20; Figure S4D, E), in keeping with a partial NOD2 colocalization with mitochondrial antiviral signaling protein MAVS (Sabbah et al., 2009), and the previously reported partial IRGM localization to mitochondria (Singh et al., 2010).

All three factors, IRGM, ATG16L1, and NOD2 co-localized in co-transfected cells (Figure 4C). Mapping of interaction domains revealed that association of IRGM with NOD2 is likely a regulated event. A region containing the two CARD domains of NOD2 was required for IRGM interaction (Figure 4D,E). A deletion of the LRR domains in NOD2 enhanced interactions between IRGM and NOD2 (Figure 4D,E). The LRR domain region is known to be inhibitory to the previously established NOD2 activities (Tanabe et al., 2004) by keeping NOD2 in a closed conformation until it is activated through stimuli such as MDP (Tanabe et al., 2004). IRGM and NOD2 interaction was confirmed by proximity ligation assay (PLA; Figure S4F), which reports direct protein-protein interactions in situ. Positive PLA readouts of direct in situ interactions between proteins appear as fluorescent dots, the products of in situ PCR that generates a fluorescent product physically attached to antibodies against the two proteins that are <16 nm (FRET distance) apart. A deletion of the CARD domains in NOD2 reduced the NOD2-IRGM PLA signal (Figure S4F), in keeping with the importance of CARDs for the interactions between IRGM and NOD2. We carried out additional interaction experiments with purified GST-IRGM protein (Singh et al., 2010), prepared from insect cells (Figure S4G, isoform d, used in all experiments in this work), and Flag-NOD2 (full length and its variants ACARD and ALRR) prepared from 293T overexpressing cells. The results show that IRGM interacts with full length NOD2 and ALRR NOD2, but not with ACARD NOD2 (Figure 4F). These findings demonstrate that the NOD2 CARD domain is key for interactions with IRGM.

Fluorescently labeled MDP co-localized with NOD2 and IRGM in the cells (Figure 4G). In the presence of MDP, interactions between IRGM and NOD2 were enhanced (Figure 4H). These findings are consistent with the inhibitory action of LRRs in the resting state of NOD2, and with the observation that following activation with MDP, NOD2 becomes available for interactions with IRGM (Figure 4I). In summary, IRGM, NOD2, and ATG16L1
form a complex, with IRGM-NOD2 assembly being controlled by MDP, thus rendering the IRGM autophagy-promoting system responsive to microbial products.

**NOD2 enhances IRGM interactions with ULK1 and Beclin 1**

NOD2 affected IRGM quaternary structure. Co-expression of NOD2 and IRGM induced IRGM oligomerization within protein complexes (Figure 5A). NOD2 furthermore promoted interactions between IRGM and ULK1 as well as between IRGM and Beclin 1 (Figure 5B,C). Incidentally, NOD2 was also found in complexes with ULK1 (Figure S5A). IRGM co-expression increased ULK1-NOD2 complexes (Figure S5A). Thus, NOD2 modulates IRGM interactions with ULK1 and Beclin 1, in contrast to the above-described (Figure S4B) absence of NOD2 effects on IRGM-ATG16L1 complex formation. Based on these and above observations, IRGM is a pivotal organizer of the core parts of the autophagy initiation machinery (ULK1/Beclin1 and ATG16L1) along with NOD2.

**Polyubiquitination of IRGM promotes its assembly with ULK1 and Beclin 1**

In the co-immunoprecipitation experiments of NOD2 with IRGM, we observed the presence of multiple GFP-IRGM bands (Figure S5B). NOD2 is known to promote ubiquitination of several target proteins (Abbott et al., 2007; Hasegawa et al., 2008). We tested whether IRGM was ubiquitinated and observed that it can be polyubiquitinated whereas NOD2 enhanced IRGM ubiquitination (Figure 5D IRGM). To determine which ubiquitination linkage was involved, we co-expressed GFP-IRGM with two HA-tagged ubiquitin variants, one that can be ubiquitinated only at K63 and another one that can be ubiquitinated only at K48. The IRGM ubiquitination showed a much stronger signal with the HA-Ub-K63 (Figure 5E). Endogenous IRGM as well as a construct with a tag smaller than GFP (V5 tag; IRGM-V5) were K63 polyubiquitinated (Figure S5C IRGM, S5D IRGM). The K63 ubiquitination of IRGM was strongly enhanced in the presence of NOD2 (Figure 5E IRGM). Overexpression or downregulation of TRAF6, an E3 ligase known to work in concert within the NOD2 pathway (Abbott et al., 2007; Yang et al., 2007) increased or decreased IRGM ubiquitination (Figure S5E and S5F) suggesting a role for TRAF6 in IRGM ubiquitination. However, TRAF6 knockdown destabilized NOD2, so it was not possible to conclude that TRAF6 was the only E3 ligase responsible for IRGM ubiquitination. Next, we mapped which of the NOD2 domains are necessary for effective ubiquitination of IRGM, and found that deletion of the CARD domain in NOD2 prevented IRGM ubiquitination, consistent with IRGM's ability to bind to that region of NOD2 (Figure 5F). Moreover, when
the CARD domain of NOD2 was expressed alone, it enhanced IRGM K63 ubiquitination (Figure 5F).

Mutation of either individual or small clusters of K (Lys) residues in IRGM did not prevent K-63 linkage ubiquitination of IRGM in the presence of NOD2 (Figure S5G). In the absence of NOD2, the low level ubiquitination (see Figure 5E) of the same series of K mutants of IRGM also persisted (Figure S5H IRGM). A similar phenomenon, i.e. an absence of a dominant ubiquitination residue, has been described for several proteins including p53 (Chan et al., 2006) and cyclins (Fung et al., 2005). Paradoxically, mutation of the K-23/K-27 cluster in IRGM, enhanced K-63 linkage ubiquitination (Figure S5H IRGM); it nevertheless reduced K-48 linked ubiquitination (Figure S5I) suggesting that K-23/K-27 cluster may be a dominant K-48 ubiquitination site, and that its elimination enhances K-63 ubiquitination of IRGM. Thus, multiple K residues in IRGM are K63-ubiquitinated. When we mutated all twelve lysine residues in IRGM (IRGM-Kmut; K residues converted to R), the GFP-IRGM fusion lost ubiquitination capacity (Figure 5G). Nevertheless, GFP-IRGM-Kmut still bound ATG16L1 equally well as the wild type IRGM (Figure 51). In contrast to its unaltered association with ATG16L1, GFP-IRGM-Kmut showed a reduced ability to oligomerize within protein complexes (revealed by using IRGM with two different tags; Figure 5H) and displayed diminished capacity for interactions with ULK1, Beclin 1 and AMBRA1 (Figure 51). In addition, NOD2 could not increase Beclin 1-IRGM-Kmut interactions, although NOD2 increased Beclin 1 interactions with wild type IRGM (Figure S5J IRGM). Thus, polyubiquitination of IRGM is important for the assembly of the core regulatory machinery centered on ULK1 and Beclin 1, and this modification of IRGM is under the control by NOD2.

**Polyubiquitinated IRGM inversely controls NOD2 and ULK1 protein levels**

We observed that co-expression of GFP-IRGM had an effect on NOD2 protein amount, by diminishing its levels relative to control (Figure 6A IRGM). IRGM promoted NOD2 degradation, which was partially blocked by bafilomycin A1, commonly used to inhibit autolysosomal degradation (Figure 6B IRGM). The IRGM-Kmut variant of IRGM displayed a decreased ability to commit NOD2 for degradation (Figure 6C IRGM). In contrast to the destabilizing effects of IRGM on NOD2, expression of IRGM increased co-expressed myc-ULK1 in a dose-dependent manner (Figure 6D IRGM). The total amount of
ULK1 was not increased when the IRGM-K\textsuperscript{mut} variant was co-expressed (Figure 6E IRGM). This effect was ULK1-specific, since Beclin 1 levels were not affected when IRGM vs IRGM-K\textsuperscript{mut} were compared, corroborating with a related finding that IRGM did not affect Beclin 1 stability (Figure S3B IRGM). Thus, polyubiquitinated IRGM protects ULK1 and promotes degradation of NOD2 (Figure 6F IRGM). This represents a negative feedback regulatory loop, which induces autophagy but at the same time limits NOD2's ability to continue unabated stimulation of this process (Figure 6G IRGM).

**IRGM affects antimicrobial and inflammatory outputs and interfaces with several innate immunity systems**

IRGM has been shown to control intracellular bacteria (Brest et al., 2011; McCarroll et al., 2008)(Singh et al., 2006). Using a model system of transfected epithelial cells previously developed by others (Brest et al., 2011; Lapaquette et al., 2010) for monitoring autophagic handling of invasive bacteria, we tested how IRGM-K\textsuperscript{mut}, the mutant form of IRGM disabled for ubiquitination and examined for its effects in molecular relationships above, affected a subset of IRGM's immune outputs. Co-expression of NOD2 with IRGM-K\textsuperscript{mut} resulted in increased NF-kB p65 nuclear translocation in response to *E. coli* LF82 (a CD isolate of adherent invasive *E. coli*) (Lapaquette et al., 2010) relative to NOD2 co-expression with IRGM wild type (Figure 7A,B, Figure S6A). Consistent with this observation, a monocytic cell line THP-1 infected with *E. coli* LF82 showed elevated pro-inflammatory response (increased IL-1\beta transcription) when IRGM was knocked down (Figure 7C IRGM, Figure S6B IRGM). The increased NF-kB response with IRGM-K\textsuperscript{mut} (Figure 7A,B IRGM) was mirrored in the effects of expressing IRGM or IRGM-K\textsuperscript{mut} on bacterial survival, reflected in the diminished ability of IRGM-K\textsuperscript{mut} to control *E. coli* LF82 (Figure S6C). Although IRGM expression on its own enhanced bacterial elimination, this was increased by co-expression with NOD2, an effect that was diminished when IRGM-K\textsuperscript{mut} was employed (Figure S6C IRGM). Although the overall magnitude of the effects on bacterial killing was subtle, it was in keeping with the known limitations of the system (Brest et al., 2011; Lapaquette et al., 2010) as reflected in its maximum output (upon starvation induction) of bacterial control by autophagy (Figure S6D). Based on the above experiments with IRGM-K\textsuperscript{mut}, the properties of IRGM that are essential for the assembly of the core autophagy machinery affect its antimicrobial and inflammatory outputs.
The inventors also tested localization of IRGM relative to the CD isolate *E. coli* LF82 (Lapaquette et al., 2010). We observed that without the co-expression of NOD2, IRGM had a diffuse cytosolic localization even when the cells were infected with bacteria (Figure S7A IRGM). However, when NOD2 was co-expressed with GFP-IRGM, IRGM was recruited to the invading bacteria (Figure S7B IRGM), in keeping the previously observed recruitment of ATG16L1 and NOD2 to bacterial entry sites (Travassos et al., 2010). While studying IRGM interacting partners, we observed a further ability of IRGM to engage other pattern recognition receptors (PRRs), such as NOD1, RIG-I, and TLR3 (Figure 7D-F). In contrast, IRGM did not interact with TLR4 (Figure S7C IRGM). Similarly to NOD2, NOD1, RIG-I, and TLR3 induced IRGM ubiquitination (Figure 7G IRGM). In conclusion, not only does IRGM assemble the core autophagy machinery to control innate immune responses to NOD2 agonists, but IRGM potentially has a broader repertoire of interactors among the PRR systems.

