(54) Title: MDM2 INHIBITOR RESPONSE PREDICTION METHOD

(57) Abstract: The present invention relates to MDM2 modulators in treatment of ER+ breast cancer characterized by decreased or abrogated function of GATA3.

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MDM2 INHIBITOR RESPONSE PREDICTION METHOD

The present invention relates to MDM2 modulators in treatment of cancer characterized by decreased or abrogated function of GATA3.

Background of the Invention

5   *GATA3* is one of the most frequently mutated genes in estrogen receptor (ER)-positive breast cancers and can be found mutated in 12% and 18% of primary and metastatic ER-positive breast cancers, respectively. GATA3 is a zinc-finger transcription factor with key functions in mammary epithelial cell differentiation. In the context of breast cancer, GATA3 suppresses epithelial-to-mesenchymal transition and acts as a pioneer factor during this process by recruiting others

10   cofactors such as ERα and FOXA1. GATA3 expression is strongly associated with ERα expression in breast cancer, and the loss of GATA3 expression, via *GATA3* somatic genetic alterations or other mechanisms, has been linked to poor prognosis in breast cancer patients. Importantly, targeted therapies directed at GATA3 deficiency are not available.

Based on the above-mentioned state of the art, the objective of the present invention is to provide

15   means and methods to treat cancer characterized by decreased or abrogated function of GATA3. This objective is attained by the subject-matter of the independent claims of the present specification.

Summary of the Invention

A first aspect of the invention relates to an agent (a pharmaceutical drug compound) for use in

20   treatment of a neoplasm, particularly a malignant neoplasm, wherein said neoplasm is characterized by decreased or abrogated function of GATA3. That agent is selected from:

a. a nucleic acid biopolymer capable of specifically suppressing expression of MDM2;

b. an MDM2 specific inhibitor, particularly an MDM2 inhibitor characterized by a molecular weight ≤700 Da,

25   c. a specific modulator of the MDM2-p53 interaction, particularly an inhibitor/antagonist, more particularly a specific modulator of the MDM2-p53 interaction characterized by a molecular weight ≤700 Da, or

d. a peptide specifically modulating the MDM2-p53 interaction, particularly an inhibitor/antagonist of that interaction.

30   A second aspect of the invention relates to a method for assigning a likelihood of responding to MDM2 modulator treatment to a patient, wherein

•   an expression and/or an enzymatic activity of GATA3 is determined in a sample of a neoplasm isolated from said patient and

- a high likelihood of responding to MDM2 modulator treatment is assigned if the sample of a neoplasm isolated from said patient is

    a. characterized by low expression of GATA3 or lack of expression of GATA3; and/or

    b. characterized by expression of a non-active GATA3 variant; and/or

    c. characterized by the presence of somatic genetic alterations in the *GATA3* gene, particularly a mutation, a copy number alteration or a genomic rearrangement;

particularly wherein the MDM2 modulator is selected from

    – a nucleic acid biopolymer capable of specifically suppressing expression of MDM2;

    – an MDM2 specific inhibitor, particularly an MDM2 inhibitor characterized by a molecular weight ≤700 Da,

    – a specific modulator of the MDM2-p53 interaction, particularly an inhibitor/antagonist, more particularly a specific modulator of the MDM2-p53 interaction characterized by a molecular weight ≤700 Da, or

    – a peptide specifically modulating the MDM2-p53 interaction, particularly an inhibitor/antagonist.

Likewise, the invention encompasses a system for determining the GATA3 status of a patient as a basis for making the assessment of likelihood of MDM2 modulator treatment response.

A third aspect of the invention relates to the agent for use in treatment or prevention of a neoplasm, particularly a malignant neoplasm, according to the first aspect, wherein a high likelihood of responding to MDM2 modulator treatment is assigned to said patient according to the method of the second aspect.

In another embodiment, the present invention relates to a pharmaceutical composition comprising at least one of the compounds of the present invention or a pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable carrier, diluent or excipient for use in treatment of a neoplasm.

Brief Description of the Figures

Fig. 1   *GATA3* and *MDM2* are synthetic lethal ER-positive breast cancer.

Fig. 2   GATA3 status determines response to MDM2 inhibitors *in vitro*.

Fig. 3   GATA3 expression determines response to MDM2 inhibitor *in vivo*.

Fig. 4   The synthetic lethality between GATA3 and MDM2 acts via the PI3K-Akt-mTOR signalling pathway.

Fig. 5   *GATA3* and *MDM2* are synthetic lethal ER-positive breast cancer.

Fig. 6.   *GATA3* and *MDM2* are synthetic lethal ER-positive breast cancer.

Fig. 7   *GATA3* and *MDM2* are synthetic lethal ER-positive breast cancer.

Fig. 8   GATA3 status determines response to MDM2 inhibitors *in vitro*.

Fig. 9   GATA3 status determines response to MDM2 inhibitors *in vitro*.

Fig. 10 GATA3 expression determines response to MDM2 inhibitor *in vivo*.

Fig. 11 The synthetic lethality between GATA3 and MDM2 acts via the PI3K-Akt-mTOR signalling pathway.

Fig. 12 The synthetic lethality between GATA3 and MDM2 acts via the PI3K-Akt-mTOR signalling pathway.

Fig. 13 The synthetic lethality between GATA3 and MDM2 acts via the PI3K-Akt-mTOR signalling pathway.

Detailed Description of the Invention

Terms and definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel et al., Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.) and chemical methods.

The term *MDM2* in the context of the present specification relates to the human MDM2 proto-oncogene, also known as the homolog of the mouse double minute 2, encoding for the E3 ubiquitin-protein ligase Mdm2.

The term GATA3 in the context of the present specification relates to the human gene GATA binding protein 3, encoding for the protein product Trans-activating-T-cell-specific transcription factor GATA3.

The TP53 in the context of the present specification relates to the human tumor suppressor gene Tumor Protein p53 (TP53), encoding for the protein product cellular tumor antigen p53.

The terms *gene expression* or *expression*, or alternatively the term *gene product,* may refer to either of, or both of, the processes - and products thereof - of generation of nucleic acids (RNA) or the generation of a peptide or polypeptide, also referred to transcription and translation, respectively, or any of the intermediate processes that regulate the processing of genetic information to yield polypeptide products. The term *gene expression* may also be applied to the transcription and processing of a RNA gene product, for example a regulatory RNA or a structural (e.g. ribosomal) RNA. If an expressed polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell. Expression may be assayed both on the level of transcription and translation, in other words mRNA and/or protein product.

The term *variant* refers to a polypeptide that differs from a reference polypeptide, but retains essential properties. A typical variant of a polypeptide differs in its primary amino acid sequence from another, reference polypeptide. Differences may be limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical.

5   Truncated variants refer to more substantial differences between the variant polypeptide and the reference polypeptide, such as a shorter version of the polypeptide is translated and an entire protein domain can be lost (e.g. DNA binding domain). A variant and reference polypeptide may differ in amino acid sequence by one or more modifications (e.g., substitutions, additions, and/or deletions). A substituted or inserted amino acid residue may or may not be one encoded by the

10   genetic code. A variant of a polypeptide may be naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. A *non-active variant* is a polypeptide, which does not retain the physiological function of the reference polypeptide inside a cell.

The term *siRNA* (small/short interfering RNA) in the context of the present specification relates to an RNA molecule capable of interfering with the expression (in other words: inhibiting or preventing

15   the expression) of a gene comprising a nucleic acid sequence complementary or hybridizing to the sequence of the siRNA in a process termed RNA interference. The term *siRNA* is meant to encompass both single stranded siRNA and double stranded siRNA. siRNA is usually characterized by a length of 17-24 nucleotides. Double stranded siRNA can be derived from longer double stranded RNA molecules (dsRNA). According to prevailing theory, the longer dsRNA is

20   cleaved by an endo-ribonuclease (called Dicer) to form double stranded siRNA. In a nucleoprotein complex (called RISC), the double stranded siRNA is unwound to form single stranded siRNA. RNA interference often works via binding of an siRNA molecule to the mRNA molecule having a complementary sequence, resulting in degradation of the mRNA. RNA interference is also possible by binding of an siRNA molecule to an intronic sequence of a pre-mRNA (an immature, non-spliced

25   mRNA) within the nucleus of a cell, resulting in degradation of the pre-mRNA.

The term *shRNA* (small hairpin RNA) in the context of the present specification relates to an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNA interference (RNAi).

The term *sgRNA* (single guide RNA) in the context of the present specification relates to an RNA

30   molecule capable of sequence-specific repression of gene expression via the CRISPR (clustered regularly interspaced short palindromic repeats) mechanism.

The term *miRNA* (microRNA) in the context of the present specification relates to a small non-coding RNA molecule (containing about 22 nucleotides) that functions in RNA silencing and post-transcriptional regulation of gene expression.

35   The term *Nucleotides* in the context of the present specification relates to nucleic acid or nucleic acid analogue building blocks, oligomers of which are capable of forming selective hybrids with RNA or DNA oligomers on the basis of base pairing. The term *nucleotides* in this context includes the classic ribonucleotide building blocks adenosine, guanosine, uridine (and ribosylthymine),

cytidine, the classic deoxyribonucleotides deoxyadenosine, deoxyguanosine, thymidine, deoxyuridine and deoxycytidine. It further includes analogues of nucleic acids such as phosphotioates, 2'O-methylphosphothioates, *peptide nucleic acids* (PNA; N-(2-aminoethyl)-glycine units linked by peptide linkage, with the nucleobase attached to the alpha-carbon of the glycine) or *locked nucleic acids* (LNA; 2'O, 4'C methylene bridged RNA building blocks). Wherever reference is made herein to a *hybridizing sequence*, such hybridizing sequence may be composed of any of the above nucleotides, or mixtures thereof.

The term *antisense oligonucleotide* in the context of the present specification relates to an oligonucleotide having a sequence substantially complimentary to, and capable of hybridizing to, an RNA. Antisense action on such RNA will lead to modulation, particular inhibition or suppression of the RNA's biological effect. If the RNA is an mRNA, expression of the resulting gene product is inhibited or suppressed. Antisense oligonucleotides can consist of DNA, RNA, nucleotide analogues and/or mixtures thereof. The skilled person is aware of a variety of commercial and non-commercial sources for computation of a theoretically optimal antisense sequence to a given target. Optimization can be performed both in terms of nucleobase sequence and in terms of backbone (ribo, deoxyribo, analogue) composition. Many sources exist for delivery of the actual physical oligonucleotide, which generally is synthesized by solid state synthesis.

The term *specific binding* in the context of the present invention refers to a property of ligands that bind to their target with a certain affinity and target specificity. The affinity of such a ligand is indicated by the dissociation constant of the ligand. A specifically reactive ligand has a dissociation constant of $\leq 10^{-7}$mol/L when binding to its target, but a dissociation constant at least three orders of magnitude higher in its interaction with a molecule having a globally similar chemical composition as the target, but a different three-dimensional structure.

A *polymer* of a given group of monomers is a homopolymer (made up of a multiple of the same monomer); a copolymer of a given selection of monomers is a heteropolymer constituted by monomers of at least two of the group.

As used herein, the term *pharmaceutical composition* refers to a compound of the invention, or a pharmaceutically acceptable salt thereof, together with at least one pharmaceutically acceptable carrier. In certain embodiments, the pharmaceutical composition according to the invention is provided in a form suitable for topical, parenteral or injectable administration.

As used herein, the term *pharmaceutically acceptable carrier* includes any solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (for example, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, and the like and combinations thereof, as would be known to those skilled in the art (see, for example, Remington: the Science and Practice of Pharmacy, ISBN 0857110624).

As used herein, the term *treating* or *treatment* of any disease or disorder (e.g. cancer) refers in one embodiment, to ameliorating the disease or disorder (e.g. slowing or arresting or reducing the

development of the disease or at least one of the clinical symptoms thereof). In another embodiment "treating" or "treatment" refers to alleviating or ameliorating at least one physical parameter including those which may not be discernible by the patient. In yet another embodiment, "treating" or "treatment" refers to modulating the disease or disorder, either physically, (e.g., stabilization of a discernible symptom), physiologically, (e.g., stabilization of a physical parameter), or both. Methods for assessing treatment and/or prevention of disease are generally known in the art, unless specifically described hereinbelow.

A first aspect of the invention relates to an agent for use in treatment of a neoplasm, particularly a malignant neoplasm, wherein said neoplasm is characterized by decreased or abrogated function of GATA3 (particularly a non-functional *GATA3* mutant or lack of expression of GATA3).

In certain embodiments of this first aspect of the invention, the agent is a nucleic acid biopolymer capable of specifically suppressing expression of MDM2. Such nucleic acid biopolymer can be, for example, an antisense oligonucleotide designed to specifically bind to MDM2 mRNA and inhibit expression of its message.

In certain embodiments of this first aspect of the invention, the agent is an MDM2 specific inhibitor, particularly an MDM2 inhibitor pharmaceutical drug molecule characterized by a molecular weight ≤700 Da.
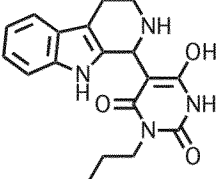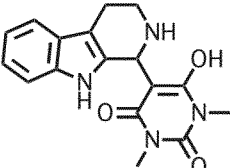
In certain embodiments of this first aspect of the invention, the agent is a specific modulator of the MDM2-p53 interaction, particularly an inhibitor/antagonist, more particularly a specific modulator of the MDM2-p53 interaction characterized by a molecular weight ≤700 Da.

In yet other certain embodiments of this first aspect of the invention, the agent is a peptide specifically modulating the MDM2-p53 interaction, particularly an inhibitor/antagonist of such interaction, wherein said agent is able to reduce the amount of functional MDM2 inside a cell.

In certain embodiments, the decreased or abrogated function of GATA3 is determined via sequencing of the *GATA3* gene, quantification of *GATA3* mRNA, particularly quantification via qRT-PCR, or evaluation of GATA3 protein expression, particularly by immunohistochemistry. Mutations in the *GATA3* gene affecting residues essential for GATA3 function or frameshift mutations leading to a truncated GATA3 may be detected via sequencing of the *GATA3* gene.

In certain embodiments, the nucleic acid biopolymer capable of specifically suppressing expression of MDM2 is selected from an siRNA, an miRNA, an antisense oligonucleotide, an shRNA, and an sgRNA.

In certain embodiments, the MDM2 specific inhibitor is selected from MEL23 and MEL24. MEL23

( ) and MEL24 ( ) are described in detail in Herman et al. (Cancer discovery 2011. 1(4); 312–25.).

In certain embodiments, the specific modulator of the MDM2-p53 interaction is a nutlin. A nutlin is a small molecule, a cis-imidazole analogue, which occupies the p53 binding pocket of MDM2. The nutlin thereby disrupts the MDM2-p53 interaction and this leads to stabilization of p53. Any nutlin is encompassed by the present invention.

In certain embodiments, the specific modulator of the MDM2-p53 interaction is selected from HLI373, NSC 66811, Nutlin-3, Nutlin-3a, RITA, SKPin C1, SMER 3, SP141, SZL P1-41, TAME hydrochloride, Thalidomide, VH 298, YH 239-EE, proTAME, Lenalidomide, MZ 1, TL 12-186, cis MZ 1, dBET1, AT 1, NSC 66811, HLI 373, NAB 2, GS 143, cis VH 298, CM 11, CMP 98, TL 13-27, Idisulam, Indasanutlin, CRBN-6-5-5-VHL, RITA NSC 652287, Tenovin-1, AT 406 (SM-406), Avadomide (CC-122), NSC 207895, JNJ-26854165 (Serdemetan), Iberdomide(CC220), CGM097, HDM201, SAR405838, RG7112, MI-77301, MI-888, MK-8242, AMG232, DS3032b, ALRN-6924 and idasanutlin (RG7388).

In certain embodiments, the specific modulator of the MDM2-p53 interaction is idasanutlin.

In certain embodiments, the peptide specifically modulating the MDM2-p53 interaction is selected from ATSP-7041, M06, SAH-p53-8, sMTide-02/02A, and ATSP-7041.

In certain embodiments, the neoplasm is characterized by expression of functional p53.

Assay of p53 function

The assay for determining the function of p53 can be a flow-cytometry-based assay described in detail in Le Garff-Tavernier et al. (Blood Cancer J. 2011 Feb; 1(2): e5). In brief, staining of p53 is accomplished by a fluorescent antibody and cells expressing p53 are detected using flow cytometry. Functionality of p53 inside the cell is assessed using etoposide and nutlin-3a. The so-called type 1 profile corresponds to normal p53 function, wherein baseline expression of p53 is undetectable, but with etoposide and nutlin-3a stimulation, a clear increase of p53 expression and its transcriptional target p21 at day 1 is observed.

In certain embodiments, the neoplasm is a neoplasm of breast tissue, urothelial tissue, stomach, uterus, ovaries, or lung, or the neoplasm is melanoma, or diffuse large B-cell lymphoma. For neoplasms of breast tissue, urothelial tissue, stomach, uterus, ovaries, or lung, and for melanoma, or diffuse large B-cell lymphoma, it is known from literature that GATA3 loss-of-function may occur. In certain embodiments, the neoplasm is a neoplasm of breast tissue. In certain embodiments, the neoplasm is an estrogen receptor (ER) positive neoplasm of breast tissue.

A second aspect of the invention relates to a method for assigning a likelihood of responding to MDM2 modulator treatment to a patient, wherein

- an expression and/or an enzymatic activity of GATA3 is determined in a sample of a neoplasm isolated from said patient; and

- a high likelihood of responding to MDM2 modulator treatment is assigned if the sample of a neoplasm isolated from said patient is

a. characterized by low expression of GATA3 or lack of expression of GATA3; and/or

b. characterized by expression of a non-active GATA3 variant; and/or

c. characterized by the presence of somatic genetic alterations in the *GATA3* gene, particularly a mutation, a copy number alteration or a genomic rearrangement.

In certain particular embodiments, the MDM2 modulator is selected from

a. a nucleic acid biopolymer capable of specifically suppressing expression of MDM2;

b. an MDM2 specific inhibitor, particularly an MDM2 inhibitor characterized by a molecular weight ≤700 Da,

c. a specific modulator of the MDM2-p53 interaction, particularly an inhibitor/antagonist, more particularly a specific modulator of the MDM2-p53 interaction characterized by a molecular weight ≤700 Da, or

d. a peptide specifically modulating the MDM2-p53 interaction, particularly an inhibitor/antagonist.