**DISCUSSION (IRGM Examples)**

In these examples, the inventors have shown that human IRGM, hitherto believed to have indirect effects on autophagy, directly governs the assembly of the principal autophagy regulators. Furthermore, it physically links the microbial sensors, including NOD2, to the core autophagic apparatus. This solves the long-standing puzzle regarding how IRGM works, and places it mechanistically at the center of action in autophagic responses to microbes. IRGM assembles ULK1 and Beclin 1 in their activated forms to promote autophagy. Of relevance for how these proteins become activated is that IRGM also stimulates AMPK by stabilizing it in its Thr-172 phosphorylated form, which is required for AMPK activation (Mihaylova and Shaw, 2011). This is likely due to effects of IRGM on mitochondria (Singh et al., 2010), which activates AMPK (Romanello et al., 2010; Turkieh et al., 2014), and may involve specific kinases upstream of AMPK including TAK1 (Criollo et al., 2011) and CAMKKβ (Hoyer-Hansen et al., 2007) that have been shown to phosphorylate AMPK at Thr-172 (Mihaylova and Shaw, 2011) and activate autophagy (Criollo et al., 2011; Hoyer-Hansen et al., 2007). The stabilization of phospho-Thr-172 AMPK likely contributes to AMPK-dependent phosphorylation and activation of ULK1 (Egan et al., 2011; Kim et al., 2011) and Beclin 1 (Kim et al., 2013). Consistent with this, IRGM increases total activated ULK1 phosphorylated at Ser-317 and Ser-555 by AMPK (Egan et al., 2011; Kim et al., 2011), and the activated form of Beclin 1 that is phosphorylated at Ser-15 by ULK1 (Kim et
al., 2013) and at Ser-93 and Ser-96 by AMPK (Kim et al., 2013). IRGM has a second effect on autophagic regulators by assembling the activated ULK1 with Beclin 1. Thus, IRGM promotes phosphorylation cascade of key autophagy regulators and assembles them into autophagy initiation complexes (Figure 7J).

Of interest is that IRGM increases levels of a number of autophagy regulators (ULK1, ATG14L, AMBRA1, and ATGL1) but does not affect the stability of others (Beclin 1 and the ATG5-ATG12 complex). The apparent absence of effects on Beclin 1 stability may be explained by the bulk of Beclin 1 being predominantly in non-autophagy related hVPS34 complexes whereas ATG14L-associated Beclin 1 represents a minority of Beclin 1 species in the cell (Kim et al., 2013). IRGM also has an effect on NOD2 levels. However, IRGM reduces NOD2 levels, in contrast to IRGM-dependent stabilization of autophagy regulators. We interpret this dichotomy as a part of the well tuned circuity in response to microbial challenge: whereas autophagy is activated as an antimicrobial effector mechanism, the stimulatory inputs into the system mediated by NOD2 are downregulated lest the system overcommits, which in turn may result in detrimental consequences for the host. PAMP (e.g. MDP) tolerance is an important mechanism to avoid septic shock, which is in part achieved by NOD2 degradation (Zurek et al., 2012).

It has been previously shown that ATG16L1 and NOD2 interact (Cooney et al., 2010; Travassos et al., 2010). This has placed two of the Crohn’s disease-genetic risk factors together, but has left the role of IRGM unexplained. The data presented here show that IRGM is in complexes with ATG16L1 and NOD2 and that IRGM enhances assembly of Atg16L1 with NOD2. Moreover, IRGM affects the stability of each of the components of this complex. Although bringing ATG16L1 to the bacterial entry site marked by NOD2 is a previously known important step (Travassos et al., 2010), how this links up with the core autophagy regulators including ULK1 and Beclin 1 has not been addressed in prior studies. In this work we show that IRGM plays that bridging role by stimulating phosphorylation and activation of key autophagy regulators and placing them together with ATG16L1 (Figure 7J). This point is not trivial, as for example it has not been easy to connect the two seemingly separate systems of autophagy initiation: ULK1-Beclin1 complexes vs. LC3-II conjugation and localized autophagosomal membrane build up. Only recently a part of this key issue has been solved for conventional (non-immunological) autophagy by showing that ATG16L1 and WIPI2 directly interact (Dooley et al., 2014), with WIPI2 recognizing the lipid modification
products of the Beclin 1-directed hVPS34 activity. We propose here that IRGM acts with a similar purpose by bridging ULK1-Beclin 1 complexes with the autophagy conjugation machinery, as shown here for ATG16L1. This can additionally explain why ATG5 is found in IRGM complexes (Gregoire et al., 2011).

Ubiquitination has been implicated in autophagy in several ways primarily in targeting of substrates for autophagic elimination (Stolz et al., 2014). However, the role of K63-linked polyubiquitination has also begun to be appreciated as a mechanism for stabilization of large autophagy-initiating complexes (Nazio et al., 2013; Shi and Kehrl, 2010). Polyubiquitination of IRGM and its role in autophagy (Figure 7J IRGM) does not play a role in targeting substrates for autophagy; instead, it stabilizes multi-protein autophagy initiation complexes. The ubiquitination of IRGM is under the control by NOD2. NOD2 enhances association of ubiquitination-competent IRGM with ULK1 and Beclin 1, whereas NOD2 has no similar effect on the ubiquitination-null mutant of IRGM (IRGM-K\textsuperscript{mut}). Importantly, IRGM-K\textsuperscript{mut} retains certain activities: it maintains the ability to bind ATG16L1 equally well as the ubiquitination-competent IRGM.

IRGM gene expression is cell-type dependent and responds to both starvation and microbial products. IRGM is particularly inducible in cells (intestinal epithelial cells and macrophages) derived from tissues affected in diseases where IRGM has been implicated as a genetic risk factor: CD and tuberculosis (Consortium, 2007; Craddock et al., 2010; Intemann et al., 2009). PAMPs induce autophagy in macrophages through IRGM linking the PAMP detection by NOD2 with the autophagic machinery activation (Figure 7J IRGM). IRGM controls not just initiation of autophagy but may also affect its maturation. IRGM complexes include UVRAG, a regulator of autophagic maturation (Itakura et al., 2008). IRGM displaces Rubicon, known to inhibit maturation complexes (Matsunaga et al., 2009). Thus, IRGM controls several points along the autophagy pathway and contributes to efficient xenophagy. In conclusion, IRGM orchestrates antimicrobial autophagic responses. We have shown here how IRGM does that and what are the exact molecular processes that IRGM controls. This explains the hitherto mysterious role of IRGM in autophagy, places it at the center of molecular complexes controlling and executing autophagy, and molecularly connects biological inputs with autophagic outputs. Finally, our findings indicate that IRGM links up not only with NOD2 but also with several other PRRs, such as NOD1, RIG-I and TLR3. Thus, IRGM and possibly its distant IRG homologs in other vertebrates may act as
transmission modules between a selective sub-repertoire of innate immune responses and the autophagy machinery.

FURTHER EXAMPLES

Second Set- Precision Autophagy Examples- All Figures for this Section are Labeled As FIGURE PRECISION for the attached figures

MATERIAL AND METHODS

Cells, plasmids, siRNA, and transfection

THP-1, HeLa and HEK293T cells were from ATCC. Human peripheral blood monocytes were from StemCell Technologies or from healthy individual donors, and cultured as described previously (Gutierrez et al., 2004). THP-1 cells were differentiated with PMA (50nM) for overnight before use. Full-length human TRIM20 was synthesized and TRIM21 was purchased from DNASU, and both were cloned by PCR into pDONR221. The TRIMs mutants were generated by site-directed mutagenesis and confirmed by sequencing. pENTR or pDONR221 vectors were generated by BP cloning and expression vectors were made by the LR reaction (Gateway; Invitrogen). Other plasmids used were Beclin 1 and its deletion mutants (from B. Levine), ULK1 (from S. Tooze), ATG16L1 and its deletion mutants (from R. Xavier), pCI-Caspase 1 (from K. Fitzgerald), IRF3 (DNASU), pUN01-hNLRP3a and pUN01-hNLRP1 (Invivogen). siRNAs were from Dharmacon, and were delivered to cells by either RNAiMax (Lifetechnologies) or nucleoporation (Amaxa). Plasmid transfections were performed by either calcium phosphate or nucleoporation (Amaxa). Herring testis (HT)-DNA (Sigma) was transfected as described previously (Gao et al., 2013).

Bacterial and viral infection

For infection studies, Escherichia coli strain LF82 (Lapaquette et al., 2010) was infected at MOI of 1:20. Single-cycle infection HIV-1 viruses were generated as previously described (Mandell et al., 2014), were infected to undifferentiated THP-1 cells (Gao et al., 2013).

Antibodies and reagents

Antibodies used were: Flag (Sigma), HA (Roche), LC3 (Sigma), AMPK, ULK1 p-Ser 317 and p-Ser 555 (Cell signalling), NLRP1 (Cell signaling), NLRP3 (Adipogen), Caspase-1
and ULK1 (Santa Cruz), and GFP, IRF3, Myc and Actin (Abeam). To determine autophagic activity by immunoblotting, cells were cultured in the presence of bafilomycin A1, and lysates were subjected to immunoblotting as described previously (Mizushima et al., 2010). The reagents used were Ultrapure LPS (Invivogen), IFN-γ (PeproTech), Cytotoxic LDH assay (Promega), TO-PRO-3 Iodide (lifetechnologies). Immunoblotting, immunostaining were conducted as described (Kyei et al., 2009). FAM-YVAD-FMK stainings (FLICA, ImmunoChemistry Technologies) were performed according to the manufacture's instruction.

**IL-ip measurement**

For IL-1β secretion, THP-1 cells that had been subjected to the differentiation with PMA (50nM) for overnight, were treated with 2^g/mL LPS for 2h, and then treated with nigericin (20µM) for 30 min. IL-1β measurements were performed using HEK-Blue IL-1β Cells (Invivogen).

**TRIM family screen**

THP-1 cells were cultured in 96-well plates containing SMARTpool siRNA (Dharmacon), RNAiMax (Lifetechnologies), and PMA. Culture media were changed after overnight incubation, and forty-eight hours after plating, cells were treated with IFN-γ or vehicle for 4hr, and then fixed and stained to detect endogeneous LC3 (Alexa Fluor 488 as a flurochrome) and nuclei (Hoechst 33342). Plates with cells were subjected to high content analysis for image acquisition and data processing. Two separate siRNA screen for induced autophagy were carried out with the cutoff (>3 SDs change relative to the mean of stimulated control) for hits.

**High content image analysis**

High content imaging and analysis was performed using a Cellomics VTI HCS scanner and iDEV software (ThermoScientific). Automated epifluorescence image collection was carried out until a minimum of 500 cells per well per siRNA knockdown per plate was acquired. Epifluorescence images were machine analyzed using present scanning parameters and object mask definitions. Hoechst 33342 staining were used to automatically detect cellular outlines based on background staining of the cytoplasm, and the mean count of LC3 puncta per cell was determined. Autophagy induction with IFN-γ resulted in a Z' value of 0.87.
High content analysis of puncta in subpopulations of transfected cells

HeLa and THP-1 cells were transfected with plasmids or siRNA, and cultured in full media for overnight (plasmids) or 48h (siRNA). Cells were then fixed and stained to detect, LC3 (Alexa Fluor 488 or 568 as fluorochromes), GFP, and nuclei. High content imaging and analysis was performed using a Cellomics V^TM HCS scanner and iDEV software (ThermoScientific). >200 cells were analyzed in more than quadruplicate manner using a 20x objective at room temperature. Hoechst 33342 staining were used to automatically detect cellular outlines based on background Hoechst staining, and the mean total count or area of punctate of LC3, or TRIM20 per cell was determined. For sub-population analyses, cells that have above the threshold of the background fluorescence were gated as successfully transfected ones.