In the context of the present invention, low expression of GATA3 is a reduction of at least 30%, particularly at least 40%, more particularly at least 50% in relation to the amount of GATA3 protein in healthy cells of the same tissue.

In the context of the present invention, a non-active GATA3 variant is a GATA3 protein with a diminished or abrogated function.

A somatic genetic alteration in the *GATA3* gene can consist of, but is not limited to a mutation, a copy number alteration and/or a genomic rearrangement. A frameshift mutation is encompassed by the term mutation. Frameshift mutations may result in truncated forms of GATA3 that e.g. lose the DNA binding domain.

In certain embodiments, the method according to the second aspect comprises additionally the following steps:

• an expression and/or a functionality of p53 is determined in said sample of a neoplasm isolated from said patient; and

• a high likelihood of responding to MDM2 modulator treatment is assigned if the sample of a neoplasm isolated from said patient is additionally characterized by expression of functional p53.

An assay for assessing the function of p53 is mentioned above.

A third aspect of the invention relates to the agent for use in treatment or prevention of a neoplasm, particularly a malignant neoplasm, according to the first aspect, wherein a high likelihood of responding to MDM2 modulator treatment is assigned to said patient according to the method of the second aspect.

MDM2 modulators (nutlins)

MDM2 modulators according to the invention include, but are not limited to,

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- 5-[[3-Dimethylamino)propyl]amino]-3,10-dimethylpyrimido[4,5-b]quinoline-2,4(3H,10H)-dione dihydrochloride (HLI373);

- 4-[[[(2R,3S,4R,5S)-3-(3-Chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)-2-pyrrolidinyl]carbonyl]amino]-3-methoxybenzoic acid (Idasanutlin);

- 2-Methyl-7-[phenyl(phenylamino)methyl]-8-quinolinol (NSC 66811);

- (±)-4-[4,5-Bis(4-chlorophenyl)-2-(2-isopropoxy-4-methoxy-phenyl)-4,5-dihydro-imidazole-1-carbonyl]-piperazin-2-one (Nutlin-3);

- 4-[[(4S,5R)-4,5-Bis(4-chlorophenyl)-4,5-dihydro-2-[4-methoxy-2-(1-methylethoxy)phenyl]-1H-imidazol-1-yl]carbonyl]-2-piperazinone (Nutlin-3a);

- 5,5'-(2,5-Furandiyl)bis-2-thiophenemethanol (RITA);

- 2-[4-Bromo-2-[[4-oxo-3-(3-pyridinylmethyl)-2-thioxo-5 thiazolidinylidene]methyl]phenoxy]acetic acid (SKPin C1);

- 9H-Indeno[1,2-e][1,2,5]oxadiazolo[3,4-b]pyrazin-9-one (SMER 3);

- 6-Methoxy-1-(1-naphthalenyl)-9H-pyrido[3,4-b]indole (SP141);

- 3-(2-Benzothiazolyl)-6-ethyl-7-hydroxy-8-(1-piperidinylmethyl)-4H-1-benzopyran-4-one (SZL P1-41);

- N^2-[(4-Methylphenyl)sulfonyl]-L-arginine methyl ester hydrochloride (TAME hydrochloride);

- N-(2,6-dioxo-3-piperidinyl)phthalimide (Thalidomide);

- (2S,4R)-1-((S)-2-(1-cyanocyclopropanecarboxamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (VH 298);

- Ethyl 3-[2-(tert-butylamino)-1-[N-(4-chlorobenzyl)formamido]-2-oxoethyl]-6-chloro-1H-indole-2-carboxylate (YH 239-EE);

- (2S)-2-[[(4-methylphenyl)sulfonyl]amino]-9,13-dioxo-14-phenyl-7-[[[[(2-phenylacetyl)oxy]methoxy]carbonyl]amino]-10,12-dioxa-6,8-diazatetradec-6-enoic acid,methylester (proTAME);

- 3-(4-Amino-1,3-dihydro-1-oxo-2H-isoindol-2-yl)-2,6-piperidinedione (Lenalidomide);

- (2S,4R)-1-((S)-2-(tert-butyl)-17-((S)-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)-4,16-dioxo-6,9,12-trioxa-3,15-diazaheptadecanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (MZ 1);

- N-(2-(2-(2-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)phenyl)piperazin-1-yl)ethoxy)ethoxy)ethyl)-2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)acetamide (TL 12-186);

- (2S,4S)-1-((S)-2-(tert-butyl)-17-((S)-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)-4,16-dioxo-6,9,12-trioxa-3,15-

diazaheptadecanoyl)-4-hydroxy-*N*-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (cis MZ 1);

- (6*S*)-4-(4-Chlorophenyl)-*N*-[4-[[2-[[2-(2,6-dioxo-3-piperidinyl)-2,3-dihydro-1,3-dioxo-1*H*-isoindol-4-yl]oxy]acetyl]amino]butyl]-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepine-6-acetamide (dBET1);

- (2*S*,4*R*)-1-(2*R*)-2-Acetamido-3-[[6-[2-[(6*S*)-4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl]acetamido]hexyl]thio]-3-methylbutanoyl]-4-hydroxy-*N*-[4-(4-methylthiazol-5-yl)benzyl)pyrrolidinine-2-carboxamide (AT 1);

- 2-Methyl-7-[phenyl(phenylamino)methyl]-8-quinolinol (NSC 66811);

- 5-[[3-Dimethylamino)propyl]amino]-3,10-dimethylpyrimido[4,5-*b*]quinoline-2,4(3*H*,10*H*)-dione dihydrochloride (HLI 373);

- *N*-[(2-Chlorophenyl)methyl]-1-(2,5-dimethylphenyl)-1*H*-benzimidazole-5-carboxamide (NAB 2);

- 4-[4-[[5-(2-Fluorophenyl)-2-furanyl]methylene]-4,5-dihydro-5-oxo-3-(phenylmethyl)-1*H*-pyrazol-1-yl]benzoic acid (GS 143);

- (2*S*,4*S*)-1-((*S*)-2-(1-cyanocyclopropanecarboxamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (*cis* VH 298);

- N^1,N^{20}-bis((*S*)-1-((2*S*,4*R*)-4-Hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)-3,6,9,12,15,18-hexaoxaicosanediamide (CM 11);

- N^1,N^{20}-bis((*S*)-1-((2*S*,4*S*)-4-Hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)-3,6,9,12,15,18-hexaoxaicosanediamide (CMP 98);

- *N*-(2-(2-(2-(4-(4-((5-Chloro-4-((2-(isopropylsulfonyl)phenyl)amino)pyrimidin-2-yl)amino)phenyl)piperazin-1-yl)ethoxy)ethoxy)ethyl)-2-((1,3-dioxo-2-(2-oxopiperidin-3-yl)isoindolin-4-yl)amino)acetamide (TL 13-27);

- N^1-(3-Chloro-1*H*-indol-7-yl)-1,4-benzenedisulfonamide (Idisulam);

- 4-[[[(2*R*,3*S*,4*R*,5*S*)-3-(3-Chloro-2-fluorophenyl)-4-(4-chloro-2-fluorophenyl)-4-cyano-5-(2,2-dimethylpropyl)-2-pyrrolidinyl]carbonyl]amino]-3-methoxybenzoic acid (Indasanutlin);

- (2*S*,4*R*)-1-((2*S*)-2-(5-((5-((6-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)hexyl)oxy)pentyl)oxy)pentanamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (CRBN-6-5-5-VHL);

- 2-Thiophenemethanol, 5,5'-(2,5-furandiyl)bis- (RITA NSC 652287);

- Benzamide, N-[[[4-(acetylamino)phenyl]amino]thioxomethyl]-4-(1,1-dimethylethyl)-(Tenovin-1);

- (5S,8S,10aR)-N-benzhydryl-5-((S)-2-(methylamino)propanamido)-3-(3-methylbutanoyl)-6-oxo- decahydropyrrolo[1,2-a][1,5]diazocine-8-carboxamide (AT 406 (SM-406));
- 2,6-Piperidinedione, 3-(5-amino-2-methyl-4-oxo-3(4H)-quinazolinyl)- (Avadomide (CC-122));
- 2,1,3-Benzoxadiazole, 4-(4-methyl-1-piperazinyl)-7-nitro-, 3-oxide (NSC 207895);
- N1-(2-(1H-indol-3-yl)ethyl)-N4-(pyridin-4-yl)benzene-1,4-diamine (JNJ-26854165 (Serdemetan));
- 2,6-Piperidinedione, 3-[1,3-dihydro-4-[[4-(4-morpholinylmethyl)phenyl]methoxy]-1-oxo-2H-isoindol-2-yl]-, (3S)- (Iberdomide(CC220)).

The skilled person is aware that any specifically mentioned drug provided for use in treatment of a neoplasm according to the invention may be present as a pharmaceutically acceptable salt of said drug. Pharmaceutically acceptable salts comprise the ionized drug and an oppositely charged counterion. Non-limiting examples of pharmaceutically acceptable anionic salt forms include acetate, benzoate, besylate, bitatrate, bromide, carbonate, chloride, citrate, edetate, edisylate, embonate, estolate, fumarate, gluceptate, gluconate, hydrobromide, hydrochloride, iodide, lactate, lactobionate, malate, maleate, mandelate, mesylate, methyl bromide, methyl sulfate, mucate, napsylate, nitrate, pamoate, phosphate, diphosphate, salicylate, disalicylate, stearate, succinate, sulfate, tartrate, tosylate, triethiodide and valerate. Non-limiting examples of pharmaceutically acceptable cationic salt forms include aluminium, benzathine, calcium, ethylene diamine, lysine, magnesium, meglumine, potassium, procaine, sodium, tromethamine and zinc.

Dosage forms may be for oral administration. Alternatively, parenteral administration may be used, such as subcutaneous, intravenous, intrahepatic or intramuscular injection forms. Optionally, a pharmaceutically acceptable carrier and/or excipient may be present.

Pharmaceutical Composition and Administration

Another aspect of the invention relates to a pharmaceutical composition for use in treatment of cancer comprising a compound of the present invention, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier. In further embodiments, the composition comprises at least two pharmaceutically acceptable carriers, such as those described herein.

In certain embodiments of the invention, the compound of the present invention is typically formulated into pharmaceutical dosage forms to provide an easily controllable dosage of the drug and to give the patient an elegant and easily handleable product.

The pharmaceutical composition can be formulated for oral administration, parenteral administration, or rectal administration. In addition, the pharmaceutical compositions of the present invention can be made up in a solid form (including without limitation capsules, tablets, pills, granules, powders or suppositories), or in a liquid form (including without limitation solutions, suspensions or emulsions).

In certain embodiments, the pharmaceutical composition or combination of the present invention can be in unit dosage of about 1-1000 mg of active ingredient(s) for a subject of about 50-70 kg. The therapeutically effective dosage of a compound, the pharmaceutical composition, or the combinations thereof, is dependent on the species of the subject, the body weight, age and individual condition, the disorder or disease or the severity thereof being treated. A physician, clinician or veterinarian of ordinary skill can readily determine the effective amount of each of the active ingredients necessary to prevent, treat or inhibit the progress of the disorder or disease.

The pharmaceutical compositions of the present invention can be subjected to conventional pharmaceutical operations such as sterilization and/or can contain conventional inert diluents, lubricating agents, or buffering agents, as well as adjuvants, such as preservatives, stabilizers, wetting agents, emulsifiers and buffers, etc. They may be produced by standard processes, for instance by conventional mixing, granulating, dissolving or lyophilizing processes. Many such procedures and methods for preparing pharmaceutical compositions are known in the art, see for example L. Lachman et al. The Theory and Practice of Industrial Pharmacy, 4th Ed, 2013 (ISBN 8123922892).

Wherever alternatives for single separable features such as, for example, a ligand type or a medical indication are laid out herein as "embodiments", it is to be understood that such alternatives may be combined freely to form discrete embodiments of the invention disclosed herein. Thus, any of the alternative embodiments for a ligand type may be combined with any of the alternative embodiments of a medical indication mentioned herein.

The invention is further illustrated by the following examples and figures, from which further embodiments and advantages can be drawn. These examples are meant to illustrate the invention but not to limit its scope.

Description of the Figures

Fig. 1   **(a)** Schematic representation of the project DRIVE shRNA screening data used to identify synthetic lethal interactors of _GATA3_. **(b)** SLIdR-derived statistical significance (-log10(P)) plotted against the difference in the mean viability scores between _GATA3_-mutant and _GATA3_-wild type breast cancer cell lines for each gene knocked-down in the shRNA screen. Middle lines of the boxplots indicate medians. Box limits are first and third quartiles. The whiskers extend to the range. **(c)** Viability scores of _MDM2_ knock-down in _GATA3_-mutant and _GATA3_-wild type cell lines. **(d-f)** Proliferation kinetics of **(d)** _GATA3_-mutant MCF-7 cells transfected with siRNA targeting _MDM2_ (gray) or control (black), **(e)** _GATA3_-wild type BT-474 cells and **(f)** _GATA3_-wild type MDA-MB134 cells transfected with siRNA targeting _GATA3_ (blue), _MDM2_ (gray), _GATA3 and MDM2_ (red) or siRNA control (black). **(g)** Apoptosis assay using Annexin V and propidium iodide co-staining. From the left: gating strategy to define apoptotic (yellow) and live (blue) cells; the percentage of apoptotic and live cells upon _MDM2_ silencing in MCF-7 cells; upon

silencing of *GATA3* or *MDM2* alone or in combination in BT-474 and MDA-MB134 cells. **(h)** Doughnut chart showing *GATA3* and *TP53* mutations in ER-positive breast cancer patients (Hoadley, K. A. *et al. Cell* **173**, 291–304.e6, 2018), Pereira, B. et al. Nat. Commun. 7, 11479, 2016). **(i)** Proliferation kinetics of *TP53*-mutant T-47D cells
5   transfected with siRNA targeting *GATA3* (blue), *MDM2* (grey), *GATA3 and MDM2* (red) or siRNA control (black). **(j)** Percentage of apoptotic and live cells upon silencing of *GATA3* and *MDM2* alone or in combination in T-47D cells. Data are mean ± s.d. **(d,e,f,g,i,j)** n ≥4 replicates. Statistical significance was determined for **(d,e,f,g,i,j)** by the two-tailed unpaired Student's t-test and for **(h)** by one-sided Fisher's Exact.

10   Fig. 2   **(a,d)** Proliferation kinetics of **(a)** *GATA3*-mutant MCF-7 cell line under increasing dosage of idasanutlin, **(d)** BT-474 cells upon *GATA3* silencing and/or treatment with 12.5µM idasanutlin. **(b,f)** Apoptosis assay using Annexin V and propidium iodide co-staining to measure the percentage of apoptotic cells **(Fig. 1g)** (b) upon increasing dosage of idasanutlin in MCF-7 cells, **(f)** upon *GATA3* silencing and treatment with 12.5µM
15   idasanutlin in BT-474 cells. **(c,g)** Immunoblot showing pro- and anti-apoptotic proteins (c) at 6, 12 and 24 hours post-treatment with DMSO, 12.5µM idasanutlin and 25µM idasanutlin in MCF-7 cells, (g) at 12 and 24 hours post-treatment with DMSO or 12.5µM idasanutlin in BT-474 cells transfected with *GATA3* siRNA or control siRNA. For all the western blots, quantification is relative to the loading control (actin) and normalized to the
20   corresponding DMSO control. Data are mean ± s.d. **(a,b,d,e,f)** n≥3 replicates. Statistical significance was determined for **(a,b,d,e,f)** by the two-tailed unpaired Student's t-test.

     Fig. 3   **(a)** Schematic representation of the zebrafish xenotransplantation assay. **(b)** Barplot shows the percentages of fish that harboured (grey) or did not harbour (blue) tumours upon transplantation with *GATA3*-silenced or control BT-474 cells pre-treated with
25   idasanutlin or DMSO. In total, 70-100 embryos were analyzed per group over two independent experiments. **(c)** Representative confocal images of tumour formation in zebrafish injected with fluorescent tracker-labelled BT-474 cells with *GATA3* siRNA or control siRNA, pretreated with idasanutlin or DMSO. **(d)** FACS analysis showing the percentage of red-tracker labelled tumour cells extracted from the embryos. Error bars
30   represent, in total, three replicates performed over two independent experiments. Each replicate represents the pooled lysate of 20-30 fish for each condition. **(e)** Schematic illustration of the CAM assay. **(f)** Photographs of *GATA3*-silenced or control BT-474 cells pre-treated with DMSO or idasanutlin implanted in CAMs and grown for 4 days post-implantation. **(g)** Volume of tumours derived from the CAM experiment (n ≥10 tumours
35   from 3 independent experiments). Values are normalized to the mean of siCTR DMSO. **(h)** Representative micrographs of BT-474 tumours extracted 4 days post-implantation. Tumoural cells (upper panel) were immunostained with GATA3 (middle panel) and the

apoptotic marker Cl. Caspase 3 (lower panel) in the different treatment conditions. Data are mean ± SEM **(d, g)** n≥4 replicates. Scale bars: 500µm **(c)**, 1cm **(f)** and 50 and 100µM **(h)**. Statistical significance was determined for **(b)** by two-sided Fisher's Exact and for **(d,g)** by the two-tailed unpaired Student's t-test.

Fig. 4   **(a)** Schematic representation of the RNA-seq experimental setup to identify gene expression changes induced by concurrent *GATA3* loss and *MDM2* inhibition. Venn diagram shows the number of pathways enriched in both MCF-7 and MDA-MB134. **(b)** Normalised enrichment scores of significantly up- and down-regulated pathways in both MCF-7 (blue) and MDA-MB134 (red). Size of the dots is proportional to the adjusted p-value as indicated in the legend. **(c,d)** Immunoblot showing markers of mTOR signalling pathway activation at 24, 48 and 72 hours post-siRNA transfection in **(c)** MCF-7 cells upon *MDM2* silencing and **(d)** BT-474 cells upon *GATA3* and/or *MDM2* silencing. **(e)** Representative immunohistochemistry micrographs of phospho-S6 stainings in BT-474 tumours extracted 4 days post-implantation in the CAM model. **(f)** Schematic representation of the mechanistic hypothesis. Scale bar **(e)** 50 and 100µM. Statistical significance was determined for **(b)** by *fgsea* (Sergushichev, A. A, BioRxiv, June 20, 2016).

Fig. 5   **(a)** Immunoblot showing *GATA3*-mutant, *GATA3*-wild type and MDM2 protein level of expression in MCF-7 cells at 72 hours post-siRNA transfection (upper panel). *MDM2* mRNA level of expression (relative expression to *GAPDH*) in MCF-7 cells at 24, 48 and 72 hours post-siRNA transfection (bottom panel). **(b)** Immunoblot showing MDM2 protein level of expression at 72 hours post-siRNA transfection (upper panel) and *MDM2* mRNA levels at 48 hours post-siRNA transfection (bottom panel) after transfection with different concentrations of siRNA (6.25nM, 12.5nM or 25nM) in MCF-7 cells. **(c)** Proliferation kinetics of MCF-7 cells transfected with *MDM2* siRNA at different concentrations. **(d)** Flow cytometry analysis of Annexin V and propidium iodide co-staining to measure the percentage of apoptotic cells (AnnV+) and live cells (AnnV-/PI-) upon *MDM2* silencing with different concentrations of siRNA in MCF-7 cells. Data are mean ± s.d. **(a,b,c,d)** n≥2 replicates. Statistical significance was determined for **(a,b,c,d)** by the two-tailed unpaired Student's t-test.

Fig. 6   **(a,b)** *MDM2* and *GATA3* mRNA level of expression (relative expression to *GAPDH*) in **(a)** BT-474 and **(b)** MDA-MB134 cells at 24, 48 and 72 hours post-siRNA transfection (left panel). Immunoblot showing *MDM2* and *GATA3* protein level of expression in **(a)** BT-474 and **(b)** MDA-MB134 cells 72 hours post-siRNA transfection (right panel). Data are mean ± s.d. **(a, b)** n ≥2 replicates. Statistical significance was determined for **(a,b)** by the two-tailed unpaired Student's t-test.

Fig. 7   **(a)** *MDM2* and *GATA3* mRNA level of expression (relative expression to *GAPDH*) in T-47D at 24, 48 and 72 hours post siRNA transfection (left panel). Immunoblot showing *MDM2* and *GATA3* protein level of expression in T-47D cells 72 hours post-siRNA transfection (right panel). **(b)** *MDM2* and *TP53* mRNA level of expression (relative to expression of *GAPDH*) in MCF-7 cells 48 hours post-siRNA transfection (left panel). Immunoblot showing MDM2 and p53 protein level of expression in MCF-7 cells 72 hours post-siRNA transfection (right panel). **(c)** Proliferation kinetics of *GATA3*-mutant MCF-7 cells transfected with siRNA targeting *MDM2* (grey), *TP53* (yellow), *TP53 and MDM2* (red) or siRNA control (black). **(d)** Flow cytometry analysis of Annexin V and propidium iodide co-staining to measure the percentage of apoptotic cells (AnnV+) and live cells (AnnV-/PI-) upon silencing of *MDM2* and *TP53* alone or in combination in MCF-7 cells. Data are mean ± s.d. **(a,b,c,d)** n≥2 replicates. Statistical significance was determined for **(a,b,c,d)** by the two-tailed unpaired Student's t-test.

Fig. 8   **(a)** Log-dose response curve of idasanutlin in MCF-7 cells. **(b)** Effect of *GATA3* silencing on proliferation upon treatment with DMSO or idasanutlin 12.5 µM in MDA-MB134 cells. **(c)** log-dose response curve of Idasanutlin in MDA-MB134 cells transfected with control siRNA or *GATA3* siRNA. **(e)** Percentage of apoptotic cells upon *GATA3* silencing and Idasanutlin treatment (12.5 µM) in MDA-MB134 cells. **(e)** Immunoblot showing pro- and anti-apoptotic proteins at 12 and 24 hours post-treatment with DMSO or Idasanutlin 12.5 µM in MDA-MB134 cells transfected with control siRNAs or GATA3 siRNAs. For all the western blots, quantification is relative to the loading control (actin) and normalized to the corresponding DMSO control. Data are mean ± s.d. **(a,b,c,d,e,g)** n≥3 replicates. Statistical significance was determined for **(c,e,g)** by the two-tailed unpaired Student's t-test.

Fig. 9   **(a)** Effect of *TP53* silencing on proliferation upon treatment with idasanutlin (12.5 µM) or DMSO in MCF-7 cells. **(b)** Percentage of apoptotic and live cells upon *TP53* silencing and idasanutlin treatment (12.5 µM) in MCF-7 cells. **(c)** Immunoblot showing MDM2 and p53 protein levels 24 hours post-treatment with DMSO or idasanutlin (12.5 µM) in MCF-7 cells transfected with control siRNAs or *TP53* siRNAs. **(d)** mRNAs levels of *BCL2* and *BAX* in control and *TP53*-silenced MCF-7 cells at 12 and 24 hours post-treatment. Data are mean ± s.d. **(a,b,d)** n≥3 replicates. Statistical significance was determined for **(a,b,d)** by the two-tailed unpaired Student's t-test.

Fig. 10   Representative micrographs of BT-474 tumours extracted 4 days post-implantation. Tumoural cells (hematoxylin/eosin; upper panel) were immunostained with the apoptotic marker Cl. Caspase 3 (lower panel) in the different treatment conditions. Scale bars 100 and 50µM.

Fig. 11   **(a)** Immunoblot showing MDM2 protein expression in MCF-7 (left) and MDM2, GATA3, p53, PARP and cleaved PARP protein expression in BT-474 cells (right). **(b)** Immunoblot showing markers of mTOR signalling pathway activation at 48 hours post siRNA transfection and 24 hours post-treatment with DMSO or Idasanutlin in BT-474 cells. Note that this immunoblot was performed using the same membrane as the immunoblot for 24hrs in Fig. 2g. **(c)** Representative immunohistochemistry phospho-Akt staining in BT-474 tumours extracted 4 days post-implantation. For all the western blots, quantification is relative to the loading control (actin) and normalized to the corresponding siCTR control or DMSO control. Scale bars 100 and 50μM.

Fig. 12   Doughnut charts showing the mutual exclusivity between *GATA3* and *PI3KCA* **(a)** and *GATA3* and *PTEN* **(b)** genetic alterations in ER-positive breast cancer patients. Statistical significance was determined for **(a, b)** by one-sided Fisher's Exact.

Fig. 13   Normalised enrichment scores of significantly up- and down-regulated pathways identified by gene set enrichment analysis **(a)** in ER-positive breast cancers with low *GATA3* versus high *GATA3* expression and **(b)** in ER-positive breast cancers with *GATA3*-mutant versus *GATA3* wild-type. Statistical significance was determined for **(a, b)** by *fgsea* (Sergushichev, A. A, BioRxiv, June 20, 2016).

Examples

Example 1: GATA3 and MDM2 are synthetic lethal in ER-positive breast cancer

To identify synthetically lethal vulnerabilities of *GATA3* in breast cancer, the authors analysed the breast cancer cell line (n=22) data from the large-scale, deep RNAi screen project DRIVE (McDonald, E. R., 3rd et al. Cell 170, 577–592.e10, 2017) (**Fig. 1a**) using our recently developed SLIdR (Synthetic Lethal Identification in R) algorithm (Srivatsa, S. *et al.* BioRxiv, 2019). SLIdR uses rank-based statistical tests to compare the viability scores for each gene knock-down between the *GATA3*-mutant and *GATA3*-wild type cell lines and identified *MDM2* as the top gene whose knock-down significantly reduced cell viability in the two *GATA3*-mutant breast cancer cell lines (**Fig. 1b-c**). *MDM2* encodes an E3 ubiquitin ligase that inhibits the tumour suppressor p53-mediated transcriptional activation and is frequently amplified and overexpressed in human cancers, including breast.

The authors first sought to validate the predicted synthetic lethality between *GATA3* and *MDM2* in the ER-positive breast cancer cell line MCF-7, one of the two *GATA3*-mutant cell lines used in the RNAi screen (McDonald, E. R., 3rd *et al. Cell* **170**, 577–592.e10, 2017). MCF-7 cells harbour the *GATA3* frameshift mutation p.D335Gfs (Barretina, J. et al. Nature 483, 603–607, 2012), a loss-of-function mutation that has been recurrently observed in breast cancer patients, and leads to a truncated GATA3 protein. Using a siRNA approach, we confirmed that silencing *MDM2*

significantly reduced cell proliferation in MCF-7 cells (**Fig. 1d** and **Fig. 5a**). *MDM2* siRNA titration analysis showed that the vulnerability induced by *MDM2* inhibition in MCF-7 cells was dose-dependent and that 50% reduction in *MDM2* expression is sufficient to inhibit proliferation in the presence of *GATA3* mutation (**Fig. 5b-c**).

5   To confirm that the effect of *MDM2* silencing is unequivocally related to GATA3 loss of function and to exclude any gain-of-function effects of the *GATA3* mutation, the authors assessed the changes in cell proliferation upon single- and dual-silencing of *GATA3* and *MDM2* using siRNA in two ER-positive *GATA3*-wild type breast cancer cell lines, the luminal A cell line MDA-MB134 and the luminal B cell line BT-474 (**Fig. 6**). Consistent with the oncosuppressor role of *GATA3* in breast
10   cancer, silencing *GATA3* led to a significant increase in cell proliferation in both BT-474 and MDA-MB134 (**Fig. 1e-f**). By contrast, dual-silencing of *GATA3* and *MDM2* significantly reduced cell proliferation compared to cells transfected with control siRNA, *GATA3* siRNA or *MDM2* siRNA alone (**Fig. 1e-f**).

To determine if *MDM2* silencing was merely inhibiting cell growth or was inducing cell death, the
15   authors assessed apoptosis using Annexin V and propidium iodide co-staining followed by flow cytometry analysis. They observed that *MDM2* silencing significantly induced apoptosis in MCF-7 cells in a dose-dependent manner (**Fig. 1g and Fig. 5d**). Similarly, dual-*GATA3*/*MDM2* silencing in BT-474 and MDA-MB134 cells led to 15-20% higher proportion of apoptotic cells than the silencing of the two genes individually (**Fig. 1g**), indicating that dual inhibition induced increased
20   apoptosis. The authors results provide evidence that MDM2 is a selected vulnerability in breast cancer with *GATA3*-mutant and/or loss of *GATA3*.

MDM2 plays a central role in the regulation of p53 and they regulate each other in a complex regulatory feedback loop. In ER-positive breast cancer, *GATA3* and *TP53* mutations are mutually exclusive (**Fig. 1h**). The authors, therefore, hypothesized that the synthetic lethal effects between
25   *GATA3* and *MDM2* may be p53-dependent. To test this hypothesis, they assessed cell growth and apoptosis in the ER-positive, *GATA3*-wild-type, *TP53*-mutant (p.L194F) T-47D breast cancer cell line. Consistent with the mutual exclusivity of *GATA3* and *TP53* mutations in patients, *GATA3* silencing in a *TP53*-mutant context resulted in a strong reduction of cell viability and induction of apoptosis (**Fig. 1i-j, Fig. 7a**). Contrary to the results obtained in cells with functional p53,
30   *GATA3*/*MDM2* dual silencing did not show synthetic lethal effect (**Fig. 1i**). The authors further observed no difference in terms of apoptosis between single *GATA3* knock-down and *GATA3*/*MDM2* dual knock-down (**Fig. 1j**). If the synthetic lethal interaction between *GATA3* and *MDM2* is *TP53*-dependent, one should expect that silencing *TP53* should partially revert the phenotype. Therefore we silenced *MDM2* alone or in combination with *TP53* in the *GATA3*-mutant
35   MCF-7 cell line. As expected, *TP53* silencing partially rescued the effect induced by *MDM2* knock-down (**Fig. 7b-d**), demonstrating the p53 dependency of the synthetic lethal interaction.

Example 2: GATA3 status determines response to MDM2 inhibitor in vitro

The selected vulnerability of MDM2 in _GATA3_-deficient ER-positive breast cancers presents MDM2 as an attractive therapeutic target in this patient cohort. To test whether the apoptotic effects of MDM2 inhibition could be achieved using an MDM2 antagonist, the authors treated the breast
5   cancer cell lines with idasanutlin (RG7388; Ding, Q. _et al._ J. Med. Chem. 56, 5979–5983, 2013; Reis, B. _et al._ Haematologica 101, e185–8, 2016). In the _GATA3_-mutant MCF7 cells, idasanutlin induced cell growth arrest and apoptosis in a dose-dependent manner (**Fig. 2a-b, Fig.8a**). To assess if idasanutlin was inducing the canonical apoptotic cascade, they assessed the expression of p53, Bax and Bcl-2, together with the canonical markers of apoptosis PARP and cleaved PARP,
10   by immunoblot at 6, 12 and 24 hours post-treatment. Idasanutlin induced an early upregulation of p53 and MDM2 proteins, together with the up- and down-regulation of pro- and anti-apoptotic proteins, respectively (**Fig. 2c**), leading to the activation of the apoptotic cascade. _TP53_ silencing partially reverted the effects of idasanutlin treatment on cell growth and apoptosis (**Fig. 9**).

To determine whether _GATA3_ expression levels would modulate response to idasanutlin, the
15   authors assessed the effect of treatment on _GATA3_-silenced BT-474 and MDA-MB134 cells. They observed that while idasanutlin treatment had no effect on the proliferation of the control cells, it significantly reduced cell proliferation upon _GATA3_ silencing **(Fig. 2d and Fig. 8b)**. In fact, both cell lines showed that _GATA3_ silencing substantially reduced the IC50 for idasanutlin **(Fig. 2e and Fig. 8c)**. Flow cytometry and immunoblot further demonstrated that idasanutlin treatment induced
20   apoptosis in both BT-474 and MDA-MB134 upon _GATA3_ silencing but not in control cells (**Fig. 2f-g and Fig. 8d-e**).

Taken together, the authors results demonstrate that _GATA3_ silencing sensitizes cells to MDM2 inhibition

Example 3: GATA3 status determines response to MDM2 inhibitor in vivo

25   To ascertain whether _GATA3_ expression levels would also modulate response to idasanutlin _in vivo_, the authors performed xenotransplantation into zebrafish embryos. As a cancer model system, human cancer xenografts in zebrafish recapitulate the response to anticancer therapies of mammalian models. To generate the zebrafish models, they treated _GATA3_-silenced and control BT-474 cells with idasanutlin (25µM) or vehicle (DMSO) 48 hours post-siRNA transfection (**Fig.
30   3a**). Twenty-four hours later, they labelled the cells with a red fluorescent cell tracker, injected them into the yolk sac of zebrafish embryos and screened embryos for tumour cell engraftment after four days.

The authors observed that _GATA3_-silenced cells injected into fish were more sensitive to idasanutlin treatment than the control (42% vs 61%, **Fig. 3b**). More importantly, idasanutlin reduced
35   tumour formation only in the context of _GATA3_-silencing (42% vs 65% treated with DMSO) but not

in control cells (61 % vs 56 % treated with DMSO, **Fig. 3b**). Tumours derived from *GATA3*-silenced, idasanutlin-treated cells, were very small, largely consisting of small clusters of tumour cells, compared to the larger solid tumour masses derived from *GATA3*-silenced cells without idasanutlin (**Fig. 3c**). To assess cell proliferation, the authors quantified the percentage of tumour cells present

5   in the fish by performing FACS analysis of the fluorescence-labelled tumour cells in whole fish extracts. Consistent with the results from the tumour formation assay, idasanutlin treatment was only effective in reducing the overall percentage of tumour cells in fish injected with *GATA3*-silenced cells (purple vs DMSO-treated in blue) but not in fish injected with control cells (yellow vs DMSO-treated in black, **Fig. 3d**), indicating that *GATA3* expression level modulates sensitivity to

10   MDM2 inhibition *in vivo*.

The zebrafish xenograft model provides insights into the tumourigenic and proliferative capability of cancer cells. However, to assess apoptosis and to quantify tumour growth, the authors employed the chicken chorioallantoic membrane (CAM), a densely vascularized, extraembryonic tissue, as a second *in vivo* model[22,23]. Similar to the zebrafish assay, we treated *GATA3*-silenced

15   and control BT-474 cells with idasanutlin (25μM) or vehicle (DMSO) for 24 hours (**Fig. 3e**). They then inoculated the cells into the CAMs and screened the eggs for tumour formation four days later. In accordance with our results in the zebrafish model, idasanutlin treatment reduced the volume of tumours formed by *GATA3*-silenced cells (purple vs DMSO-treated in blue) but not in control cells (yellow vs DMSO-treated in black, **Fig, 3f-g**), suggesting that *GATA3* expression

20   modulates response to MDM2 inhibitors in the CAM model as well. The authors then evaluated apoptosis induction by staining tumour sections with the apoptotic marker cleaved caspase 3. Notably, only *GATA3*-silenced idasanutlin-treated tumours showed a strong positive signal for cleaved caspase 3, as well as morphological features of apoptosis (e.g. nuclear fragmentation, hypereosinophilic cytoplasm, "apoptotic bodies", **Fig. 3h** and **Fig. 10**). demonstrating that

25   idasanutlin induces apoptosis in the context of *GATA3* silencing *in vivo*. Taken together, their results show that *GATA3* expression modulates response to idasanutlin in two independent *in vivo* models.