Fluorescence confocal microscope image acquisition

Fluorescence confocal microscopy was carried out as described previously (Kyei et al., 2009). In brief, Images were acquired using a Zeiss META microscope equipped with a 63x/1.4 NA oil objective, LSM META camera and AIM software (Zeiss) at room temperature. Fluorochromes associated with secondary antibodies were Alexa Fluor 488, 568, or 647. The images were adjusted for brightness and contrast using ImageJ.

IRF3 dimerization assay and quantitative RT-PCR.

Detection of IRF3 dimerization was performed by native polyacrylamide gel electrophoresis (PAGE) as previously described (Takahasi et al., 2003). Quantitative RT-PCR was performed as previously described (Kimura et al., 2013) using the following primer sets: ULK1, (AGATGTTCAGACAAGGTAGAG, AATGACAGTGCACACTTGG); BECN1, (GGGACACCTAGCCGAGAC, ACGTGAGCTGACTGACAG); ACTIN, (GGGCATGGGTCAGCAAGAC, ACTGTTGACTGACTGACAG); TRIM1, (AAGAATGTGACGAGTTGGTAGAG, ATGAGGACTGTTAGCCACAG); TRIM5, (CATGCCTCAGCAACACCAC, GGTAACCTGATCCGCAACACT); TRIM8, (ATCCTGTGAGGGCGGACCCA, CTCCCCTTGGCCACTTC); TRIM16, (GTAAGCCAGCAACCAATATG, TCCAGGGCTGAACTTCTATT); TRIM20, (CTGATAGAGAGGGGACAG, GCTGCTCCTGTCCTGATT); TRIM21, (CAGTGATTGACAGTGGTAGAG, ATGAGGACTGTTAGCCACAG); TRIM22, (CCATACTGTGCGCTCTACTG, GGTTCATGCTTGTGTCAG); TRIM28, (CTCCTGCTGCGCTCTACTG, GGTTCATGCTTGTGTCAG); TRIM56,
Co-immunoprecipitation and GST pull-down

Co-immunoprecipitations were performed as previously described (Kyei et al., 2009) with slight modification. In brief, cells were lysed with NP-40 buffer (lifetechnologies) containing ImM PMSF and protease inhibitor cocktail (Roche) for 45 min, followed by centrifugation. Supernatants were incubated for 2 h with antibodies at 4°C. The immune complexes were captured with Dynabeads (lifetechnologies). Immunoprecipitates were washed three times with PBS, eluted with Laemmli SDS-PAGE sample buffer, and subjected to immunoblots analysis.

GST and GST-tagged proteins were expressed in Escherichia coli BL21 (DE3) or SoluBL21 (Amsbio). GST and GST-fusion proteins were purified and immobilized on glutathione-coupled sepharose beads (Amersham Bioscience, Glutathione-sepharose 4 Fast Flow) and pull-down assays with in vitro translated [35S]-labeled proteins were done as described previously (Pankiv et al., 2007). The [35S] labeled proteins were produced using the TNT T7 Quick Coupled Transcription/Translation System (Promega) in the presence of [35S] L-methionine. The proteins were eluted from washed beads by boiling for 5 min in SDS-PAGE gel loading buffer, separated by SDS-PAGE, and radiolabeled proteins detected in a Fujifilm bioimaging analyzer BAS-5000 (Fuji).

Peptide array overlay assay

Peptide arrays were synthesized on cellulose membrane using a MultiPep automated peptide synthesizer (INTAVIS Bioanalytical Instruments AG, Germany) as described previously (Kramer et al., 1996). Interaction analyses between peptide and recombinant protein were probed by overlaying the membranes with recombinant protein, and bound proteins were detected with HRP-conjugated anti-GST antibody (clone RPN1236; GE Healthcare).

Statistical analyses

Either a two-tailed Student’s t test or ANOVA were used. Statistical significance was defined as $P < 0.05$. 

(TTCTTCGTCAATGGGCTGCT, AAGTCATCGGCACAGTCCAG); and TRIM65, (GATCTACCTGAACCTGCTCTG, GAGGAGGGAGGAATCTGTCT). For IFN-β and GAPDH, Taqman probes and real-time PCR master mixes were used.
Cell culture

Cell lines were maintained and primary human peripheral blood-monocyte-derived macrophages were isolated and maintained as described (Gutierrez et al., 2004).

Transfections

Plasmid transfections in HEK293T were performed using ProFection Mammalian Transfection System from Promega; siRNAs were delivered to cells by nucleoporation (Amaxa).

Microscopy analyses and quantification

Immunofluorescence was performed as described earlier (Kyei et al., 2009). For quantification of puncta, images from different fields were captured and analyzed. For quantification of total cell fluorescence, image J was used as described previously (Chauhan et al., 2013).

Gene expression analysis

Total RNA was isolated from cell culture using Trizol as per the manufacturer's instruction (Invitrogen). For quantitative real-time PCR: TURBO DNA-free kit (Ambion) was used to remove contaminating residual DNA; cDNA was prepared using the high capacity cDNA reverse transcription kit as per the manufacturer's instruction (Applied Biosytem). Taqman probes (Applied Biosystem) and real-time PCR master mixes (Applied Biosystem) were used for real-time PCR as per the manufacturer's instruction. Data were normalized using GAPDH.

Cytokine and NF-kB responses

For NFkB-p65 nuclear localization assay, HeLa cells were platted on cover slips a day before infection. Cells were infected with AIEC LF82 strain at MOI of 1:20 for 2 h followed by washings with PBS and fixing the cells with 4% paraformaldehyde. Cells were visualized using a laser confocal microscope and images were captured using LSM510 software. For IL-1β measurement, IL-1β transcription was determined using qRT-PCR in THP-1 cells.

Bacterial survival analyses

AIEC LF82 survival assay was performed as described previously (Lapaquette et al., 2010). HEK293T cells were infected with AIEC LF82 of MOI of 1:20 for 3 h. Cells were
treated with gentamycin (100 \( \mu \text{g/ml} \)) for 1 h followed by incubation in fresh media for 2 h. Cells were lysed and surviving bacteria quantified by plating and determining colony forming units.

**Proximity ligation assay (PLA)**

HEK293T cells transiently expressing the plasmid constructs were fixed and PLA (Soderberg et al., 2006) performed according to the manufacturer's protocol (Olink Bioscience). Samples were then imaged and analyzed by high content microscopy using a CellomicsArrayScan (Thermo Scientific) with images analyzed using pre-set parameters for cell and PLA puncta identification within iDev software (Thermo Scientific). The average total area of red PLA puncta was determined per cell for a minimum of 500 GFP-IRGM positive cells.

**Flag pull-down assay**

Lysates of HEK293T cells transiently expressing the Flag-NOD2 constructs were incubated with anti-Flag magnetic beads (Sigma) for 2 h. Beads were washed thoroughly (5X) to remove unbound contaminants. The collected beads were incubated with purified recombinant proteins (GST or GST-IRGMd (Singh et al., 2010)) for 2 h and then washed again (5X). The beads were boiled in SDS-PAGE buffer and subjected to Western blotting

IFN-\( \gamma \) induces autophagy (Fabri et al., 2011; Gutierrez et al., 2004; Inbal et al., 2002) and influences cytokine networks and polarization of immune systems (Ghezzi and Dinarello, 1988; Mishra et al., 2013; Schroder and Tschopp, 2010), whereas TRIMs are involved in immune responses (Kawai and Akira, 2011) and, through an assortment of proposed mechanisms affect autophagy (Barde et al., 2013; Khan et al., 2014; Mandell et al., 2014; Niida et al., 2010; Pineda et al., 2015; Pizon et al., 2013; Tomar et al., 2012; Yang et al., 2013). IFN-\( \gamma \) can induce expression of a subset of TRIMs (Carthagea et al., 2009). We wondered whether TRIMs might be contributing mediators to autophagy induction by IFN-\( \gamma \). We employed an image-based high content (HC) analysis of LC3 puncta (Fig. 1A) to screen for effects of TRIM knockdowns on IFN-\( \gamma \)-induced autophagy in human myelomonocytic cells. IFN-\( \gamma \)-induced autophagy in THP-1 (Fig. 1A), also showing dose dependence (Fig. S1A PRECISION), and in primary human macrophages (Fig. S1B). For standardization, we used THP-1 cells for the screen (Fig. 1B and Fig. SIC). Out of the 70 human TRIMs tested,
knockdowns of 24 different TRIMs reduced endogenous LC3 puncta per cell under IFN-\(\gamma\) treatment (Fig. IB, open circles; Fig. SIC shows average± range values from two independent screens). We followed this up by individual knockdowns of a subset of 6 positive and 4 neutral TRIMs from the screen (Fig. IC). All 6 TRIMs that were positive hits from the screen, TRIM1, TRIM8, TRIM20, TRIM21, TRIM22, and TRIM65 (knockdowns were evaluated in Fig. SID), were required for optimal induction of autophagy by IFN-\(\gamma\) (Figure IC). Of the neutral TRIMs, TRIM56 that was marginally positive in the screen, showed a borderline but statistically significant effect (Fig. IC). Thus, TRIMs contribute to autophagy induction in response to IFN-\(\gamma\) (Fig. ID).

**TRIM20 induces autophagy.**

The inventors focused on TRTM20 as a TRIM strongly induced by IFN-\(\gamma\) (Carthagena et al., 2009; Chae et al., 2011). We confirmed that TRIM20 expression was responsive to IFN-\(\gamma\) in our system and tested its kinetics and dose-response (Fig. S1E, F). The inventors next used HC analysis to establish in a dose response setting that TRIM20 was required for IFN-\(\gamma\)-induced autophagy (Fig. S1G, H). This was confirmed in immunoblot assays of LC3 lipidation in the presence of bafilomycin A1, an inhibitor of autophagic flux (LC3-II conversion; Fig. IE). Mirroring these findings, overexpression of GFP-TRIM20 increased LC3 puncta (Fig. IF), and enhanced LC3-II conversion in immunoblots (Fig. S1I); as expected, the LC3-II band was revealed only in bafilomycin A1-treated cells, which protects it from degradation through autophagic flux. These results indicate that activation of autophagy by IFN-\(\gamma\) depends on TRIM20 and that elevated expression of TRIM20, a TRIM whose transcription is known to be strongly activated by IFN-\(\gamma\) (Carthagena et al., 2009; Chae et al., 2011), induces autophagy.
TRIM20 interacts with ULK1, Beclin 1 and ATL16L1

The inventors next examined how TRIM20 induced autophagy. Autophagy requires ULK1 and Beclin 1, both of which play pivotal roles in autophagy initiation in mammalian cells [He and Levine, 2010; Mizushima et al., 2001]. We detected GFP-TRIM20 in immunoprecipitates with co-expressed Myc-ULK1 and Flag-Beclin 1 (Fig. 2A and B) and with endogenous ULK1 and Beclin 1 (Fig. 2A and B). TRIM20 puncta colocalized with ULK1 in the cytoplasm (Fig. 2C). Induction of autophagy depends on a phosphorylation cascade, which includes activation of ULK1 by phosphorylation at Ser-317 (Kim et al., 2011). Active p-ULK1 (Ser-317) co-immunoprecipitated with TRIM20 (Fig. 2D). We next mapped ULK1-binding regions within TRIM20 (Fig. 2E). Like the majority of TRIMs (Kawai and Akira, 2001; Reymond et al., 2001), TRIM20 has B box, CCD and PRY/SPRY domains, but lacks an E3 ligase RING domain, and is uniquely endowed with a pyrin (PYD) domain. TRIM20 constructs lacking PYD and PRY/SPRY domains still bound ULK1 in immunoprecipitation assays (Fig. 2F). Direct binding between TRIM20 and ULK1 was established in GST pull-down experiments (Fig. 2G). Both in vivo and in vitro experiments pointed to the middle portion (including B-box and CCD) of TRIM20 as being critical for association with ULK1, whereas the N-terminal PYD and the C-terminal SPRY domains were dispensable (Fig. 2E).

Beclin 1 showed a more complex domain-requirement for inclusion in TRIM20 complexes, with either the middle portion (including B-box and CCD) or the C-terminal region (PRY/SPRY) displaying an independent capacity to bring down Beclin 1 in immunoprecipitates (Fig. 3A and B). We also examined Beclin 1 for regions required for the ability to co-immunoprecipitate with TRIM20 (Fig. S2C and D). Two Beclin 1 regions appeared to be required: the first one between BH3 and CCD and the second one overlapping with the ECD domain of Beclin 1 (Fig. S2C and D). Furthermore, in the presence of TRIM20, the immunoprecipitated Beclin 1 complexes were enriched for ULK1 (Fig. 3C). Thus, TRIM20 can interact simultaneously with multiple autophagy factors and serves as a platform for co-assembly of ULK1 and Beclin1.

The inventors also found that TRIM20 co-immunoprecipitated with ATG16L1 (Fig. 3D). TRIM20 displayed a complex domain requirement for inclusion in ATG16L1 complexes, with either the middle portion (including B-box and CCD) or the C-terminal region (PRY/SPRY) showing an independent capacity to bring down ATG16L1 in immunoprecipitates (Fig. 3E and F). TRIM20 primarily interacted with the WD repeat of
ATG16L1 (Fig. 3, G and H). Thus, the TRIM20 platform_(Fig. 3I)_contains other autophagy regulators, such as ATG16L1, a component of the autophagy E3-like complex that regulates LC3 conjugation and autophagosome formation (Mizushima et al., 2003).

**TRIM20 interacts with a subset of mammalian Atg8 paralogs**

The inventors examined whether TRIM20 possessed the ability to interact with mammalian Atg8 paralogs (niAtg8s), factors required for autophagosomal membrane formation (Mizushima et al., 2011). Although no binding was detected with LC3B, the commonly used marker for autophagic membrane (Kabeya et al., 2000), GST pull-down experiments revealed interactions of TRIM20 with GABARAP and GABARAPL1, and to a lesser extent with LC3A, LC3C, and GABARAPL2 (Fig. 4A). GABARAP colocalized with TRIM20 (Fig. S2E). Albeit TRIM20 did not directly interact with LC3B, mCherry-TRIM20 profiles were closely juxtaposed to conventional LC3-positive puncta (Fig. S2F). The region of TRIM20 (Fig. 4B) responsible for the interaction with mAtg8s, GABARAP and LC3A, was mapped. A TRIM20 deletion construct spanning residues 375-595 retained capacity to bind GABARAP or LC3A (Fig. 4B and C). To delimit further the TRIM20 sequence required for mAtg8s binding we used GST-GABARAP as bait in a binding assay with an array of TRIM20 peptides (Fig. 4D). Three series of TRIM20 peptides (regions of primary sequence staggered by 3 amino acid residues), with either three or four positive consecutive binding signals, were identified (Fig. 4D). The most upstream region (397-ICSLSHQEH-404; Region I) did not contain a recognizable LIR motif, whereas Region II (470-YYFLEQQEHFFVSL-498) and Region III (523-SEWELLQD-530) contained potential LIR motifs^®Birgisdottir et al., 2013). In follow-up mutational analyses, no single or double alterations of the Regions I-III abrogated GABARAP binding (Fig. S2G). Only when all three regions (I, II and III) were mutated, did this cause loss of GABARAP binding (Fig. 4E and Fig. S2G). Thus, all three regions contribute to the binding of TRIM20 to mAtg8s. Collectively the above findings and experiments described in previous sections demonstrate that TRIM20 assembles both the key regulators of autophagy (ULK1, Beclin 1, ATG16L1) and a subset of effector factors (mAtg8s).

**TRIM20 is a receptor for selective autophagy of inflammasome components**

TRIM20, encoded by the *MEFVg&ae*, is a risk locus for familial Mediterranean fever (FMF) French FMF Consortium, 1997, The International FMF Consortium, 1997. TRIM20
has 305 FMF-associated variants website fmf.igh.cnrs.fr/ISSAID/infevers/, with frequent mutations in its PRY/SPRY domain (Masters et al., 2009). The PYD domain of TRIM20 has been the primary focus of interest in inflammasome regulation due to its potential to bind the cognate PYD domain of ASC (Schroder and Tschopp, 2010). However, it has been reported that the PRY/SPRY domain, located at the other end of TRIM20, recognizes and binds to NLRP3 (Papin et al., 2007). The latter relationship has remained obscure despite the frequency of mutations in the PRY/SPRY domain (Masters et al., 2009). We explored the significance of the interactions between the TRIM20 PRY/SPRY domain and NLRP3 in the context of the above recognized function of TRIM20 in autophagy. The full length TRIM20 and a TRIM20 construct containing only the PRY/SPRY domain both interacted with NLRP3 (Fig. S3A). A knockdown of TRIM20 spared NLRP3 from degradation in cells treated with IFN-γ and LPS (Fig. 5A; Fig. S3B and C). When cells were treated with LPS alone, a knockdown of TRIM20 had no effect on NLRP3 levels (Fig. S3D), albeit LPS alone increased NLRP3 levels (Fig. S3E) as expected (Bauernfeind et al., 2009), in keeping with TRIM20 acting to transduce the effects of IFN-γ. Bafilomycin A1 (an inhibitor of autophagic degradation) protected NLRP3, whereas TRIM20 knockdown increased amounts of NLRP3 and eliminated the protective effects of bafilomycin A1 (Fig. 5B). Similar effects were observed with THP-1 cells exposed to pathogens (adherent-invasive Escherichia coli LF82 (Lapaquette et al., 2010)) and with primary human peripheral blood monocyte-derived macrophages (MDMs) treated as above (Fig. 5C and D).

Degradation of NLRP3 depended on ULK1 and Beclin 1, establishing that disposal of NLRP3 was through autophagy (Fig. 5E; and Fig. S3F). Conversely, expression of TRIM20 decreased levels of co-expressed NLRP3 (Fig. 5F). The destabilizing effect of TRIM20 overexpression on NLRP3 levels was suppressed by bafilomycin A1 (Fig. 5F). Additionally, TRIM20 was protected by bafilomycin A1 from degradation in the presence of NLRP3 (Fig S3G and H), indicating that TRIM20 is degraded along with the delivery of its substrate to autolysosomal compartments.

In addition to NLRP3, other inflammasome components, pro-caspase 1 (Chae et al., 2006; Papin et al., 2007) and NLRP1 (Papin et al., 2007), have been previously shown to interact with the PRY/SPRY domain of TRIM20. When pro-caspase 1 and NLRP1 were co-expressed with TRIM20, they too were subject to degradation inhabitable by bafilomycin A1.
These data show that TRIM20 acts as an autophagy receptor for degradation of inflammasome components and that TRIM20 is responsible for delivery of NLRP3 and other tested inflammasome components for autophagic degradation.

**Presence of target substrate potentiates assembly of activated autophagic components on the TRIM20 platform**

The inventors tested whether the availability of substrate, NLRP3, influenced TRIM20 assembly with ULK1. Although ULK1 was enriched in NLRP3 immunoprecipitates when cells expressed TRIM20 (Fig. 6A) this was reduced when cells were subjected to TRIM20 knockdown (Fig. S3I), the presence of NLRP3 did not affect levels of total ULK1 in TRIM20 immunoprecipitates (Fig. 6B). However, the presence of NLRP3 increased the amount of active p-ULK1 (Ser-317 and Ser-555) (Egan et al., 2011; Kim et al., 2013) associated with TRIM20 (Fig. 6B). Because these two sites of ULK1 are phosphorylated by AMPK (Egan et al., 2011; Kim et al., 2011), we tested whether AMPK is recruited to the TRIM20 complex. AMPK was found in TRIM20 complexes with or without NLRP3 (Fig. S3J). These data indicate that modulation of TRIM20 action, in the presence of its cognate autophagic target, is reflected in ULK1 phosphorylation state and not in ULK1 or AMPK levels. These and above data suggest a model in which not only does TRIM20 organize autophagic machinery by serving as a platform for the assembly of ULK1, Beclin 1, ATG16L1, and mAtg8s, but it also recognizes autophagic substrates via its PRY/SPRY domain, and that this substrate recognition enriches ULK1 in its activated state on the TRIM20 platform (Fig. 6C).

**Disease-associated mutations in TRIM20 diminish its autophagic potency**

A physiologically relevant consequence of TRIM20 mutations in FMF is excessive IL-1β production (Chae et al., 2011; Meinzer et al., 2011; Omenetti et al., 2014). In patients (Omenetti et al., 2014), albeit not in murine systems (Chae et al., 2011), this is dependent on NLRP3 in the context of TRIM20 mutations. A knockdown of ULK1 or TRIM20 elevated IL-1β responses (Fig. 6E; specifically for IL-1β since LDH release was unaffected, S4B). When the cells knocked down for either ULK1 or TRIM20 were also subjected to knockdowns of NLRP3, the latter normalized IL-1β expression (Fig. S4C-E). When cells were subjected to inflammasome activation with LPS and nigericin, FLICA staining (based on a fluorogenic probe FAM-YVAD-FMK for detection of *in situ* caspase 1 activity)
revealed active caspase-1 puncta, as reported previously (Broz et al., 2010). The number of FLICA-positive cells increased when cells were subjected to a TRIM20 knockdown (Fig. 6D and S4A). Thus, TRIM20 suppresses caspase-1 activation and IL-1β production.

We then tested whether the disease-causing variants of TRIM20 affected autophagy and clearance of inflammasome components. We chose the three most frequent variants found in FMF patients (Masters et al., 2009), M680I, M694V and V726A (Fig. 6F). Compound (double or triple) mutant variants of TRIM20 formed fewer TRIM20 puncta (Fig. S4F and G). Whereas expression of wild type TRIM20 resulted in degradation of NLRP3, overexpression of TRIM20 single (M694V), double (M680I and M694V) and triple (M680I, M694V and V726A) mutants showed diminished degradation of NLRP3 (Fig. 6G).

Furthermore, protein complexes with the M694V, double (M680I+M694V), and triple (M680I+M694V+V726A) TRIM20 mutants harbored less ULK1, a trend that was paralleled by phospho-ULK1 levels (Fig. 6H). Consistent with this, there were fewer LC3 puncta per cell induced through expression of the triple mutant TRIM20 (M680I+M694V+V726A) than by the wild type TRIM20 (Fig. S4H). Thus, the disease-associated mutations in the PRY/SPRY domain of TRIM20 perturb ULK1 recruitment and autophagic degradation of NLRP3 and hence may contribute to the inflammatory phenotype associated with FMF mutations (Fig. 6I).

TRIM21 interacts with autophagy factors

The IFN-γ screen with TRIM family of proteins yielded additional hits beside TRIM20 (Fig. 1B), several of which were validated in follow-up analyses (Fig. 1C). Among these was TRIM21 (also known as Ro52/SSA associated with Sjogren syndrome), which is transiently induced by IFN-γ (Fig. S5A). Incidentally, TRIM20 and 21 could be co-immunoprecipitated (Fig. S5B). The IFN-γ induction of TRIM21 expression was in agreement with previous reports (Carthagena et al., 2009; Espinosa et al., 2009). TRIM21 has an acknowledged role in regulating type I interferon responses (Espinosa et al., 2009; Higgs et al., 2008; McEwan et al., 2013; Yoshimi et al., 2009; Zhang et al., 2013). In one mechanism, TRIM21 has been reported to cause IKKβ degradation most likely through autophagy, based on its 3-methyladenine protection and LC3 localization (Niida et al., 2010). Based on our detailed studies with TRIM20 described above, we wondered whether TRIM21 might also act as a platform for assembly of autophagic regulatory factors. Indeed, TRIM21 bound both regulators, ULK1 and Beclin 1 (Fig. 7A and B), and a subset of mAtg8s, most
prominently GABARAP (Fig. 7C). GABARAP binding to TRIM21 did not require the SPRY domain of TRIM21 (Fig. 7D and E). Unlike TRIM20, which does not bind Sequestosome/p62 (p62) (Mandell et al., 2014), a well-known autophagic receptor (Birgisdottir et al., 2013), TRIM21 did bind p62 (Fig. 7F and G). The TRIM21-binding region within p62 was delimited to the residues 170-256 of p62 (Fig. 7F and G). The regions of TRIM21 binding p62 excluded its SPRY domain (Fig. 7D and E). Thus, TRIM21 interacts with multiple regulators and effectors of autophagy.