Example 4: The synthetic lethality between GATA3 and MDM2 acts via the PI3K-Akt-mTOR signaling pathway

30   To investigate the putative mechanisms driving the synthetic lethality, the authors analysed the gene expression changes induced by concurrent *GATA3* loss and *MDM2* silencing. RNA-sequencing analysis of the *MDM2*-silenced MCF-7 cells and dual *GATA3/MDM2*-silenced MDA-MB134 cells revealed 20 commonly dysregulated pathways (**Fig. 4a**). As expected, p53 related pathways and apoptosis were significantly up-regulated in both cell lines, while many proliferation-

35   related pathways such as *E2F* and *MYC* targets were down-regulated (**Fig. 4b**). Interestingly, the mTORC1 signalling pathway was among the most significantly down-regulated pathways in both cell lines. Indeed, the authors confirmed that *MDM2* silencing in the *GATA3*-mutant MCF7 cells

reduced phospho-Akt, phospho-S6, as well as phospho-GSK3β, compared to control cells (**Fig. 4c and Fig. 11**), indicating the downregulation of the mTOR pathway. Similarly, in BT-474 cells, dual *GATA3/MDM2* silencing reduced levels of phospho-Akt, phospho-S6 and phospho-GSK3β and induced apoptosis (**Fig. 4d and Fig. 11a**). By contrast, phospho-Akt levels were higher when only
5 *GATA3* was silenced. Pharmacological inhibition of MDM2 in *GATA3*-silenced BT-474 cells also resulted in a reduction in phospho-Akt, phospho-S6 and phospho-GSK3β (**Fig. 11b**). To determine whether activation of the mTOR signalling could also be observed *in vivo*, the authors stained the tumours in our CAM model with phospho-S6 and phospho-Akt. Indeed, in tumours derived from *GATA3*-silenced BT-474 cells, both phospho-S6 and phospho-Akt were drastically reduced upon
10 treatment with idasanutlin, while in tumours derived from control cells, idasanutlin treatment did not have an effect on mTOR signalling (**Fig. 4e and Fig. 11c**).

The authors, therefore, hypothesized that GATA3 loss may induce addiction to mTOR signalling in breast cancer cells. In support of their hypothesis, they observed that, in ER-positive breast cancers, genetic alterations in *GATA3* are significantly mutually exclusive with those in both
15 *PI3KCA* and *PTEN* (**Fig. 12**). Furthermore, differential gene expression and pathway enrichment analyses between *GATA3*-mutant and *GATA3*-wild type ER-positive breast cancers and between ER-positive breast cancers with low and high *GATA3* expression levels also showed significant enrichment for the mTORC1 signalling pathway (**Fig. 13**). Taken together, their results show that synthetic lethality between GATA3 and MDM2 acts via the PI3K-Akt-mTOR signalling pathway.

20 Methods

Cell lines

ER-positive breast cancer-derived cell lines MCF-7 (*GATA3* mutant p.D335Gfs; *TP53* wild-type), BT-474 (*GATA3* wild-type, *TP53* mutant p.E285K with retained transactivation activity (Jordan, J. J. et al. Mol. Cancer Res. 8, 701–716, 2010)), MDA-MB-134 (*GATA3* wild-type; *TP53* wild-type)
25 and T47D (*GATA3* wild-type, *TP53* mutant p.L194F) authenticated by short tandem repeat profiling. All the cells were monitored regularly for mycoplasma contamination by PCR using specific primers as previously described (Geyer, F. C. et al. Nat. Commun. 9, 1816, 2018). All cell lines were maintained under the condition as recommended by the provider. Briefly, all cell lines were culture in DMEM supplemented with 5% Fetal Bovine Serum (FBS), non-essential amino-acids (NEAA)
30 and antibiotics (Penicillin/Streptomycin). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. Exponentially growing cells were used for all *in vitro* and *in vivo* studies.

Transient gene knockdown by siRNAs

Transient gene knockdown was conducted using ON-TARGET plus siRNA transfection. ON-
35 TARGET plus SMARTpool siRNAs against human *GATA3* (Dharmacon, CO; #L-003781-00-0005), *MDM2* (Dharmacon, CO; #L-003279-00-0005), TP53 (Dharmacon, CO; # L-009625-00-

0005), ON-TARGET plus SMARTpool non-targeting control and DharmaFECT transfection reagent (Dharmacon, CO; #T-2001-03) were all purchased from GE Dharmacon. Transfection was performed according to the manufacturer's protocol. Briefly, log-phase ER positive breast cancer cells were seeded at approximately 60% confluence. Because residual serum affects the

5 knockdown efficiency of ON-TARGET plus siRNAs, growth medium was removed as much as possible and replaced by serum-free medium (Opti-MEM). siRNAs were added to a final concentration of 25 nM. (Note: siRNAs targeting different genes can be multiplexed). Cells were incubated at 37°C in 5% CO_2 for 24-48-72 hours (for mRNA analysis) or for 48-72 hours (for protein analysis). To avoid cytotoxicity, transfection medium was replaced with complete medium

10 after 24 hours.

RNA extraction and relative expression by qRT-PCR

Total RNA was extracted from cells at 75% confluence using TRIZOL (ThermoFisher Waltham, Massachusetts, USA) according to manufacturer's guidelines. cDNA was synthesized from 1 µg of total RNA using SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen). All reverse

15 transcriptase reactions, including no-template controls, were run on an Applied Biosystem 7900HT thermocycler. The expression for all the genes was assessed by using FastStart Universal SYBR Green Master Mix (Merk, CO; #4913850001) and all qPCR performed were conducted at 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min on a QuantStudio 3 Real-Time PCR System (Applied Biosystems). The specificity of the

20 reaction was verified by melt curve analysis. Measurements were normalized using *GAPDH* level as reference. The fold change in gene expression was calculated using the standard ΔΔCt method as previously described (Livak, K. J. & Schmittgen, Methods 25, 402–408, 2001). All samples were analyzed in triplicate.

List of primers used:

25 *GATA3*_Forward (TCGCAGAATTGCAGAGTCGT) (SEQ ID 001)

*GATA3*_Reverse (GAGTTTCCGTAGTAGGGCGG) (SEQ ID 002)

*MDM2*_Forward (GGCGAGCTTGGCTGCTTC) (SEQ ID 003)

*MDM2*_Reverse (TGAGTCCGATGATTCCTGCTG) (SEQ ID 004)

*TP53*_Forward (TGCTCAAGACTGGCGCTAAA) (SEQ ID 005)

30 *TP53*_Reverse (TTTCAGGAAGTAGTTTCCATAGGT) (SEQ ID 006)

*BCL2*_Forward (TCTTTGAGTTCGGTGGGGTC) (SEQ ID 007)

*BCL2*_Reverse (GACTTCACTTGTGGCCCAGAT) (SEQ ID 008)

*BAX*_Forward (GCCCTTTTCTACTTTGCCAGC) (SEQ ID 09)

*BAX*_Reverse (AGACAGGGACATCAGTCGC) (SEQ ID 010)

35 *PUMA*_Forward (CTGCCAGATTTGTGGTCCTC) (SEQ ID 011)

*PUMA*_Reverse (CCTTCCGATGCTGAGTCCAT) (SEQ ID 012)

GAPDH83U (AGGTGAAGGTCGGAGTCAACG) (SEQ ID 013)

GAPDH28L (TGGAAGATGGTGATGGGATTT) (SEQ ID 014)

Immunoblot

Immunoblot total protein was harvested by directly lysing the cells in Co-IP lysis buffer (100 mmol/L NaCl, 50 mmol/L Tris pH 7.5, 1 mmol/L EDTA, 0.1% Triton X-100) supplemented with 1x protease inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Roche, CO; #4693159001) and 1x phosphatase inhibitors (PhosSTOP, CO; #4906845001). Cell lysates were then treated with 1x reducing agent (NuPAGE Sample Reducing Agent), 1x loading buffer (NuPAGE LDS Sample Buffer), boiled and loaded onto neutral pH, pre-cast, discontinuous SDS-PAGE mini-gel system (NuPAGE 10% Bis-Tris Protein Gels). After electrophoresis, proteins were transferred to nitrocellulose membranes using Trans-Blot Turbo Transfer System(Bio-Rad). The transblotted membranes were blocked for 1 hr in TBST 5% milk and then probed with appropriate primary antibodies (from 1:200 to 1:1000) overnight at 4°C. Next, the membranes were incubated for 1 hour at room temperature with fluorescent secondary goat anti-mouse (IRDye 680) or anti-rabbit (IRDye 800) antibodies (both from LI-COR Biosciences). Blots were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences) and band intensity was quantified using ImageJ software. The ratio of proteins of interest/loading control in idasanutlin-treated samples were normalized to their DMSO-treated control counterparts. All experiments were performed and analysed in triplicate.

List of antibodies and working concentrations:

Antibodies

Immunoblot

GATA3 (EPR16651)	abcam	ab199428	1 : 1000
MDM2	MerkMillipore	MABE281	1 : 50
p53 (DO-1)	abcam	ab1101	1 : 250
BCL-2	Cell signalling (CST)	2872S	1 : 1000
PARP/cl.PARP	Cell signalling (CST)	9542	1 : 1000
BAX (D2E11)	Cell signalling (CST)	5023	1 : 1000
AKT	Cell signalling (CST)	9272S	1 : 1000
phospho-AKT (Ser473) (D9E)	Cell signalling (CST)	4060S	1 : 1000
S6 ribosomal protein (5G10)	Cell signalling (CST)	2217S	1 : 1000
phospho-S6 ribosomal protein (Ser235/236) (D57.2.2E)	Cell signalling (CST)	4858S	1 : 2000

GSK-3beta (D5C5Z)	Cell signalling (CST)	12456S	1 : 1000
phospho-GSK-3beta (Ser21/9)	Cell signalling (CST)	9331S	1 : 1000
Actin	Sigma	A5441	1 : 2000

Drug treatment

10×10^3 exponentially growing cells were plated in a 96-well plate. After 24hrs, cells were treated with serial dilution of RG7388-idasanutlin (Selleckchem, CO; #S7205) or dimethyl sulfoxide (DMSO). DMSO served as the drug vehicle, and its final concentration was no more than 0.1%. Cell viability was measured after 72 hours using CellTiter-Glo Luminescent Cell Viability Assay reagent (Promega, CO; #G7570). Results were normalized to vehicle(=100%DMSO). Curve fitting was performed using Prism (GraphPad) software and the nonlinear regression equation. All experiments were performed at least two times in triplicate. Results are shown as mean ± SD.

Proliferation assay

Cell proliferation was assayed using the xCELLigence system (RTCA, ACEA Biosciences, San Diego, CA, USA) as previously described (Andreozzi, M. et al. Neoplasia 18, 724–731, 2016). Background impedance of the xCELLigence system was measured for 12 s using 50 µl of room temperature cell culture media in each well of E-plate 16. Cells were grown and expanded in tissue culture flasks as previously described (Andreozzi, M. et al. Neoplasia 18, 724–731, 2016). After reaching 75% confluence, the cells were washed with PBS and detached from the flasks using a short treatment with trypsin/EDTA. 5 000 cells were dispensed into each well of an E-plate 16. Growth and proliferation of the cells were monitored every 15 min up to 120 hours via the incorporated sensor electrode arrays of the xCELLigence system, using the RTCA-integrated software according to the manufacturer's parameters. In the case of transient siRNA transfection, cells were detached and plated on xCELLigence 24 hours post transfection. For all the experiments with idasanutlin (RG7388), the drug or DMSO were added to the cells 24hrs post-seeding on the xCELLigence system, as indicated on the figures. All experiments were performed in triplicate. Results are shown as mean ± SD.

Apoptosis analysis by flow cytometry

Cells were collected 72 hours post siRNA transfection and 48 hours post treatment with RG7388 respectively, stained with annexin V (Annexin V, FITC conjugate; Invitrogen, CO; #V13242) and propidium iodide (PI; Invitrogen, CO; #V13242), and analyzed by flow cytometry using the BD FACSCanto II cytometer (BD Biosciences, USA). Briefly, cells were harvested after incubation period and washed twice by centrifugation (1,200 g, 5 min) in cold phosphate-buffered saline (DPBS; Gibco, CO; #14040133). After washing, cells were resuspended in 0.1 mL AnnV binding buffer 1X (ABB 5X, Invitrogen, CO; #V13242; 50 mM HEPES, 700 mM NaCl, and 12.5 mM CaCl2 at pH 7.4) containing fluorochrome-conjugated AnnV and PI (PI to a final concentration of 1 ug/mL) and incubated in darkness at room temperature for 15 min. As soon as possible cells were analyzed

by flow cytometry, measuring the fluorescence emission at 530 nm and >575 nm. All experiments were performed in triplicate. Data were analyzed by FlowJo software version 10.5.3.

Zebrafish Xenograft

Animal experiments and zebrafish husbandry were approved by the "Kantonales Veterinaeramt Basel-Stadt" (haltenewilligung: 1024H). Zebrafish were bred and maintained as described previously (Nusslein-Volhard, C. & Dahm, R. Ze *Zebrafish*, Oxford University Press, 2002). Staging was done by hours post-fertilization (hpf) as described previously (Kimmel, C. B., *et al.* Development 108, 581–594, 1990). and according to FELASA and Swiss federal law guidelines. Zebrafish wild-type Tuebingen strains were used in this study. 48 hours post siRNA transfection, *GATA3* silenced and control BT-474 cells were pre-treated for 24 hours with idasanutlin (25 µM). After harvesting, the cells were labeled with a lipophilic red fluorescent dye (CellTracker™ CM-Dil #C7000; Life Technologies, Darmstadt, Germany), according to the manufacturer's instructions. Wild-type zebrafish were maintained, collected, grown and staged in E3 medium at 28.5°C according to standard protocols. For xenotransplantation experiments, zebrafish embryos were anesthetized in 0.4% tricaine (Sigma) at 48 hours post fertilization (hpf) and 200 control or *GATA3* silenced BT-474 cells were micro-injected into the vessel-free area of the yolk sac. After injection, embryos were incubated for 1hr at 28.5–29°C for recovery and cell transfer verified by fluorescence microscopy. Embryos were examined for the presence of a fluorescent cell mass localized at the injection site in the yolk sac or hindbrain ventricle. Fish harbouring red cells were incubated at 35°C as described previously. (Konantz, M. *et al.* Zebrafish xenografts as a tool for in vivo studies on human cancer. *Annals of the New York Academy of Sciences* **1266**, 124–137 (2012)., Haldi, M., Ton, C., Seng, W. L. & McGrath, P. Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. *Angiogenesis* **9**, 139–151 (2006).). On assay day 4, embryos were screened by fluorescence microscopy for (a) normal morphology, (b) a visible cell mass in the yolk or hindbrain ventricle, using a Zeiss SteREO Discovery V20 microscope and the number of tumor-bearing fish quantified. The screening was performed independently by two scientists. For each condition, 70 to 100 fish were analysed over two experiments. Representative pictures were taken using a Nikon CSU-W1 spinning disk microscope. To assess cell proliferation, fish were further dissociated into single cells as described previously (; Carapito, R. *et al.* Mutations in signal recognition particle SRP54 cause syndromic neutropenia with Shwachman-Diamond-like features. *J. Clin. Invest.* **127**, 4090–4103 (2017).) and cells analyzed on a BD FACSCanto II cytometer for CM-Dil–positive cells. For each condition, 20 to 30 fishes were analyzed. Each experiment was repeated twice., 1007–1020 (2016).; Carapito, R. *et al. J. Clin. Invest.* **127**, 4090–4103 (2017).) and the number of fluorescence-labeled cells was then determined using flow cytometry on a BD FACSCanto II cytometer for CM-Dil–positive cells. For each condition, 20 to 30 fishes were analyzed. Each experiment was repeated twice.

Chorioallantoic membrane (CAM) assays

Fertilized chicken eggs were obtained from Gepro Geflügelzucht AG at day 1 of gestation and were maintained at 37°C in a humidified (60%) incubator for 10 days (Zijlstra, A. *et al.* Cancer Res. 62, 7083–7092, 2002). At this time, an artificial air sac was formed using the following procedure: a

5   small hole was drilled through the eggshell into the air sac and a second hole near the allantoic vein that penetrates the eggshell membrane. A mild vacuum was applied to the hole over the air sac in order to drop the CAM. Subsequently, a square 1cm window encompassing the hole near the allantoic vein was cut to expose the underlying CAM (Zijlstra, A. *et al.* ibid.). After the artificial air sac was formed, BT-474 cells growing in tissue culture were inoculated on CAMs at 2×10^6 cells

10   per CAM, on three to four CAMs each. Specifically, 48 hrs post-siRNA transfection, *GATA3-*silenced and control BT-474 cells were treated with idasanutlin (25μM). 24 hrs post-treatment, cells were detached from the culture dish with Trypsin, counted, suspended in 20μl of medium (DMEM) and mixed with an equal volume of Matrigel. To prevent leaking and spreading of cells, a 8mm (inner diameter) sterile teflon ring (removed from 1.8ml freezing vials, Nunc, Denmark) was placed

15   on the CAMs and the final mixture was grafted onto the chorioallantoic membranes inoculating the cells with a pipette inside the ring (Kim, J., Yu, W *et al.* Cell 94, 353–362,1998). Embryos were maintained at 37 °C for 4 days after which tumors at the site of inoculation were excised using surgical forceps. Images of each tumor were acquired with a Canon EOS 1100D digital camera. Surface measurements were performed by averaging the volume (height*width*width) of each

20   tumor using ImageJ, as previously described (Lauzier, A. *et al.* Sci. Rep. 9, 11316, 2019). Total n≥10 tumours for each condition were analysed over three independent experiments.

Immunohistochemistry

Tumors were fixed in 10% Paraformaldehyde (PFA) immediately after excision from the CAM. PFA-fixed and paraffin-embedded tumors were cut as 3.5μm thick sections. Hematoxylin

25   and eosin (H&E) staining was performed according to standard protocols. Tissue sections were rehydrated and immunohistochemical staining was performed on a BOND-MAX immunohistochemistry robot (Leica Biosystems) with BOND polymer refine detection solution for DAB, using anti-GATA3, cleaved caspase 3, phospho-Akt or phospho-S6 primary antibodies as substrate. Photomicrographs of the tumors were acquired using an Olympus BX46 microscope. All

30   stained sections were evaluated blindly by two independent pathologists.

<u>*List of antibodies and working concentrations:*</u>

Immunoistochemistry

GATA3	abcam	EPR16651	1 : 500
Cleaved Caspase 3 (D175)	Cell signalling (CST)	9661S	1 : 200
phospho-S6 rib. prot. (Ser235/236) (D57.2.2E)	Cell signalling (CST)	4858S	1 : 400

| phospho-AKT (Ser473) (D9E) | Cell signalling (CST) | 4060S | 1 : 100 |

RNA sequencing and pathway analysis

Biological duplicates were generated for all the samples analyzed. Total RNA was extracted from cells at 75% confluence using TRIZOL (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to manufacturer's guidelines. RNA samples were treated with Turbo Dnase (AM 1907,
5   Thermo Fisher Scientific) and quantified using a Qubit Fluorometer (Life Technologies). RNA integrity was measured using the Agilent Bioanalyzer 2100 (Agilent Technologies).