**TRIM21 is a regulator-receptor for autophagic degradation of activated IRF3**

TRIM21 is known to interact with the transcription factor IRF3 through its SPRY domain (Higgs et al., 2008). It has been proposed that TRIM21 can suppress type I IFN response (Espinosa et al., 2009; Higgs et al., 2008; Yoshimi et al., 2009; Zhang et al., 2013), albeit an activation effect (McEwan et al., 2013) has also been reported. The proposed mechanism for negative regulation of IRF3 is mainly focused on proteasomal degradation of IRF3 (Higgs et al., 2008; Saitoh et al., 2006). However, autophagy is also known to play a suppressive role on type I IFN (Deretic et al., 2015; Jounai et al., 2007; Mathew et al., 2014; Saitoh et al., 2009). We thus wondered if TRIM21 could cause autophagic degradation of IRF3, analogous to what we observed with TRIM20 and NLRP3. IRF3 colocalized with TRIM21 in LC3-positive dots (Fig. 8A). Furthermore, IRF3+TRIM21+ profiles were also ULK1 positive (Fig. 8B). Moreover, IRF3 was found in protein complexes with ULK1 when TRIM21 was present (Fig. 8C).

Cytosolic DNA (during viral infection, e.g. with HIV) induces type I interferon response through endogenous second messenger (cyclic GMP-AMP) by utilizing its adaptor protein STING that results in IRF3 dimerization/activation (Gao et al., 2013). It is the dimerized form of IRF3 that activates type I IFN responses (Takahasi et al., 2003). A knockdown of TRIM21 increased levels of IRF3 dimers in IFN-γ-treated cells stimulated with double stranded DNA (HT-DNA) transfected into the cells (Fig. 8D) but not in cells treated with HT-DNA alone, i.e. in the absence of IFN-γ (Fig. S5D), in keeping with the role of TRIM21 in acting as an effector of IFN-γ. A knockdown of TRIM21 also increased IRF3 dimers in cells infected with a single-cycle infection HIV-1 virus under conditions when cells were treated with INF-γ (Fig. S5E). Bafilomycin A1 protected dimerized IRF3 from degradation; this protection was no longer apparent in cells knocked down for TRIM21 (Fig.
indicating that dimerized IRF3 was routed for autophagic degradation by TRIM21. As a physiologically relevant consequence, knockdown of TRIM21 resulted in increased levels of IFN-β expression after DNA transfection or infection with HIV-1 (Fig. 8F; and Fig. S5G). These data show that TRIM21 acts as a platform for IRF3 degradation, connecting it with the autophagic regulators (ULK1) and effectors (mAtg8s)(Fig. S5I). A knockdown of TRIM21 resulted in increased levels of IFN-β response to LPS (Fig. S5H), in keeping with the proposed autophagic targeting of IKKβ (Niida et al., 2010) within a parallel pathway to IRF3-dependent activation of type I interferon responses.

Collectively, the present findings show that multiple TRIMs participate in autophagic response to IFN-γ. Specifically, TRIM20 and TRIM21 organize autophagic apparatus to degrade their cognate targets and downregulate responses via inflammasome/IL-1β and IRF3/type I IFN (Fig. 8G). Tapering of such responses may be essential to prevent excessive inflammation.

Discussion Precision Autophagy

The inventors’ findings show that a subset of TRIMs act as receptors and regulators for selective autophagy targeting components of the inflammasome and type I interferon response systems. TRIM20 recognizes the inflammasome components, NLRP1, NLRP3, and pro-caspase 1, and leads to their autophagic degradation. A similar principle is at work with TRIM21, which targets activated (dimerized) IRF3 for autophagy. Not only do TRIM20 and TRIM21 directly bind their respective cargo, but they also recruit autophagic machinery thus coordinating target recognition with assembly of the autophagic apparatus and initiation of autophagy. These studies increase the repertoire of currently known autophagic receptors (Birgisdottir et al., 2013; Johansen and Lamark, 2011), and expand the target-receptor role of TRIMs in autophagy, previously indicted only for TRIM5a (Mandell et al., 2014). Thus, direct target recognition and assembly of autophagic machinery to conduct a process referred to as precision autophagy (Deretic et al., 2015) is a more general feature of the TRIM family of proteins.

The recognition of cognate targets by TRIM20 and TRIM21 is reminiscent of direct retroviral capsid recognition by TRIM5a (Stremlau et al., 2006) (Stremlau et al, 2006),
which, as recently shown (Mandell et al., 2014) leads to autophagic degradation of HIV. The principles of precision autophagy (Deretic et al., 2015) may differ fundamentally from targeting of a variety of ubiquitinated cargo earmarked for autophagy by ubiquitin-binding receptors (Stolz et al., 2014). Incidentally, TRIM20 does not possess the RING E3 ubiquitin ligase domain, and does not bind p62 (Mandell et al., 2014). The absence of a RING domain and absence of binding to p62 underscores the ubiquitin-independent nature of target recognition by TRIM20. However, engagement of other Sequestosome 1-like receptors, a class (Deretic et al., 2013) of ubiquitin and galectin recognizing receptors (Gomes and Dikic, 2014; Randow and Youle, 2014) may not be ruled out, as well as a non-targeting role for ubiquitination in stabilizing autophagy initiation complexes (Chauhan et al., 2015; Nazio et al., 2013; Shi and Kehrl, 2010). Furthermore, inclusion of additional cytoplasmic material along with specific targets during TRIM-directed autophagy may not be ruled out.

Importantly, these findings indicate that substrate recognition by TRIM20 also directs precision autophagy machinery assembled by TRIM20. Thus, in their role in autophagy, TRIM20 and TRIM21 act not only as receptors for autophagy but also as platforms for assembly of regulators (ULK1, Beclin 1) and effectors (mAtg8s; p62 in the case of TRIM21), into initiation complexes. The presence in TRIM20 complexes of ATG16L1 may reflect direct association or reinforcement of indirect links between ULKI and ATG16L1 (Gammoh et al., 2013; Nishimura et al., 2013). Other TRIMs may function similarly, as observed with TRIM5a and preliminarily with TRIM6, TRIM17, TRIM22, TRIM49, and TRIM55 (Mandell et al., 2014). The concept of platforms for assembly of autophagic machinery in mammalian cells also extends to generic, starvation induced autophagy, which utilizes exocyst components specifically endowed with Exo84 (Bodemann et al., 2011). However, TRIM engagement with autophagy may entail other mechanisms, as for example TRIM28 has multiple (both positive and negative) proposed mechanisms of action (Barde et al., 2013; Pineda et al., 2015; Yang et al., 2013), whereas the mechanism of autophagy induction for TRIM13 in response to the ER stress has not been fully delineated (Tomar et al., 2012) although it shows a relationship with p62 and DFCP, an ER-derived autophagy precursor compartment termed omegasome (Axe et al., 2008).

A further major biological finding reported here is that TRIMs are mediators of IFN-γ induced autophagy. The engagement of multiple TRIMs reveled in our screen should not be
surprising, as multiple systems can trigger INF-γ-induced autophagy, such as the previously described DAPK phosphorylation of Beclin 1 (Inbal et al., 2002; Zalckvar et al., 2009) and immunity related GTPases (IRG)-dependent induction of autophagy (Gutierrez et al., 2004), which has recently been shown to act through a co-assembly of ULK1 and Beclin 1 (Chauhan et al., 2015). Additional upstream mechanisms may be controlled by TRIMs detected in our screen, as in the case of TRIM8, which is known to be inducible by IFN-γ (Toniato et al., 2002). TRIM8 activates TAK1 (Li et al., 2011), which is proposed to occur through K63 polyubiquitination. TAK1, in turn, activates AMPK-dependent autophagy (Criollo et al., 2011; Herrero-Martin et al., 2009; Kanayama et al., 2004) by phosphorylating AMPK (Xie et al., 2006). Hence, TRIM8 affects upstream pathways known to activate autophagy. This may explain why TRIM8 was identified as a hit in our IFN-γ-dependent autophagy induction screen. Furthermore, it is likely that TRIMs, known to hetero-oligomerize (Bell et al., 2012) as supported by our observations with TRIM20 and TRIM21, cooperate in IFN-γ induction of autophagy.

The finding that TRIM20 is a mediator of IFN-γ suppression of inflammasome activation provides a mechanism for this important IFN-γ effect in prevention of excessive inflammasome activation and associated pathology in infectious and autoimmune diseases (Minguela et al., 2007; Nandi and Behar, 2011), for which a satisfactory definition has been lacking albeit indirect mechanisms have been proposed (Mishra et al., 2013). The TRIM20-dependent direct recognition and autophagic degradation of the inflammasome components NLRP3, pro-caspase 1, and NLRP1, differs from the previous reports of indirect effect on inflammasome activation via mitophagy (Nakahira et al., 2011; Zhou et al., 2011), and is more akin to the proposed autophagic degradation of AIM2, a sensory component of the DNA-reactive specialized inflammasome, albeit AIM2 has been proposed to be eliminated by ubiquitin-tag recognizing receptor (Shi et al., 2012). We furthermore demonstrated that FMF disease-associated mutations in the PRY/SPRY domain of TRIM20 (Masters et al., 2009), alter the capacity of TRIM20 to direct autophagic degradation of inflammasome components. These mutations reduced the binding of ULK1, thus explaining in part how the common mutations associated with FMF work. We propose that IFN-γ-TRIM20-autophagy axis normally suppress excessive inflammasome and IL-1β activation, and that this ability is blunted by common disease-associated TRIM20 polymorphisms occurring in FMF.
The reported TRIM21-dependent suppression of type I IFN activation by autophagic degradation of IRF3 dimers mirrors the action of TRIM20 in suppressing inflammasome activation. TRIM21, an autoantigen associated with Sjogren syndrome and systemic lupus erythematosus, suppresses type I IFN response (Espinosa et al., 2009; Higgs et al., 2008; Yoshimi et al., 2009; Zhang et al., 2013), albeit this has been ascribed to proteasomal degradation of IRF3 (Higgs et al., 2008) and IRF7 (Higgs et al., 2010). Nevertheless, type I IFN can also be activated by NF-κB, and autophagy has been implicated in degradation of the upstream NF-κB activating kinase, IKKβ (Niida et al., 2010). The TRIM21-directed autophagic degradation of activated IRF3 shown here complements the action of TRIM21 on NF-κB (Niida et al., 2010). Although the mechanism is not fully known, activation of type I IFN system is one major feature of Sjogren syndrome and systemic lupus erythematosus (Banchereau and Pascual, 2006). We thus raise the possibility that perturbations of IFN-γ-TRIM21-autophagy axis may cause activation of type I IFN in autoimmune diseases.

The inventors’ findings reported here broaden the concept of TRIMs acting as autophagic receptors and as platforms for assembly of autophagy initiation complexes. Our findings also link cargo recognition by a TRIM, acting as an autophagic receptor, with the function of the same TRIM in the assembly of autophagic machinery triggering the execution of autophagy of a very specific cytoplasmic targets. This brand of autophagy, termed precision autophagy, is guided by TRIMs and has important biological functions. For example, the TRIM20- and TRIM21-precision autophagy uncovered here balances key innate immunity responses, potentially serving as a guardian against excessive inflammation, which in turn may cause pathology during autoimmune processes or in infections causing cytokine storms. We propose that the large family of TRIMs with 70 members in humans endows cells with a precision in deploying autophagy.

References (IRGM)


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Supplemental References (IRGM)


References (Precision Autophagy)


**Supplemental References (Precision Autophagy)**


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|        |               | QAClsenKQFARQPSASQKNAERAVAMASSQVLPISPINFNDAFNAFALDSREKRL EGDLYLTAPNPSIEKCRLASTXDITVHWIDDDFGSIESSYELQITFTQGANFISK SWCCSWLWPPEIRKKEAVSCSRLAGARPGLYNSVSMWIPNQKNHVYTHGLQGSTR YIFIVKAINQAGSMNSEPRTLKNQSFPFQKPKMTHKLKISNDGLQMEKDESSLKKH LPERFSGSCTGCGYAGNNFIDSGCHYEWVMEGTSWAYIAIGAISAPKNENWIGKNAW VFSRSCRSNNFVRRHMKLVLKPDVFHLKRKLVLVLDXNMLSYFDPSNLHHTFDVT FILVQCPFTIIWNSLIMLSGLAPDFIDYPERCECNRCPQESFVYSMKTCH      |
|        |               | **SEQ ID NO: 1**                                                                                                                                                                                       |               |
| TRIM2  | NM_015271     | MHS弹性GRYQTQORAGSKTAGPFPCOWSRMASEGTINPSFVQVID KQFLICSLERYKNNPKVLPCLHTFCERCLQNYIPARSILTSCLPCVRQTSILPEKGV ALOQNNFPTILINMDVQRTGQSVRNAESESSILEETVTAAYAKPLSCPNIDGNVMFECQSC ETAMCRECTEGEHAHPVLPKDVQUEHQAASLQVQQLADVYNKLPLFIDEALQFIEIIH QLNTQKSAVQLADVNLHSTFDE1QLTVRKSVMLMELVEYNGLKHKLQVQSLQDLTTLQCQK SIKSCSNFTAQOLLNVAQAVLLANLQADQFPFLHPRNEDQLDFIVETEC LKKSMHSNHTLITNNAVSETVAGLQRTIQGPMSTTTTKDKDELCTGNAYL TAELESTPDGSVDAEGDLNKNQNYFETYFLQVEKQEGDFTLSLRLYDPHQRGFSFPRKVVIR SADVSTPTTEVCRVSFGPGSHGQKAVKRPSMYSTGKRENKEDIDLFRVGTGRK NKGFEFNLQVQAATSNXKLNDAMSNQCQVIFSQNDQFQRSFGIRGRSPQGQRTGV AVHPSGDIILIIADYDNKWSIIFSSDGGKFKRTGKGLMKPGKVSVDANHIHEDVYDKNAC CVFIFQPGNKIVTRGPRSGNRQFAPQFHAPAANSNLEGITIITDFHHHSKVQNFQGEGF MFLKSFSNENQGQNFFAPTVAYVDSNGNIIADANGNRIQVFGSGSFLSYINTSADFL YGPEGLATSLSGHDVVDASGNHCDFKYYRLQ | **SEQ ID NO: 6** |
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|        |               | **SEQ ID NO: 13**                                                                                                                                                                                       |               |
|        |               | **SEQ ID NO: 5**                                                                                                                                                                                       |               |
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|        |           | LSQAALGNLSRDAKEAEFGLVQRLNMQIQESNVVEFAACLVAQCDALIDAQNRRKQQL | |
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|        |             | CLSASTAVKRPVFCDAEFTTYQGLGLNLNQRSNEHLEGPSFSGVPDQLRQIS |             |               |
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|                |        |            | RINMYCELCRRFVCHLCKLGGNHANHVTTMSAYKTLKELSKIDYLIGKESQVKS |
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|                |        |            | QMEYQQGGLNNLGVLQAQVLKDQSCFYQATKQLHLRIKATESLKSFRPAQTS |
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| NM_198853 | MAWQVSLLELEDRLQPCICELFKESEMLQCGHSYCKGCLVSLS | GGACCGAAAUCGUAAUGA | SEQ ID NO: 329 |
|           | YHLDYTKRCFMQWVGGSSLPNVSALWIEALRLPGDEFKVCVHRRNLPLSFCEK | CAAGGAGUCUCAAUGCUA | SEQ ID NO: 330 |
|           | DQELICGCLGGLGSHQHPVTSPVCFSRMKKEELAALFSELKQEQQKVDELIAKLKNRTRIVNESDFSWFVIRREQFELRHPVEEKARCLEGIIIGGTHRLVASLDMQLEQAQGTRERLQAACVEQVLQENDEHHEFIWKFHSMASR | UGCAGCCUCUUCUCUGA | SEQ ID NO: 331 |
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**PCT/US2016/042899**
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```
Claims:

1. A pharmaceutical composition comprising:
   (a) an IRGM modulator in an effective amount and optionally one or more of the following:
   (b) an autophagy modulator in an effective amount;
   (c) a pharmaceutically-acceptable carrier, additive and/or excipient; and
   (d) at least one additional bioactive agent,
   or a pharmaceutically acceptable salt of any one or more of the above agents thereof.

2. The composition according to claim 1 wherein said IRGM modulator is a double stranded RNA or a muramyl peptide or a pharmaceutically acceptable salt thereof.

3. The composition according to claim 2 wherein said double stranded RNA is poly I:C, poly U-G or poly ICLC, or a pharmaceutically acceptable salt thereof.

4. The composition according to claim 2 wherein said muramyl peptide is a compound

   \[
   \begin{align*}
   &\text{O} \\
   &\text{C} \quad \text{R} \\
   &\text{R^3-CH} \\
   &\text{CH_2OR^1} \\
   &\text{R^2O} \\
   &\text{H} \\
   &\text{OR^5} \\
   &\text{NHCOR^4}
   \end{align*}
   \]

   wherein:
   R^1 represents a hydrogen atom or a C1-C22 acyl group;
   R^2 represents a hydrogen atom or a C1-C22 acyl group;
   R^3 represents a hydrogen atom or a C1-C6 alkyl group;
   R^4 represents a C1-C21 alkyl group or a C5 or C10 aryl group;
   R^5 represents a hydrogen atom; and
R represents the residue of an amino acid or a linear peptide built up of from 2 to 6 amino acid residues, at least one of the residues being optionally substituted with a lipophilic group including muramyl dipeptide and desmethylmuramyl dipeptide.

5. The composition according to claim 2 wherein said muramyl peptide is muroctasin;
MTP-PE;
murabutide;
t-MDP;
GMDP
GMDP-LL;
GMDP-Obu;
GMDO-Lys;
GMDB-Lys(St);
GMDBA-Lys(St);
GMDPA(OBzl)$_2$;
MeGMDP;
(GMDP)$_2$;
(GMDPA)$_2$;
(GMDPLys)$_2$;
[GMDP-Lys(St)]$_2$;
GMDP-Ad;
GMDP-tuftsin E;
GMDP-tuftsin A;
GMDP-tuftsin lipophilic;
GMDP-bursin;
GMDP-thymogen I;
GMDP-thymogen II;
GMDP-thymogen III;
Thr-MDP, and mixtures thereof.

6. The composition according to claim 1 wherein said autophagy modulator is selected from the group consisting of flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine,
dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon, nortriptyline, tetrachlorisopthalonitrile, phenylmercuric acetate, benzethonium, niclosamide, monensin, bromperidol, levobunolol, dehydroisoandrosterone 3-acetate, sertraline, tamoxifen, reserpine, hexachlorophene, dipyridamole, harmaline, prazosin, lidoflazine, thiethylperazine, dextromethorphan, desipramine, mebendazole, canrenone, chlorprothixene, maprotiline, homochlorcyclizine, loperamide, nicardipine, dexfenfluramine, nilvadipine, dosulepin, biperiden, denatonium, etomidate, toremifene, tomoxetine, clorgyline, zotepine, beta-escin, tridihexethyl, ceftazidime, methoxy-6-harmalan, melengestrol, albendazole, rimantadine, chlorpromazine, pergolide, cloperastine, prednicarbate, haloperidol, clotrimazole, nitrofural, iopanoic acid, naftopidil, methimazole, trimeprazine, ethoxyquin, clocortolone, doxycycline, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon, nortriptyline pharmaceutically acceptable salts thereof and mixtures thereof.

7. The composition according to any of claims 1-6 wherein said autophagy moldulator is selected from the group consisting of flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon, nortriptyline pharmaceutically acceptable salts thereof and mixtures thereof.

8. The composition according to any of claims 1-7 wherein said autophagy modulator is selected from the group consisting of benzethonium, niclosamide, monensin, bromperidol, levobunolol, dehydroisoandrosterone 3-acetate, sertraline, tamoxifen, reserpine, hexachlorophene, dipyridamole, harmaline, prazosin, lidoflazine, thiethylperazine, dextromethorphan, desipramine, mebendazole, canrenone, chlorprothixene, maprotiline, homochlorcyclizine, loperamide, nicardipine, dexfenfluramine, nilvadipine, dosulepin, biperiden, denatonium, etomidate, toremifene, tomoxetine, clorgyline, zotepine, beta-escin, tridihexethyl, ceftazidime, methoxy-6-harmalan, melengestrol, albendazole, rimantadine, chlorpromazine, pergolide, cloperastine, prednicarbate, haloperidol, clotrimazole, nitrofural, iopanoic acid, naftopidil, methimazole, trimeprazine, ethoxyquin, clocortolone, doxycycline, pirlindole mesylate, doxazosin, depotropine, nocardazole, scopolamine, oxybenzone,
halcinonide, oxybutynin, miconazole, clomipramine, cyproheptadine, doxepin, dyclonine, salbutamol, flavoxate, amoxapine, fenofibrate, pimethixene, pharmaceutically acceptable salts thereof and mixtures thereof.

9. The composition according to any of claims 1-8 wherein said additional bioactive agent is an antibiotic or an antiviral agent.

10. The composition according to claim 9 wherein said antibiotic is an anti-tuberculosis agent.

11. The composition according to claim 9 wherein said antiviral agent is an anti-HIV agent, an anti-HBV agent, anti-influenza agent, an anti-herpes agent or an anti-HCV agent.

12. The composition according to claim 11 wherein said antiviral agent is a nucleoside reverse transcriptase inhibitor (NRTI), a non-nucleoside reverse transcriptase inhibitor (NNRTI), a protease inhibitor, a fusion inhibitor or a mixture thereof.

13. The composition according to claim 11 wherein said anti-HIV agent is 3TC (Lamivudine), AZT (Zidovudine), (-)-FTC, ddl (Didanosine), ddC (Zalcitabine), abacavir (ABC), tenofovir (PMPA), D-D4FC (Reverset), D4T (Stavudine), Racivir, L-FddC, L-FD4C, NVP (Nevirapine), DLV (Delavirdine), EFV (Efavirenz), SQVM (Saquinavir mesylate), RTV (Ritonavir), IDV (Indinavir), SQV (Saquinavir), NFV (Nelfinavir), APV (Amprenavir), LPV (Lopinavir), fusion inhibitors such as T20, among others, fuseon and mixtures thereof.

14. The composition according to any of claims 9-13 wherein said bioactive agent includes an anticancer agent.

15. The composition according to claim 14 wherein said anticancer agent is an antimetabolite, an inhibitor of topoisomerase I and/or II, an alkylating agent, a microtubule inhibitor, a tyrosine kinase inhibitor, an EGF kinase inhibitor or an ABL kinase inhibitor.