Library generation was performed using the TruSeq Stranded mRNA (Illumina). Paired-end RNA sequencing was performed on the Illumina NovaSeq 6000 platform using the 2x100bp protocol according to the manufacturer's guidelines. Reads were aligned to the GRCh37 human reference
10   genome using STAR 2.7.1 (Dobin, A. *et al.* Bioinformatics 29, 15–21, 2013), and transcript quantification was performed using RSEM 1.3.2 (Li, B. & Dewey, C. N. BMC Bioinformatics vol. 12, 2011). Genes without at least 10 assigned reads in at least 2 samples were discarded. Counts were normalized using the median of ratios method from the DESeq2 package (Love, M. I., Huber, W. & Anders, S. Genome Biol. 15, 550, 2014) in R version 3.6.1 (https://www.R-project.org/).
15   Differential expression analysis was performed using the DESeq2 Wald test. Gene set enrichment analysis was performed using the *fgsea* R package (Sergushichev, A. A, BioRxiv, June 20, 2016) and the Hallmark gene set from the Molecular Signatures Database (Liberzon, A. *et al.* Cell Systems vol. 1 417–425, 2015) using the ranked t statistics from the DESeq2 Wald test. Pathways with false discovery rate (FDR) < 0.05 were considered to have significantly altered expression.
20   Results were visualised using ggplot2 (Wickham, H. ggplot2, 2009).

Analysis of The Cancer Genome Atlas (TCGA) data

ER-positive breast cancer mutation annotation file for variant calling pipeline mutect2, FPKM gene expression data and raw read counts of the TCGA BRCA project were downloaded using *TCGAbiolinks* (Colaprico, A. et al. Nucleic Acids Res. 44, e71, 2016) package. Tumor samples
25   were classified as *GATA3*-mutant (n=122) and *GATA3*-wild type (n=596) according to the *GATA3* mutation status. Samples with *GATA3* mRNA expression in the bottom and top quartile were classified as *GATA3*-low (n=200) and *GATA3*-high (n=204), respectively. *edgeR* package (Robinson, M. D. *et al.* Bioinformatics vol. 26 139–140, 2010) was used for differential expression analysis and the genes with low expression (<1 log-counts per million in ≥ 30 samples) were
30   filtered out. Normalization was performed using the "TMM" (weighted trimmed mean) method (Robinson, M. D. & Oshlack, A. Genome Biol. 11, R25, 2010) and differential expression was assessed using the quasi-likelihood F-test. Gene set enrichment analysis of all analyzed genes ranked based on signed p-value according to the direction of the log-fold change was performed using the *fgsea* package (Sergushichev, A. A, BioRxiv, June 20, 2016) Hallmark gene sets from

Molecular Signatures Database (Liberzon, A. *et al.* Cell Systems vol. 1 417–425, 2015) were used to identify significantly upregulated/downregulated pathways. Pathways with FDR < 0.05 were considered significantly regulated.

Mutual exclusive / co-occurrence event analysis

5   ER-positive breast cancer mutational data for the *GATA3, TP53, PIK3CA* and *PTEN* genes and copy number status for *PTEN* derived from the TCGA PanCancer Atlas (Hoadley, K. A. *et al.* Cell 173, 291–304.e6, 2018) and the METABRIC dataset Pereira, B. *et al.* Nat. Commun. 7, 11479, 2016) were obtained using cBioportal (Cerami, E. *et al.* Cancer Discov. 2, 401–404, 2012). A total of 2379 samples were used for the analysis. Mutual exclusivity and co-occurrence of somatic

10   mutations in *GATA3, TP53, PIK3CA* and *PTEN* and deep deletions for *PTEN* were calculated using one-sided Fisher's Exact and $P<0.05$ was considered statistically significant.

Quantification and statistical analysis

Statistical analyses were conducted using Prism software v7.0 (GraphPad Software, La Jolla, CA, USA). For *in vitro* studies, statistical significance was determined by the two-tailed unpaired

15   Student's t-test. For comparison involving multiple time points, statistical significance was determined by the two-tailed unpaired multiple Student's t-test. A *P* value < 0.05 was considered statistically significant. For all figures, ns, not significant. For *in vivo* studies two-sided Fisher's Exact was used to compare the number of tumour-harboring fish (Figure 3b). For the CAM assay a two-tailed unpaired Student's t-test was used. Mutual exclusivity and co-occurrence of somatic

20   mutations were calculated using one-sided Fisher's Exact. The statistical parameters (i.e., exact value of n, p values) have been noted in the figures. Unless otherwise indicated, all data represent the mean ± standard deviation from at least three independent experiments.

Claims

1. An agent for use in treatment of a neoplasm, particularly in treatment of a malignant neoplasm, wherein said neoplasm is characterized by decreased or abrogated function of GATA3, and wherein the agent is selected from
   a. a nucleic acid biopolymer capable of specifically suppressing expression of MDM2;
   b. an MDM2 specific inhibitor, particularly an MDM2 inhibitor characterized by a molecular weight ≤700 Da,
   c. a specific modulator of the MDM2-p53 interaction, particularly a specific modulator of the MDM2-p53 interaction characterized by a molecular weight ≤700 Da, or
   d. a peptide specifically modulating the MDM2-p53 interaction.

2. The agent for use in treatment of a neoplasm according to claim 1, wherein said nucleic acid biopolymer capable of specifically suppressing expression of MDM2 is selected from an siRNA, an miRNA, an antisense oligonucleotide, an shRNA, and an sgRNA.

3. The agent for use in treatment of a neoplasm according to claim 1, wherein said MDM2 specific inhibitor is selected from MEL23 and MEL24.

4. The agent for use in treatment of a neoplasm according to claim 1, wherein said specific modulator of the MDM2-p53 interaction is a nutlin, particularly wherein said specific modulator of the MDM2-p53 interaction is selected from HLI373, NSC 66811, Nutlin-3, Nutlin-3a, RITA, SKPin C1, SMER 3, SP141, SZL P1-41, TAME hydrochloride, Thalidomide, VH 298, YH 239-EE, proTAME, Lenalidomide, MZ 1, TL 12-186, cis MZ 1, dBET1, AT 1, NSC 66811, HLI 373, NAB 2, GS 143, cis VH 298, CM 11, CMP 98, TL 13-27, Idisulam, Indasanutlin, CRBN-6-5-5-VHL, RITA NSC 652287, Tenovin-1, AT 406 (SM-406), Avadomide (CC-122), NSC 207895, JNJ-26854165 (Serdemetan), Iberdomide(CC220), CGM097, HDM201, SAR405838, RG7112, MI-77301, MI-888, MK-8242, AMG232, DS3032b, ALRN-6924 and idasanutlin (RG7388).

5. The agent for use in treatment of a neoplasm according to claim 1 or 4, wherein said specific modulator of the MDM2-p53 interaction is idasanutlin.

6. The agent for use in treatment of a neoplasm according to claim 1, wherein said peptide specifically modulating the MDM2-p53 interaction is selected from ATSP-7041, M06, SAH-p53-8, sMTide-02/02A, and ATSP-7041.

7. The agent for use in treatment of a neoplasm according to any one of the preceding claims, wherein the neoplasm is characterized by expression of functional p53.

8. The agent for use in treatment of a neoplasm according to any one of the preceding claims, wherein the neoplasm is a neoplasm of breast tissue, urothelial tissue, stomach, uterus, ovaries, or lung, or the neoplasm is melanoma, or diffuse large B-cell lymphoma, particularly the neoplasm is a neoplasm of breast tissue, more particularly the neoplasm is an estrogen receptor (ER) positive neoplasm of breast tissue.

9. A method for assigning a likelihood of responding to MDM2 modulator treatment to a patient, wherein

   • an expression and/or an enzymatic activity of GATA3 is determined in a sample of a neoplasm isolated from said patient, particularly a malignant neoplasm, of said patient; and

   • a high likelihood of responding to MDM2 modulator treatment is assigned if the sample of a neoplasm isolated from said patient is

      a. characterized by low expression of GATA3 or lack of expression of GATA3; and/or

      b. characterized by expression of a non-active GATA3 variant; and/or

      c. characterized by the presence of somatic genetic alterations in the *GATA3* gene, particularly a mutation, a copy number alteration or a genomic rearrangement.

10. The method for assigning a likelihood of responding to MDM2 modulator treatment to a patient, wherein additionally

    • an expression and/or a functionality of p53 is determined in said sample of a neoplasm isolated from said patient; and

    • a high likelihood of responding to MDM2 modulator treatment is assigned if the sample of a neoplasm isolated from said patient is additionally characterized by expression of functional p53.

11. The agent for use in treatment or prevention of a neoplasm, particularly a malignant neoplasm, according to any one of claims 9 or 10, wherein a high likelihood of responding to MDM2 modulator treatment is assigned to said patient according to the method of claims 1 to 8.
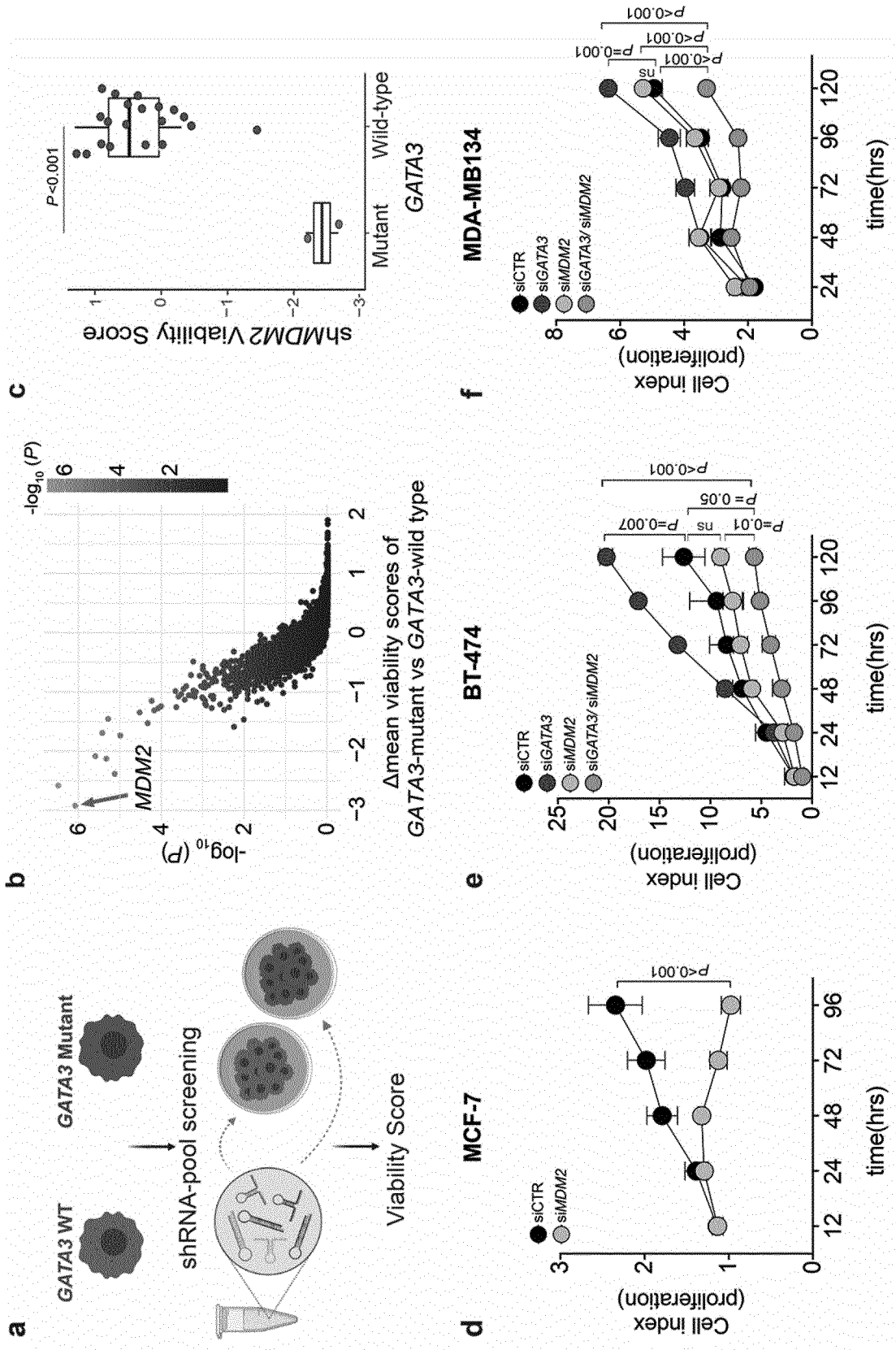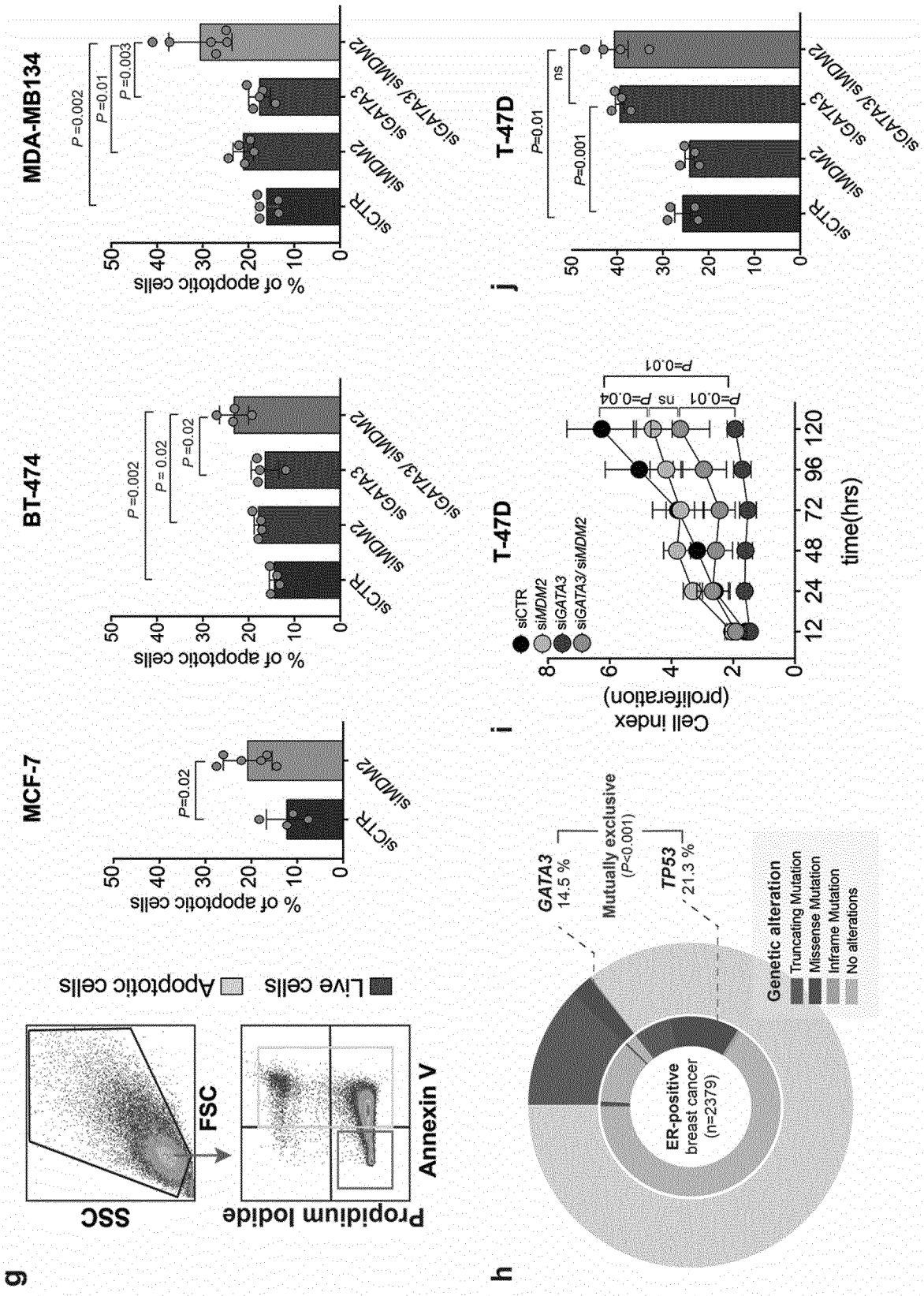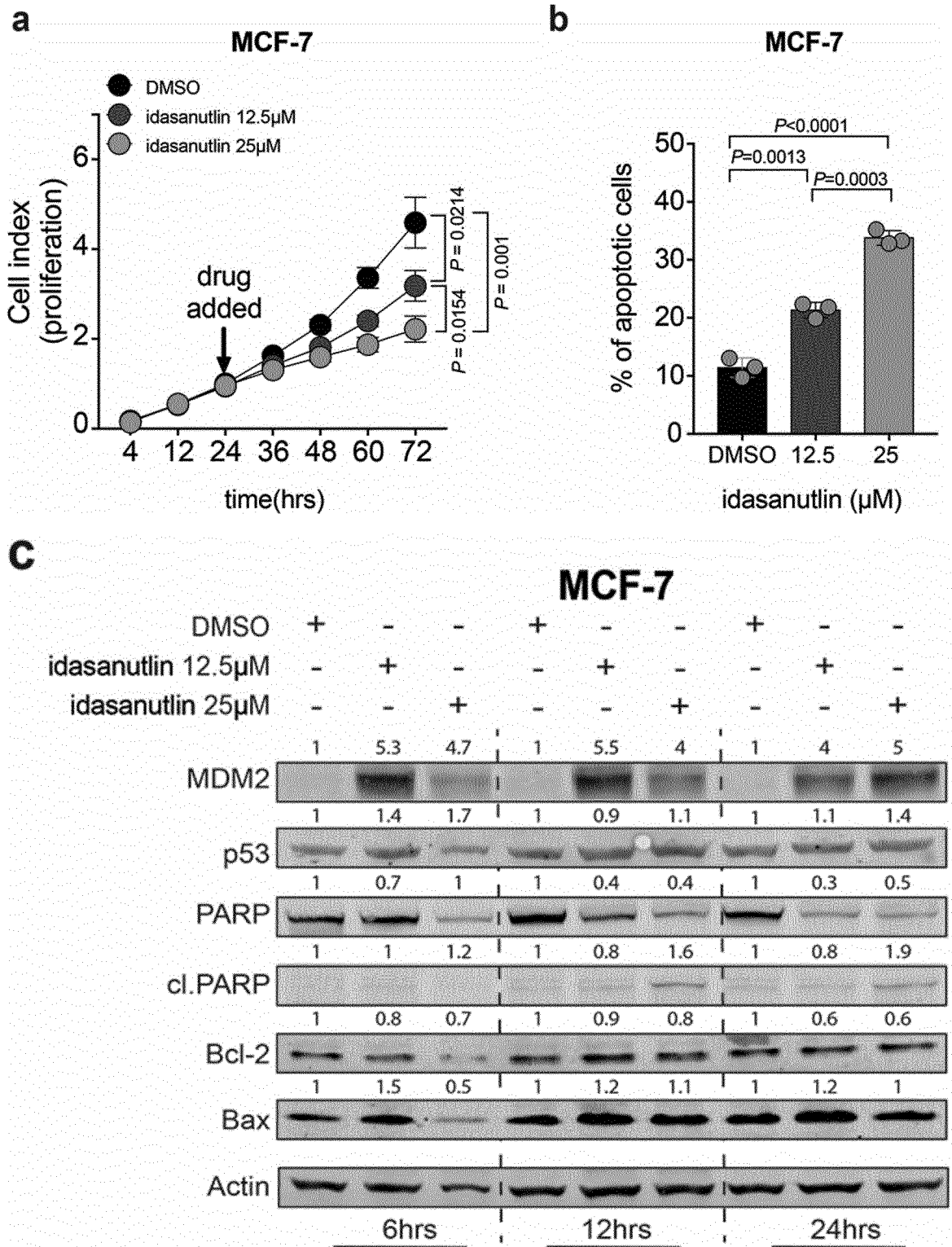
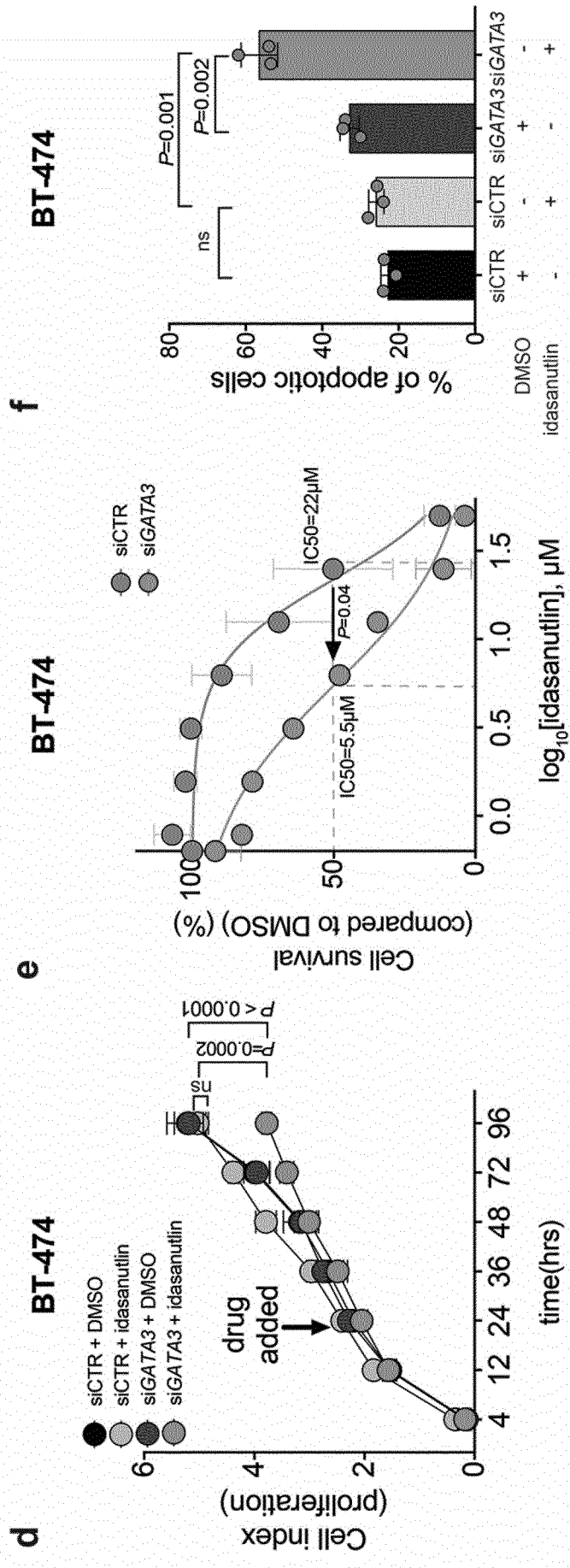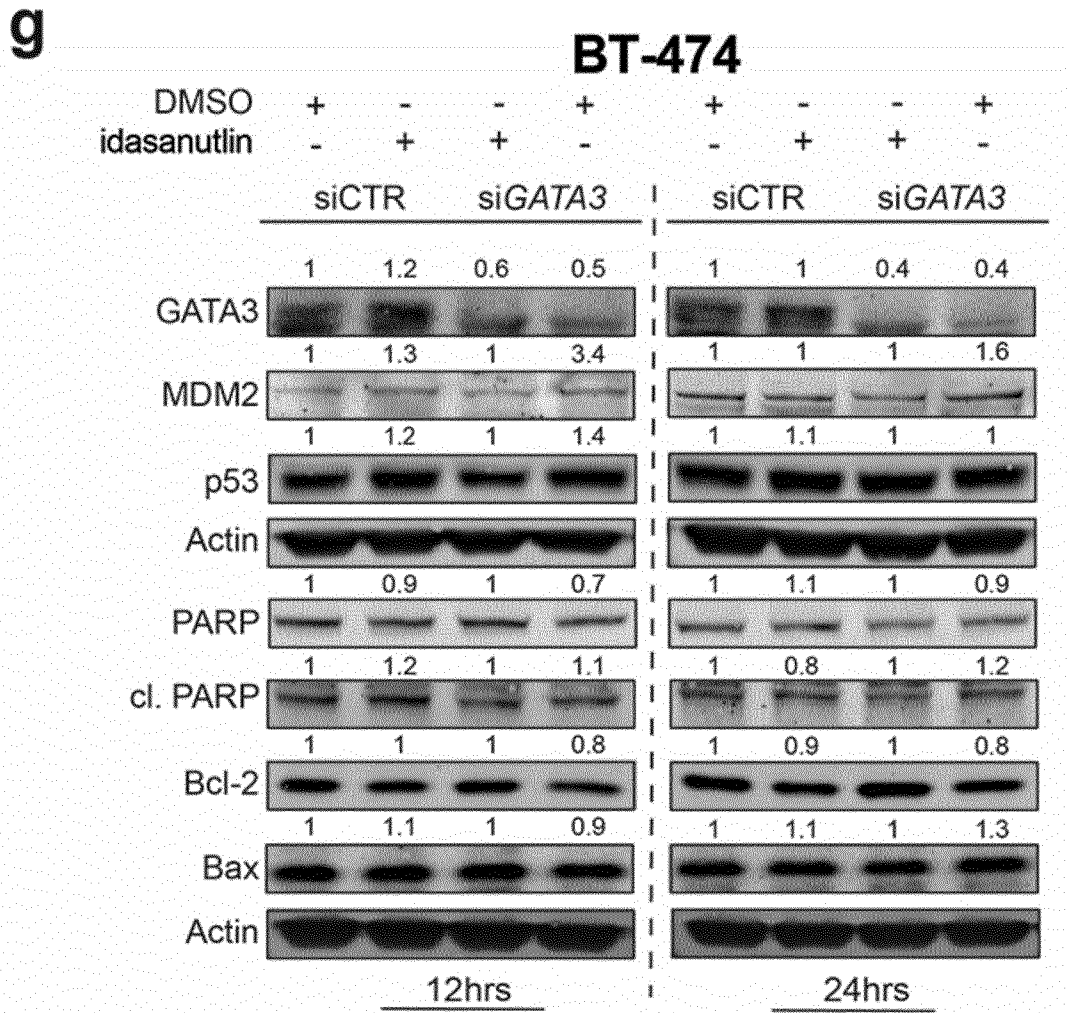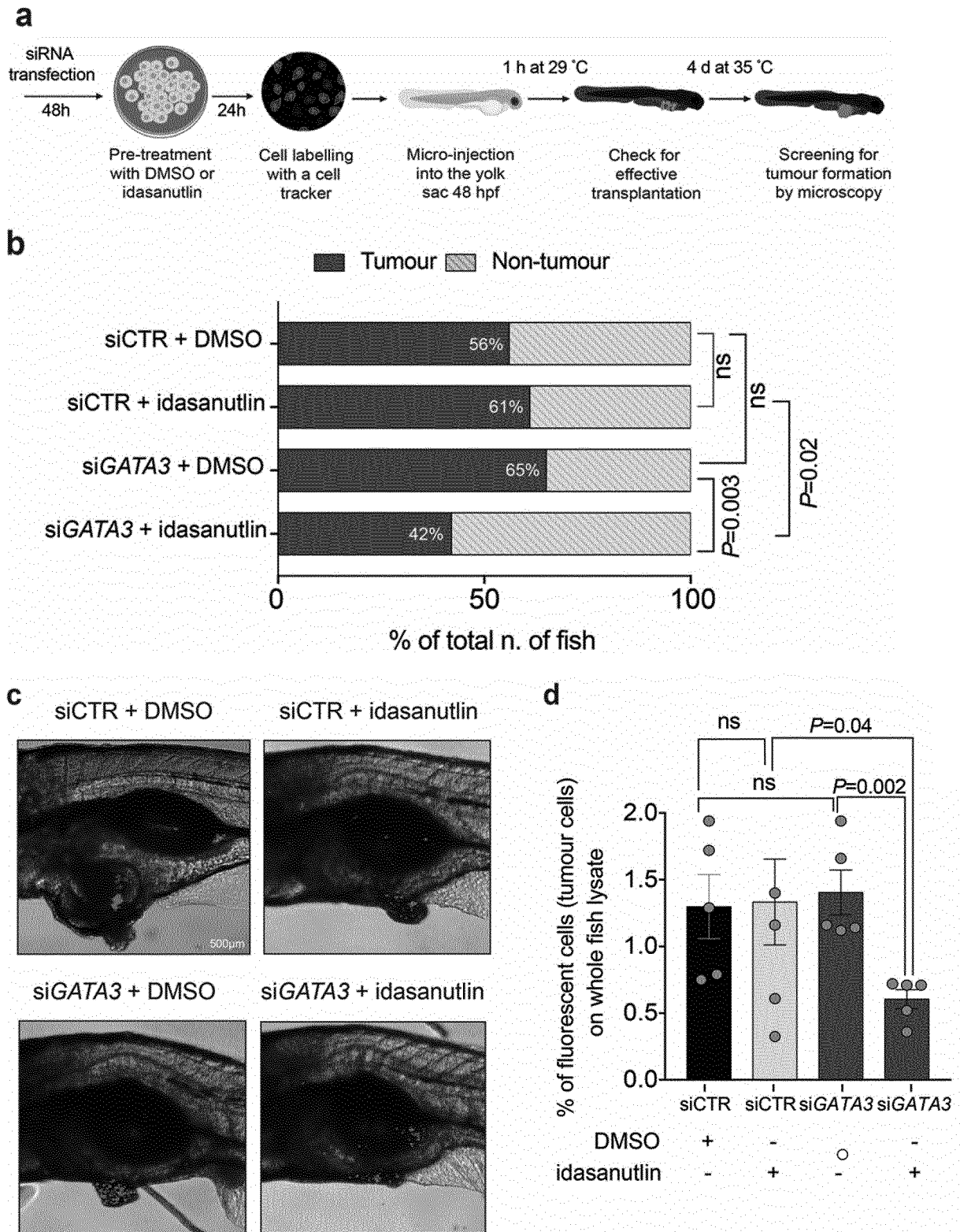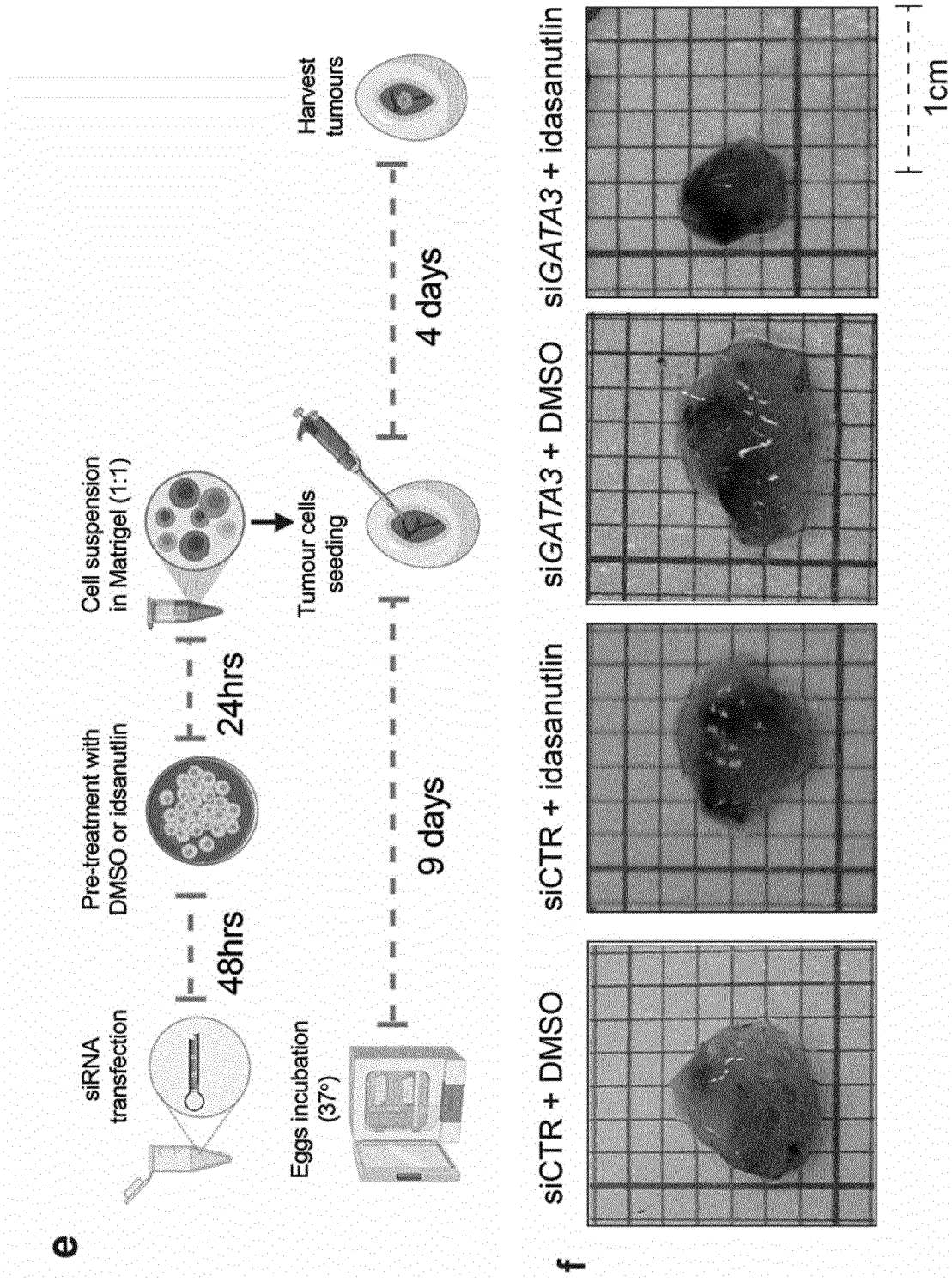Fig. 1