16. The composition according to claim 14 wherein said anticancer agent is Aldesleukin; Alemtuzumab; alitretinoin; allopurinol; altretamine; amifostine; anastrozole; arsenic trioxide; Asparaginase; BCG Live; bexarotene capsules; bexarotene gel; bleomycin; busulfan
intravenous; busulfan oral; capecitabine; carboplatin; carmustine; cyclophosphamide; cytarabine; dacarbazine; dactinomycin; actinomycin D; Darbepoetin alfa; daunorubicin liposomal; daunorubicin; daunomycin; Denileukin difitox; dexrazoxane; docetaxel; doxorubicin; doxorubicin liposomal; Dromostanolone propionate; Elliott's B Solution; epirubicin; Epoetin alfa; estramustine; etoposide phosphate; etoposide (VP-16); exemestane; Filgrastim; fludarabine; fluorouracil (5-FU); fulvestrant; gemtuzumab ozogamicin; goserelin acetate; hydroxyurea; Ibritumomab Tiuxetan; idarubicin; ifosfamide; imatinib mesylate; Interferon alfa-2a; Interferon alfa-2b; irinotecan; letrozole; leucovorin; levamisole; lomustine (CCNU); mecloretamine (nitrogen mustard); megestrol acetate; melphalan (L-PAM); mercaptopurine (6-MP); mesna; methotrexate; methoxsalen; mitomycin C; mitotane; mitoxantrone; nandrolone phenpropionate; Nofetumomab; LOddC; Oprelvekin; oxaliplatin; paclitaxel; pamidronate; Pegaspargase; Pegfilgrastim; pentostatin; pipobroman; plicamycin; mitoxamycin; porfimer sodium; procarbazine; quinacrine; Rasburicase; Rituximab; Sargramostim; streptozocin; surafenib; talbuvidine (LDT); tcalc; tamoxifen; tarceva (erlotinib); temozolomide; teniposide (VM-26); testolactone; thioguanine (6-TG); thiopeta; topotecan; toremifene; Tositumomab; Trastuzumab; tretinoin (ATRA); Uracil Mustard; valnibicin; valtorcitabine (monoval LDC); vinblastine; vinorelbine; zoledronate or a mixture thereof.

17. A method of treating an autophagy-mediated disease in a patient in need thereof comprising administering to said patient an effective amount of a composition according to any of claims 1-16.

18. The method according to claim 17 wherein said autophagy-mediated disease is cancer, lysosomal storage diseases, Alzheimer's disease, Parkinson's disease; a chronic inflammatory disease, Crohn's disease, diabetes I, diabetes II, metabolic syndrome, an inflammation-associated metabolic disorder, liver disease, renal disease, cardiovascular disease, muscle degeneration and atrophy, symptoms of aging (including the amelioration or the delay in onset or severity or frequency of aging-related symptoms and chronic conditions including muscle atrophy, frailty, metabolic disorders, low grade inflammation, atherosclerosis and associated conditions such as cardiac and neurological both central and peripheral manifestations including stroke, age-associated dementia and sporadic form of
Alzheimer's disease, pre-cancerous states, and psychiatric conditions including depression), spinal cord injury, infectious disease and developmental disease.

19. The method according to claim 17 wherein said autophagy-mediated disease is selected from the group consisting of Type I and Type II diabetes, severe insulin resistance, hyperinsulinemia, hyperlipidemia, obesity, insulin-resistant diabetes, Mendenhall's Syndrome, Werner Syndrome, leprechaunism, lipoatrophic diabetes, acute and chronic renal insufficiency, end-stage chronic renal failure, glomerulonephritis, interstitial nephritis, pyelonephritis, glomerulosclerosis, GH-deficiency, GH resistance, Turner's syndrome, Laron's syndrome, short stature, increased fat mass-to-lean ratios, decreased CD4+ T cell counts and decreased immune tolerance, chemotherapy-induced tissue damage, congestive heart failure, Alzheimer's disease, Parkinson's disease, multiple sclerosis, Crohn's disease, peripheral neuropathy, muscular dystrophy, myotonic dystrophy, anorexia nervosa, a viral infection, and a bacterial infection.

20. The method according to claim 17 wherein said autophagy-mediated disease is selected from the group consisting of activator deficiency/GM2 gangliosidosis, alpha-mannosidosis, aspartylglucoaminuria, cholesteryl ester storage disease, chronic hexosaminidase A deficiency, cystinosis, Danon disease, Fabry disease, Farber disease, fucosidosis, galactosialidosis, Gaucher Disease (Types I, II and III), GM1 Gangliosidosis, including infantile, late infantile/juvenile and adult/chronic), Hunter syndrome (MPS II), I-Cell disease/Mucolipidosis II, Infantile Free Sialic Acid Storage Disease (ISSD), Juvenile Hexosaminidase A Deficiency, Krabbe disease, Lysosomal acid lipase deficiency, Metachromatic Leukodystrophy, Hurler syndrome, Scheie syndrome, Hurler-Scheie syndrome, Sanfilippo syndrome, Morquio Type A and B, Maroteaux-Lamy, Sly syndrome, mucolipidosis, multiple sulfate deficiency, Niemann-Pick disease, Neuronal ceroid lipofuscinoses, CLN6 disease, Jansky-Bielschowsky disease, Pompe disease, pycnodysostosis, Sandhoff disease, Schindler disease, Tay-Sachs or Wolman disease.

21. A method of treating of cancer in a patient in need, comprising administering to said patient an effective amount of a composition according to any of claims 1-16.
22. A method of treating Crohn's disease or tuberculosis, the method comprising administering an effective amount of a composition according to any of claims 1-16.

23. A method of treating excessive inflammation associated with an autophagy-related diseases in a patient in need thereof, the method comprising administering an effective amount of a compound or composition selected from the group consisting of at least one TRIM protein or a variant thereof having at least 90% sequence identity to said TRIM protein, at least one inhibitor of a TRIM protein or a mixture thereof, and optionally at least one additional bioactive agent.

24. The method according to claim 23 wherein said TRIM protein is TRIM1 (SEQ ID NO:1), TRIM3 (SEQ ID NO:11), TRIM8 (SEQ ID NO:36), TRIM10 (SEQ ID NO:46), TRIM13 (SEQ ID NO:56), TRIM17 (SEQ ID NO:81), TRIM19 (SEQ ID NO:91), TRIM20 (SEQ ID NO:96), TRIM21 (SEQ ID NO:101), TRIM22 (SEQ ID NO:106), TRIM38 (SEQ ID NO:172), TRIM 41 (SEQ ID NO:187), TRIM43 (SEQ ID NO:97), TRIM44 (SEQ ID NO:202), TRIM45 (SEQ ID NO:207, TRIM46 (SEQ ID NO:212), TRIM54 (SEQ ID NO:247), TRIM55 (SEQ ID NO:252), TRIM56 (SEQ ID NO:257), TRIM58 (SEQ ID NO:262), TRIM59 (SEQ ID NO:267), TRIM60 (SEQ ID NO:272), TRIM65 (SEQ ID NO:297), TRIM66 (SEQ ID NO:302) and TRIM75 (SEQ ID NO:338).

25. The method according to claim 23 wherein said TRIM protein is TRIM 1 (SEQ ID NO:1), TRIM 8 (SEQ ID NO:236), TRIM 20 (SEQ ID NO:20), TRIM 21 (SEQ ID NO:101), TRIM 22 (SEQ ID NO:106), TRIM 56 (SEQ ID NO:257), TRIM 65 (SEQ ID NO:297) or a mixture thereof.

26. The method according to any of claims 23-26 wherein said TRIM protein inhibitor is a siRNA of about 9-30 nucleotide units in length.

27. The method according to any of claims 23-26 wherein said autophagy-related disease is an inflammatory disease, an autoimmune disease, an infectious disease, a cardiovascular disease or a metabolic disease.
28. The method according to any of claims 23-27 wherein said autophagy-related disease is selected from the group consisting of a lysosomal storage disease, neurodegeneration, autoimmune diseases and chronic inflammatory diseases resulting in excessive inflammation, hyperglycemic disorders, liver disease, renal disease, cardiovascular disease, muscle degeneration and atrophy, symptoms of aging, pre-cancerous states, psychiatric conditions, stroke, spinal cord injury, arteriosclerosis and infectious diseases.

29. The method according to any of claims 23-26 wherein said autophagy-related disease is a lysosomal storage disease, Alzheimer's disease, Parkinson's disease, Huntington's disease, inflammatory bowel disease, rheumatoid arthritis, lupus, multiple sclerosis, chronic obstructive pulmony disease/COPD, pulmonary fibrosis, cystic fibrosis, Sjogren's disease; hyperglycemic disorders, diabetes (I and II), affecting lipid metabolism islet function and/or structure, excessive autophagy may lead to pancreatic β-cell death and related hyperglycemic disorders, including severe insulin resistance, hyperinsulinemia, insulin-resistant diabetes (e.g. Mendenhall's Syndrome, Werner Syndrome, leprechaunism, and lipoatrophic diabetes) and dyslipidemia (e.g. hyperlipidemia as expressed by obese subjects, elevated low-density lipoprotein (LDL), depressed high-density lipoprotein (HDL), and elevated triglycerides) and metabolic syndrome, liver disease (excessive autophagic removal of cellular entities- endoplasmic reticulum), renal disease (apoptosis in plaques, glomerular disease), cardiovascular disease (especially including ischemia, stroke, pressure overload and complications during reperfusion), muscle degeneration and atrophy, symptoms of aging (including amelioration or the delay in onset or severity or frequency of aging-related symptoms and chronic conditions including muscle atrophy, frailty, metabolic disorders, low grade inflammation, atherosclerosis and associated conditions such as cardiac and neurological both central and peripheral manifestations including stroke, age-associated dementia and sporadic form of Alzheimer's disease, pre-cancerous states, and psychiatric conditions including depression), stroke and spinal cord injury, arteriosclerosis, infectious diseases (microbial infections, removes microbes, provides a protective inflammatory response to microbial products, limits adaption of autophagy of host by microbe for enhancement of microbial growth, regulation of innate immunity) including bacterial, fungal, cellular and viral (including secondary disease states or conditions associated with infectious diseases, especially including heptatis B and C and HIV I and II), including AIDS and tuberculosis.
30. The method according to any of claims 23-29 wherein said compound or composition is further co-administered with at least one additional bioactive agent.

31. The method according to claim 30 wherein said additional bioactive agent is interferon-γ or pegylated interferon.

32. The method according to claim 30 wherein said additional bioactive agent is at least one compound selected from the group consisting of flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon, nortriptyline, tetrachlorisopthalonitrile and phenylmercuric acetate, benzethonium, niclosamide, monensin, bromperidol, levobunolol, dehydroisoandrosterone 3-acetate, sertraline, tamoxifen, reserpine, hexachlorophene, dipyridamole, harmaline, prazosin, lidoflazine, thiethylperazine, dextromethorphan, desipramine, mebendazole, canrenone, chlorprothixene, maprotiline, homochlorcyclizine, loperamide, nicardipine, dexamfluramine, nilvadipine, dosulepin, biperiden, denatonium, etomidate, toremifene, tomoxetine, clorgyline, zotepine, beta-escin, tridihexethyl, ceftazidime, methoxy-6-harmalan, melengestrol, albendazole, rimantadine, chlorpromazine, pergolide, cloperastine, prednicarbate, haloperidol, clotrimazole, nitrofural, iopanoic acid, naftopidil, methimazole, trimeprazine, ethoxyquin, clocortolone, doxycycline, pirlindole mesylate, doxazosin, depotpine, nocodazole, scopolamine, oxybenzone, halcinonide, oxybutynin, miconazole, clomipramine, cyproheptadine, doxepin, dyclonine, salbutamol, flavoxate, amoxapine, fenofibrate, pimethixene, TRIM2 (SEQ ID NO:6), TRIM 4 (SEQ ID NO:16), TRIM5 (TRIM5a) (SEQ ID NO:21), TRIM6 (SEQ ID NO:26), TRIM 7 (SEQ ID NO:31), TRIM9 (SEQ ID NO:41), TRIM 1 1 (SEQ ID NO:51), TRIM 1 4 (SEQ ID NO:61), TRIM 1 5 (SEQ ID NO:66), TRIM 1 6 (SEQ ID NO:71), TRIM 1 8 (SEQ ID NO:86), TRIM 2 3 (SEQ ID NO:11), TRIM 2 4 (SEQ ID NO:16), TRIM 2 5 (SEQ ID NO:121), TRIM 2 7 (SEQ ID NO:126), TRIM 2 8 (SEQ ID NO:131), TRIM 2 9 (SEQ ID NO:136), TRIM 3 0, TRIM 3 1 (SEQ ID NO:141), TRIM 3 2 (SEQ ID NO:146), TRIM 3 3 (SEQ ID NO:151), TRIM 3 4 (SEQ ID NO:156), TRIM 3 5 (SEQ ID NO:161), TRIM 3 6 (SEQ ID NO:166), TRIM 3 7 (SEQ ID NO:167), TRIM 3 9 (SEQ ID NO:177), TRIM 4 0 (SEQ ID NO:182), TRIM 4 2 (SEQ ID NO:192), TRIM 4 7 (SEQ ID NO:217), TRIM 4 8 (SEQ ID NO:222), TRIM 4 9 (SEQ ID NO:227), TRIM 5 0 (SEQ ID NO:232), TRIM 5 1 (SEQ ID NO:237), TRIM 5 5 (SEQ ID NO:252), TRIM 6 8 (SEQ ID NO:312), TRIM 7 2 (SEQ ID NO:323), TRIM 7 3 (SEQ ID
NO:328), TRIM74 (SEQ ID NO:333), TRIM76 (SEQ ID NO:343), a triglyceride, a diglyceride, a monoglyceride, a glycolated mono- or diacylglyceride, dolichol, polyprenol, polyprenal, very long chain fatty acids or a pharmaceutically acceptable salt thereof.

33. The method according to claim 30 wherein said additional bioactive agent is selected from the group consisting of pp242, rapamycin, envirolimus, everolimus, cidaforoUimus, epigallocatechin gallate (EGCG), caffeine, curcumin, reseveratrol, digoxin, xylazine, hexetidine, sertindole and mixtures thereof.

34. The method according to claim 30 wherein said additional bioactive agent is an antiviral agent.

35. A pharmaceutical composition comprising an effective amount of at least one TRIM protein selected from the group consisting of TRIM1 (SEQ ID NO:1), TRIM3 (SEQ ID NO:1), TRIM8 (SEQ ID NO:36), TRIM10 (SEQ ID NO:46), TRIM13 (SEQ ID NO:56), TRIM17 (SEQ ID NO:81), TRIM19 (SEQ ID NO:91), TRIM20 (SEQ ID NO:96), TRIM21 (SEQ ID NO:101), TRIM22 (SEQ ID NO:106), TRIM38 (SEQ ID NO:172), TRIM41 (SEQ ID NO:187), TRIM43 (SEQ ID NO:97), TRIM44 (SEQ ID NO:202), TRIM45 (SEQ ID NO:207), TRIM46 (SEQ ID NO:212), TRIM54 (SEQ ID NO:247), TRIM55 (SEQ ID NO:252), TRIM56 (SEQ ID NO:257), TRIM58 (SEQ ID NO:262), TRIM59 (SEQ ID NO:267), TRIM60 (SEQ ID NO:272), TRIM65 (SEQ ID NO:297), TRIM66 (SEQ ID NO:302) and TRIM75 (SEQ ID NO:338) or a pharmaceutically acceptable salt thereof in combination with an additional bioactive agent.

36. The composition according to claim 35 wherein said additional bioactive agent is interferon-gamma (IFN-gamma), pegylated interferon (PEG-IFN), a siRNA or a mixture thereof.

37. The composition according to claim 35 further including at least one compound selected from the group consisting of flubendazole, hexachlorophene, propidium iodide, bepridil, clomiphene citrate (Z,E), GBR 12909, propafenone, metixene, dipivefrin, fluvoxamine, dicyclomine, dimethisoquin, ticlopidine, memantine, bromhexine, norcyclobenzaprine, diperodon, nortriptyline, tetrachlorisophthalonitrile and phenylmercuric acetate, benzethonium, niclosamide, monensin, bromperidol, levobunolol,
dehydroisoandrosterone 3-acetate, sertraline, tamoxifen, reserpine, hexachlorophene, dipyridamole, harmaline, prazosin, lidoflazine, thiethylperazine, dextromethorphan, desipramine, mebendazole, canrenone, chlorprothixene, maprotiline, homochlorcyclizine, loperamide, nicardipine, dexamfetamine, nilvadipine, dosulepin, biperiden, denatonium, etomidate, toremifene, tomoxetine, clorgyline, zotepine, beta-escin, tridihexethyl, ceftazidime, methoxy-6-harmalan, melengestrol, albendazole, rimantadine, chlorpromazine, pergolide, cloperastine, prednicarbate, haloperidol, clotrimazole, nitrofurazone, ipanoic acid, naftopidil, methimazole, trimeprazine, ethoxyquin, clocortolone, doxycycline, pirlindole mesylate, doxazosin, depropine, nocodazole, scopolamine, oxybenzone, halcinonide, oxybutynin, miconazole, clomipramine, cyproheptadine, doxepin, dyclonine, salbutamol, flavoxate, amoxapine, fenofibrate, pimethixene, a TRIM protein selected from the group consisting of TRIM2 (SEQ ID NO:6), TRIM4 (SEQ ID NO:16), TRIM5 (TRIM5a) (SEQ ID NO:21), TRIM6 (SEQ ID NO:26), TRIM7 (SEQ ID NO:31), TRIM9 (SEQ ID NO:41), TRIM11 (SEQ ID NO:51), TRIM14 (SEQ ID NO:61), TRIM15 (SEQ ID NO:66), TRIM16 (SEQ ID NO:71), TRIM18 (SEQ ID NO:86), TRIM23 (SEQ ID NO:111), TRIM24 (SEQ ID NO:116), TRIM25 (SEQ ID NO:121), TRIM27 (SEQ ID NO:126), TRIM28 (SEQ ID NO:131), TRIM29 (SEQ ID NO:136), TRIM30, TRIM31 (SEQ ID NO:141), TRIM32 (SEQ ID NO:146), TRIM33 (SEQ ID NO:151), TRIM34 (SEQ ID NO:156), TRIM35 (SEQ ID NO:161), TRIM36 (SEQ ID NO:166), TRIM37 (SEQ ID NO:167), TRIM39 (SEQ ID NO:177), TRIM40 (SEQ ID NO:182), TRIM42 (SEQ ID NO:192), TRIM47 (SEQ ID NO:217), TRIM48 (SEQ ID NO:222), TRIM49 (SEQ ID NO:227), TRIM50 (SEQ ID NO:232), TRIM51 (SEQ ID NO:237), TRIM55 (SEQ ID NO:252), TRIM68 (SEQ ID NO:312), TRIM72 (SEQ ID NO:323), TRIM73 (SEQ ID NO:328), TRIM74 (SEQ ID NO:333) and TRIM76 (SEQ ID NO:343), a triglyceride, a diglyceride, a monoglyceride, a glycolated mono- or diacylglyceride, dolichol, polyenol, polyenal, very long chain fatty acids or a pharmaceutically acceptable salt thereof or a pharmaceutically acceptable salt thereof.

38. The composition according to any one of claims 35-37 wherein said composition includes at least one additional bioactive agent selected from the group consisting of pp242, rapamycin, envirolimus, everolimus, cidaforollimus, epigallocatechin gallate (EGCG), caffeine, curcumin, reseveratrol, digoxin, xylazine, hexetidine, sertindole and mixtures thereof.
39. The composition according to any one of claims 35-38 wherein said composition further comprises an antiviral agent.

40. The composition according to claim 36 wherein said siRNA is a siRNA according to any one of the sequences set forth in the table on pages 92-116 hereof or an oligonucleotide which contains plus or minus up to 5 nucleotide units upstream or downstream of any of said siRNA sequences.

41. The composition according to claim 40 wherein said siRNA is between 19 and 23 nucleotide units.

42. The method according to claim 23 or 26 wherein said siRNA is a siRNA according to any one of the sequences set forth in the table on pages 92-116 hereof or an oligonucleotide which contains plus/minus up to 5 nucleotide units upstream or downstream of any of said siRNA sequences.

43. The method according to claim 42 wherein said siRNA is between 19 and 23 nucleotide units.
FIGURE 1 IRGM (CONT'D)
FIGURE 3 PRECISION (CONT'D)

IFN-γ → S317 → ULK1 → TRIM20 → ATG16L1 → Autophagy

Flag-ATG16L1

Vector control

IP: GFP

WB:

Flag-ATG16L1

Flag-ATG16L1

GFP-TRIM20

Cells: HEK293

H

Input

ATG16L1

1-N-term

85-607

286-341

WD repeat

WD repeat

WD repeat

WD repeat

CDD

CDD

CDD

CDD

85

85

85

85

607

607

607

607

607

607

607

607

607

607

607

607

607
**Figure F**

<table>
<thead>
<tr>
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<th>HT-DNA</th>
<th>IFN-γ</th>
<th>Scr</th>
<th>siTRIM21</th>
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<td>IFN-β RNA (fold)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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</table>

**Figure G**

- Precision autophagy
- Inflammasome
- Type I IFN response

Diagram showing regulatory interactions involving TRIM20 and TRIM21.

(WO 2016/138236)

(Figure 8: Precision (Cont'd))
FIGURE S2 PRECISION (CONT'D)

A

GST-LC3A
GST-GABARAP

GST

Input (10%)

Wild type

Pull-down of

[35S] Myc-TRIM20

GST-LC3A
GST-GABARAP

Input (CBB)

B

Fluorescence

Intensity

GFP-LC3
mCherry-TRIM20

HeLa cells

Distance (µm)

Distance (µm)
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)

A61K 31/713, 31/00, 38/17, A61P 25/16, 25/28, 31/06, 35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>Y</td>
<td>WO 201 1/133879 A2 (UNIVERSITY OF MASSACHUSETTS et al.) 27.10.201 1, abstract, claims</td>
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Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier document but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "&" document member of the same patent family

Date of the actual completion of the international search
29 June 2016 (29.06.2016)

Date of mailing of the international search report
14 July 2016 (14.07.2016)

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Form PCT/ISA/210 (second sheet) (January 2015)
## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Uncheck box if not applicable. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Uncheck box if not applicable. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Check box if applicable. Claims Nos.: 8-16, 28, 30-34, 39 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Uncheck box if not applicable. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. Uncheck box if not applicable. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. Uncheck box if not applicable. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. Uncheck box if not applicable. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- [ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- [ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- [ ] No protest accompanied the payment of additional search fees.
INTERNATIONAL SEARCH REPORT
Classification of subject matter

A61K 31/713 (2006.01)
A61K 31/00 (2006.01)
A61P 25/16 (2006.01)
A61P 25/28 (2006.01)
A61P 31/06 (2006.01)
A61P 35/00 (2006.01)
A61K 38117 (2006.01)

Form PCT/ISA/210 (extra sheet) (January 2015)