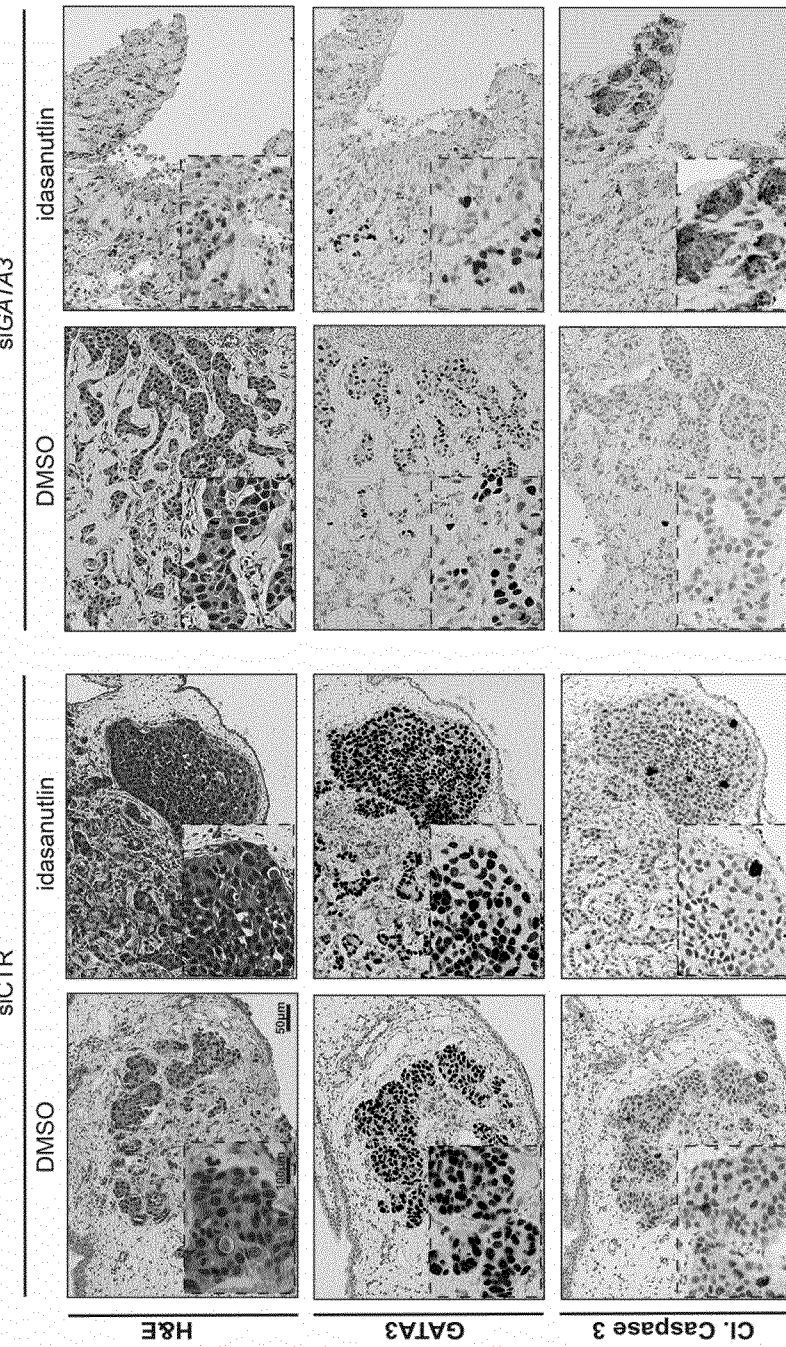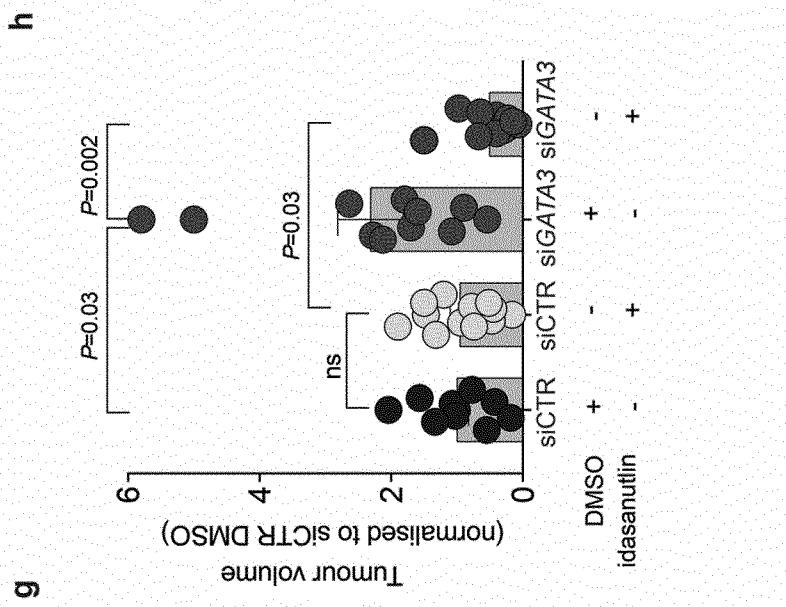Fig. 1 (continued)

Fig. 2

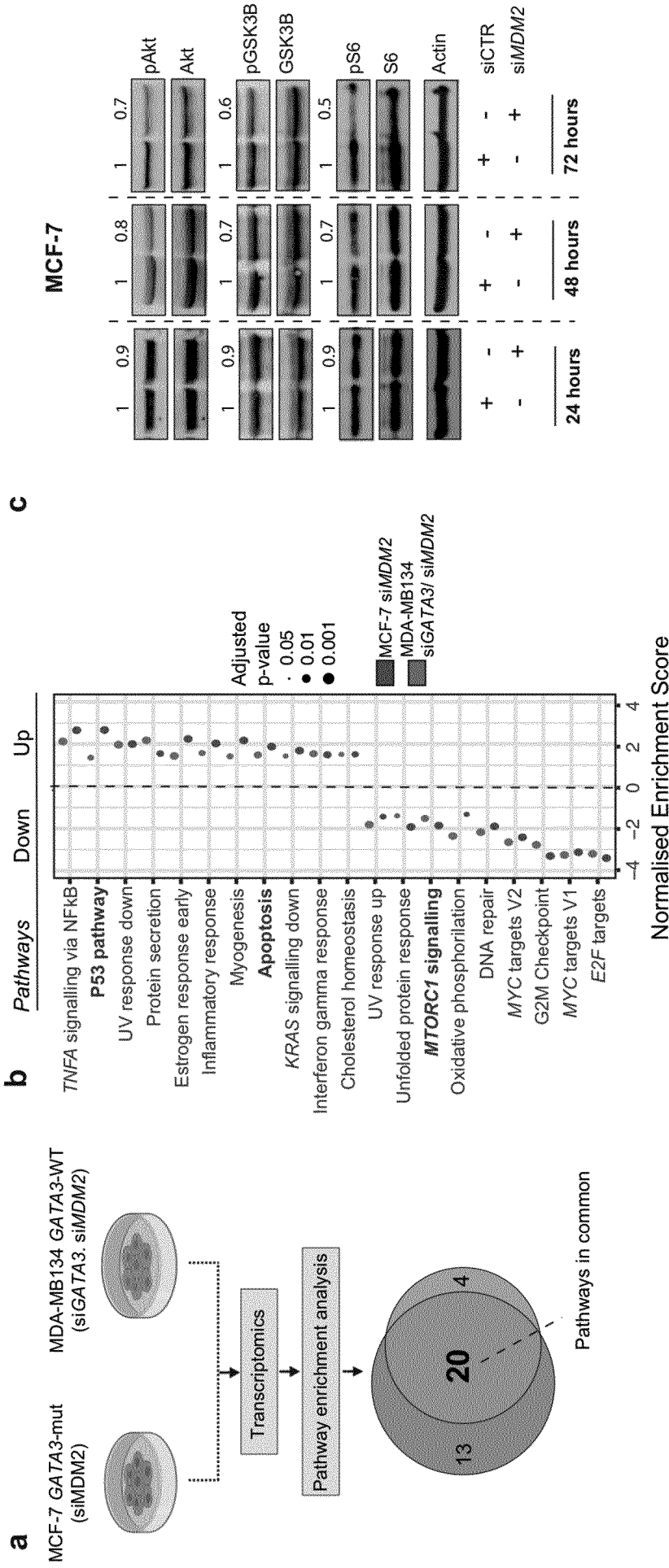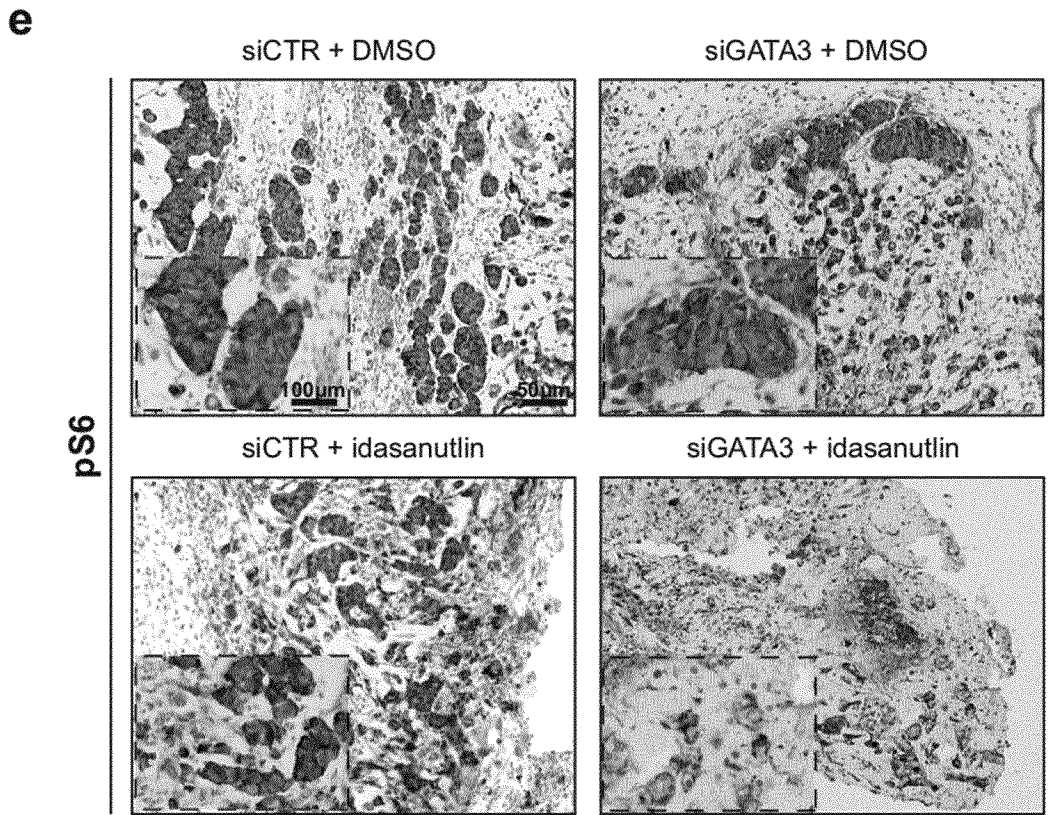Fig. 2 (continued)

Fig. 2 (continued)

Fig. 3

a

| siRNA transfection 48h | Pre-treatment with DMSO or idasanutlin | 24h | Cell labelling with a cell tracker | Micro-injection into the yolk sac 48 hpf | 1 h at 29 °C | Check for effective transplantation | 4 d at 35 °C | Screening for tumour formation by microscopy |

b

Tumour | Non-tumour

% of total n. of fish

c

siCTR + DMSO   siCTR + idasanutlin

siGATA3 + DMSO   siGATA3 + idasanutlin

d

% of fluorescent cells (tumour cells) on whole fish lysate

	siCTR	siCTR	siGATA3	siGATA3
DMSO	+	−	○	−
idasanutlin	−	+	−	+

Fig. 3 (continued)

Fig. 3 (continued)

Fig. 4

Fig. 4 (continued)

d

BT-474

	1	1.1	0.9	0.9		1	1.2	0.8	0.6		1	1.1	0.7	0.6	pAkt
															Akt
	1	0.9	0.8	0.7		1	0.9	1	0.7		1	0.8	0.8	0.6	pGSK3B
															GSK3B
	1	0.9	0.9	0.8		1	1	1	0.8		1	0.9	0.9	0.7	pS6
															S6
															Actin

+	−	−	−		+	−	−	−		+	−	−	−	siCTR
−	−	+	+		−	−	+	+		−	−	+	+	si*MDM2*
−	+	−	+		−	+	−	+		−	+	−	+	si*GATA3*

24 hours   **48 hours**   **72 hours**

e

pS6

siCTR + DMSO   siGATA3 + DMSO

siCTR + idasanutlin   siGATA3 + idasanutlin

Fig. 4 (continued)

Fig. 5

Fig. 5 (continued)

c

MCF-7

Cell index (proliferation)

○ siCTR
○ siMDM2 6.25nM
● siMDM2 12.5nM
● siMDM2 25nM

time(hrs): 24, 48, 72, 96, 120

$P=0.018$
$P<0.001$
$P<0.001$
$P<0.001$

d

MCF-7

% of apoptotic cells

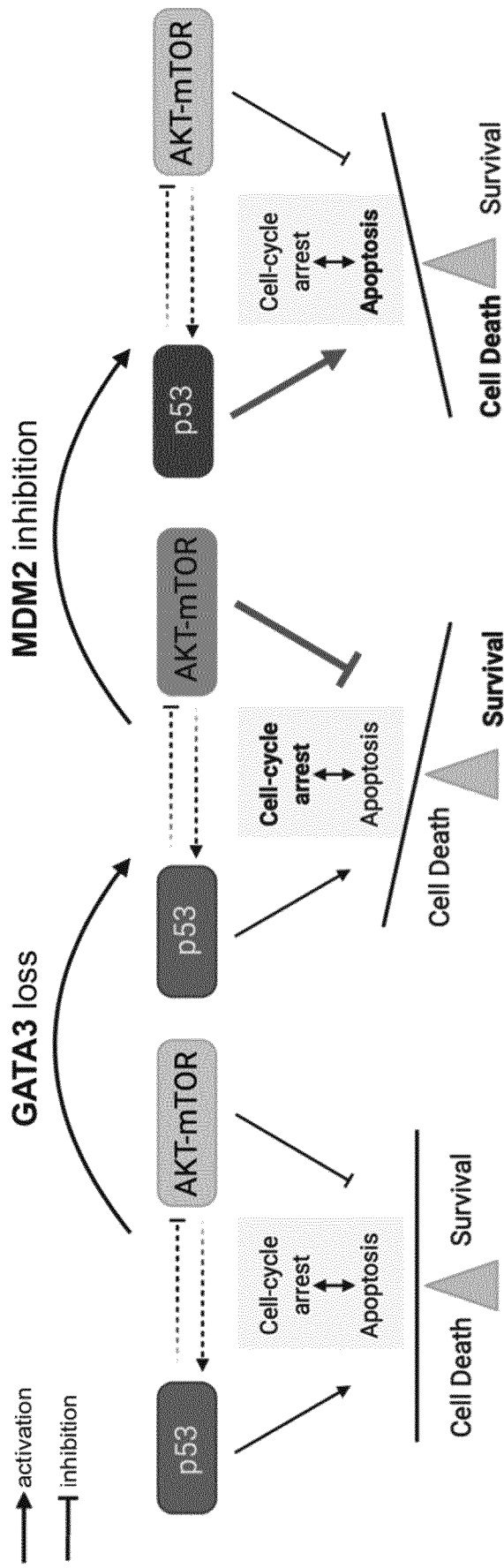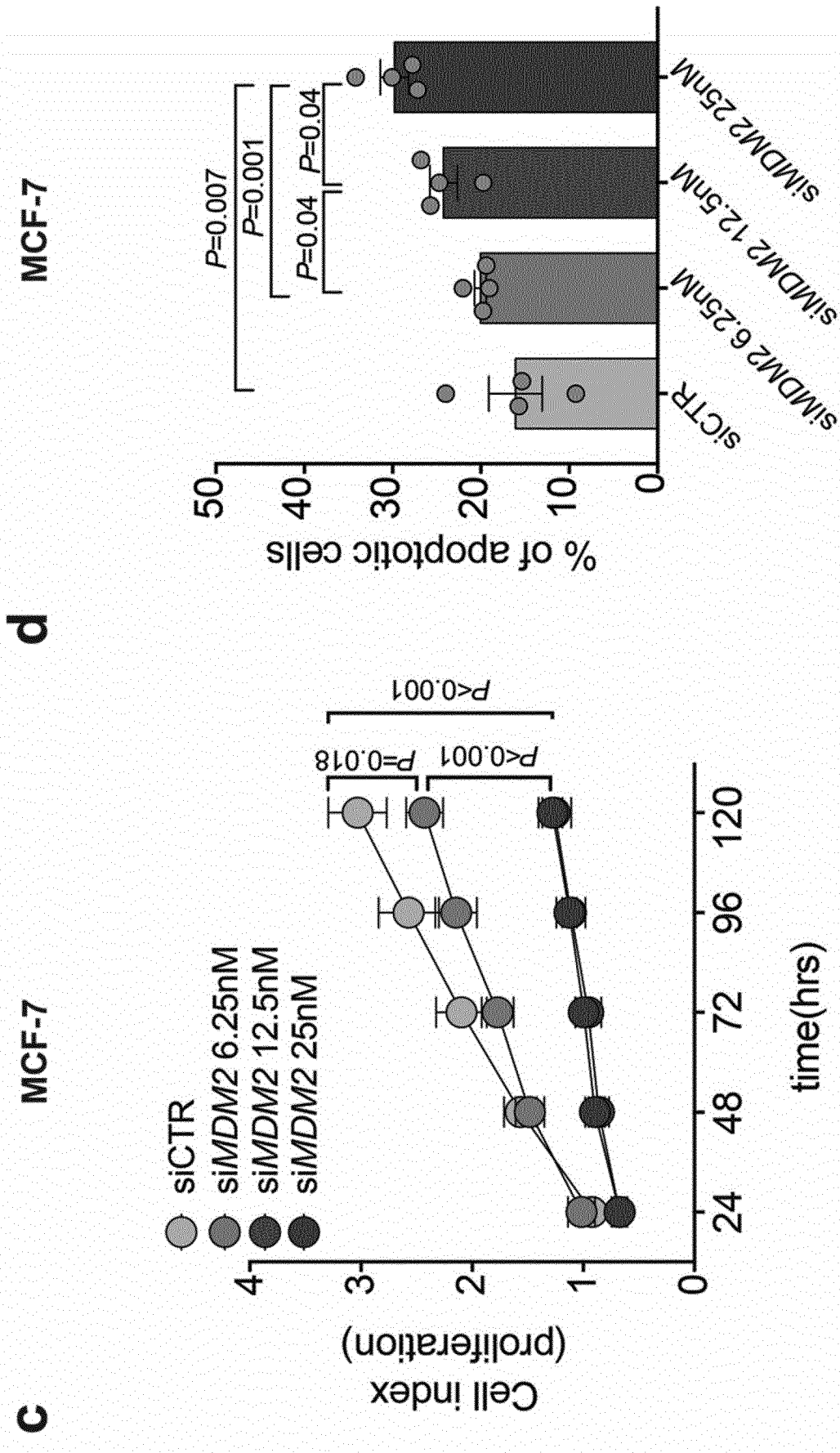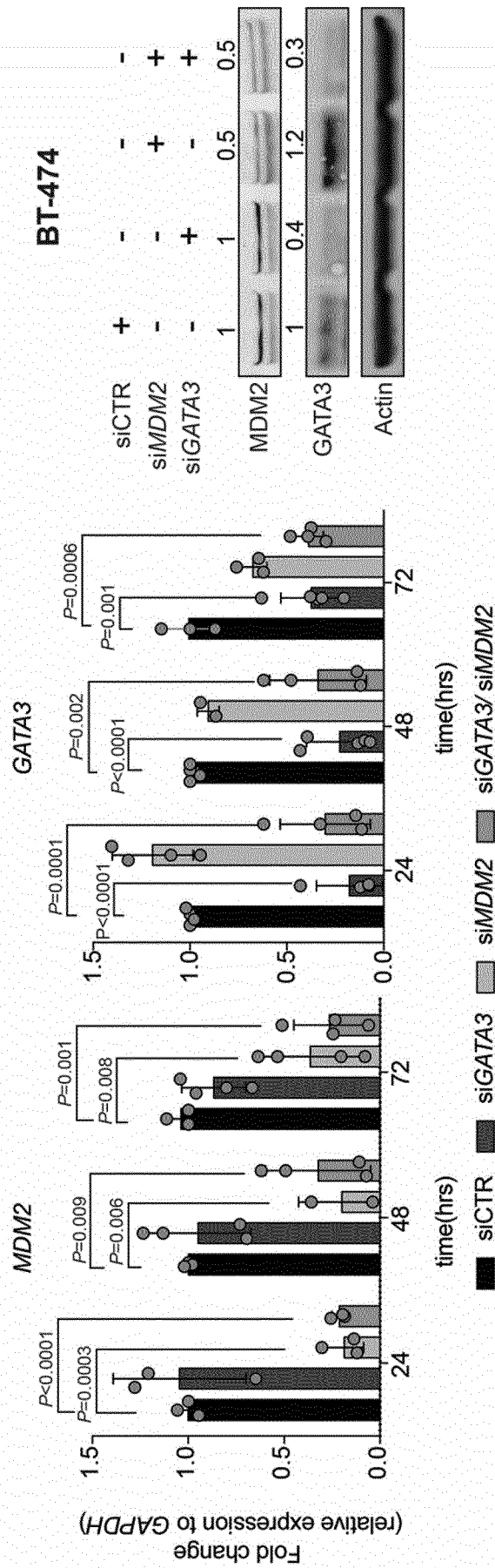siCTR, siMDM2 6.25nM, siMDM2 12.5nM, siMDM2 25nM
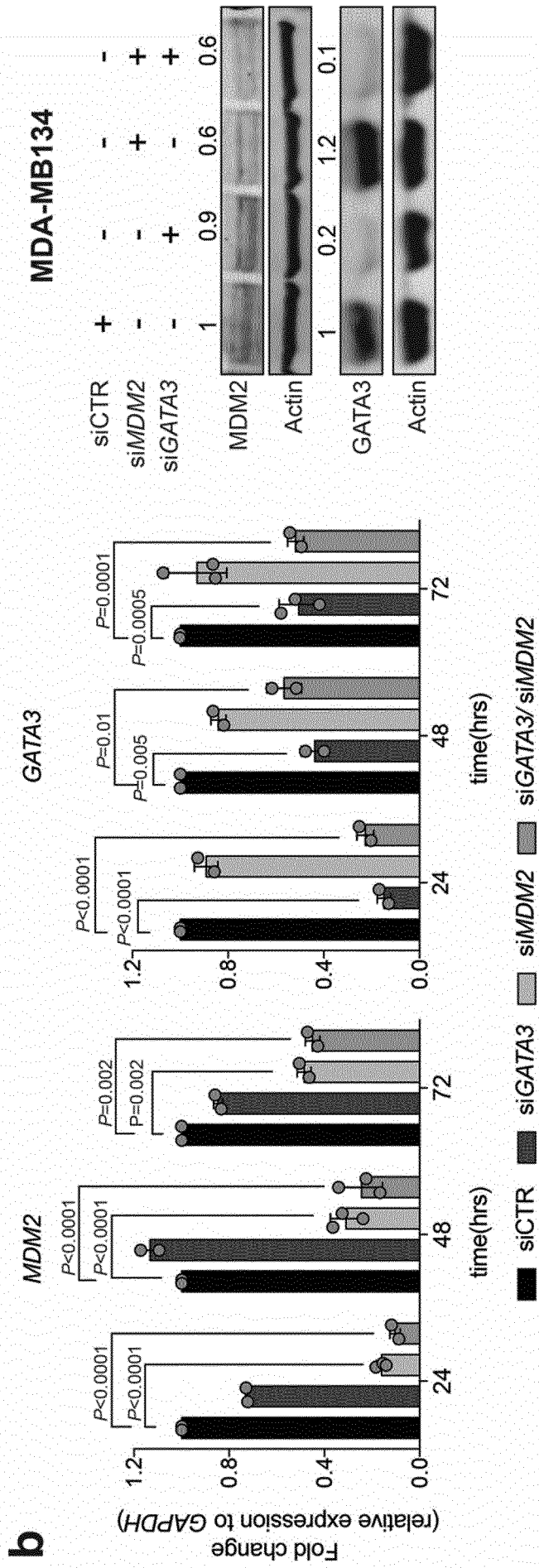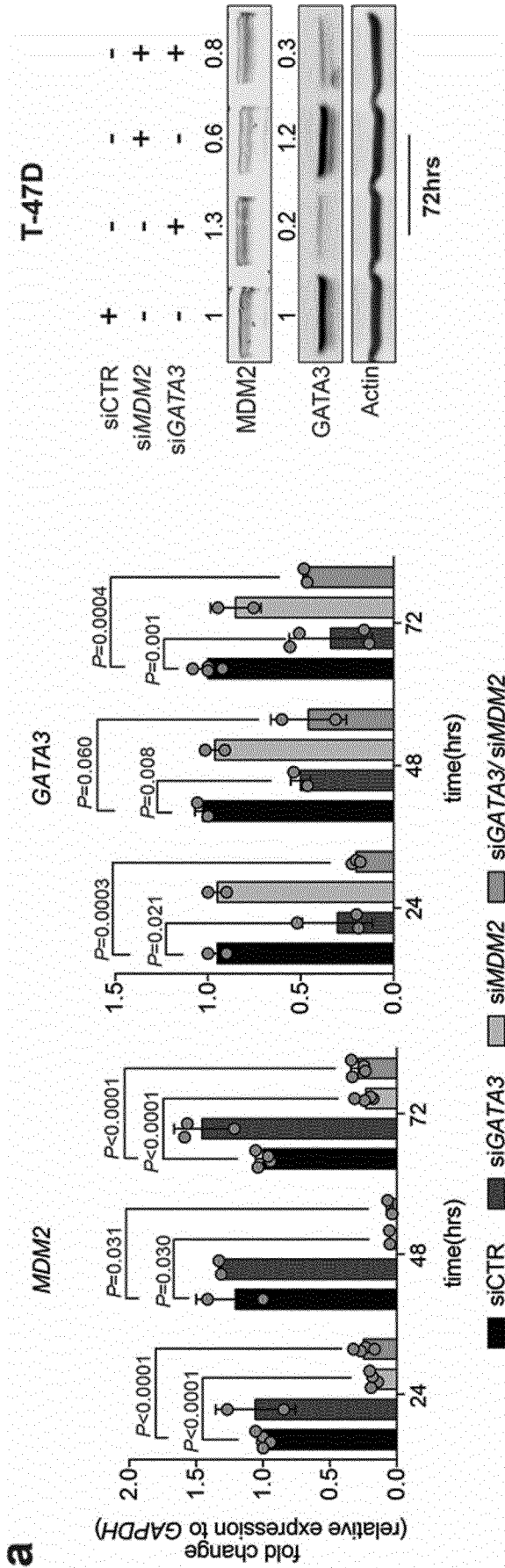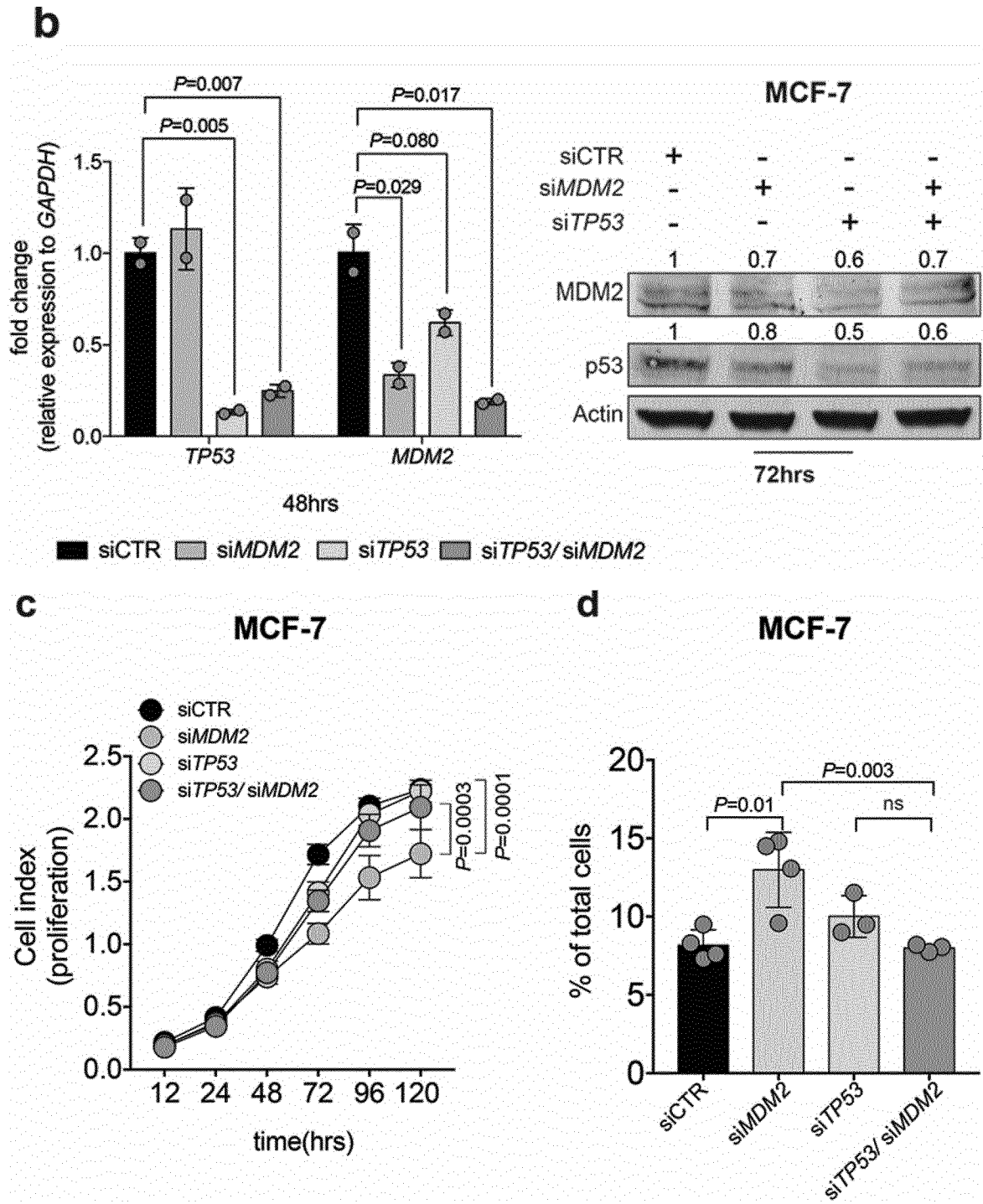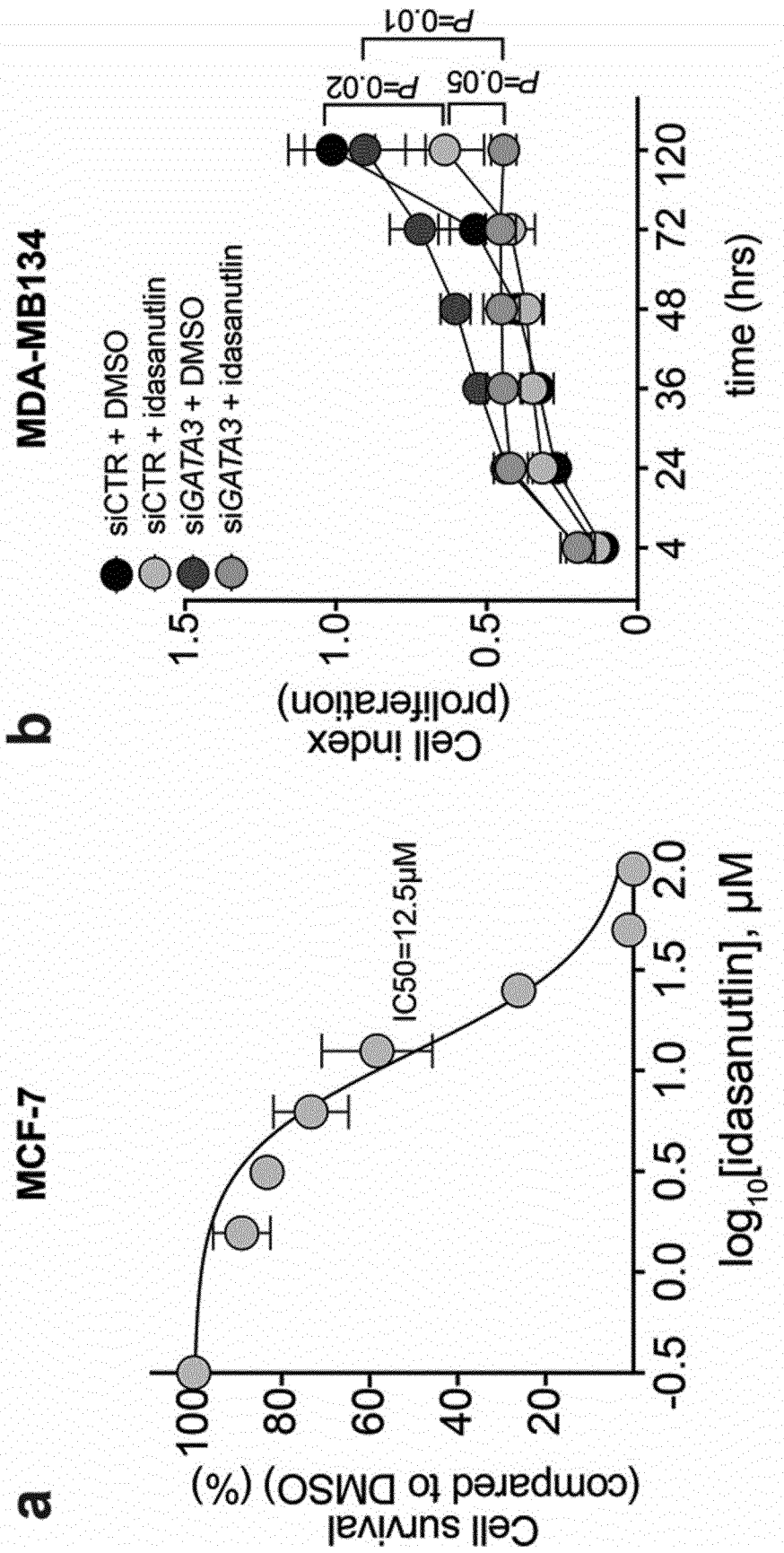
$P=0.007$
$P=0.001$
$P=0.04$
$P=0.04$
$P=0.04$

Fig. 6

Fig. 6 (continued)

Fig. 7

Fig. 7 (continued)

b

c

d

Fig. 8

Fig. 8 (continued)

c   **MDA-MB134**   **d**   **MDA-MB134**
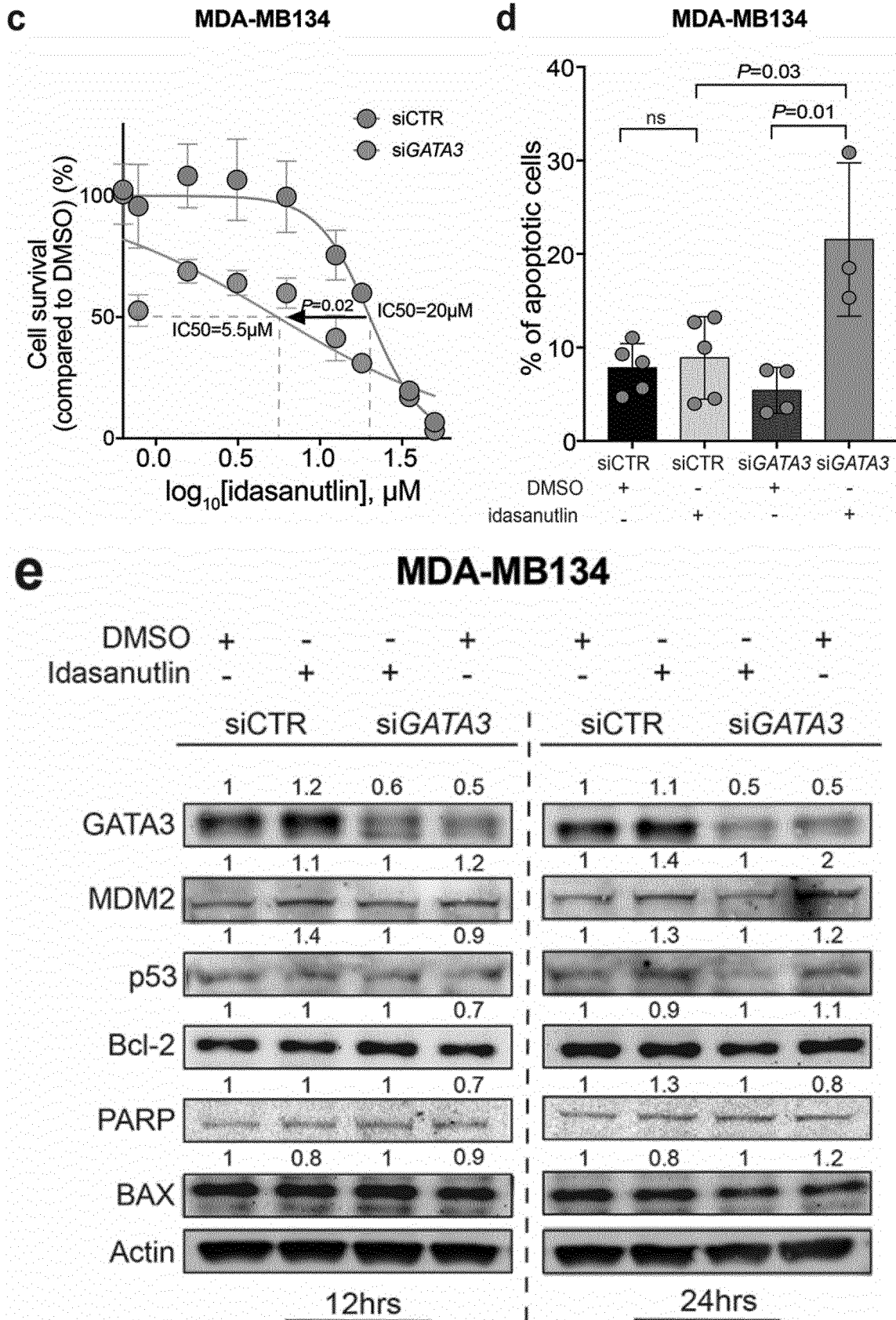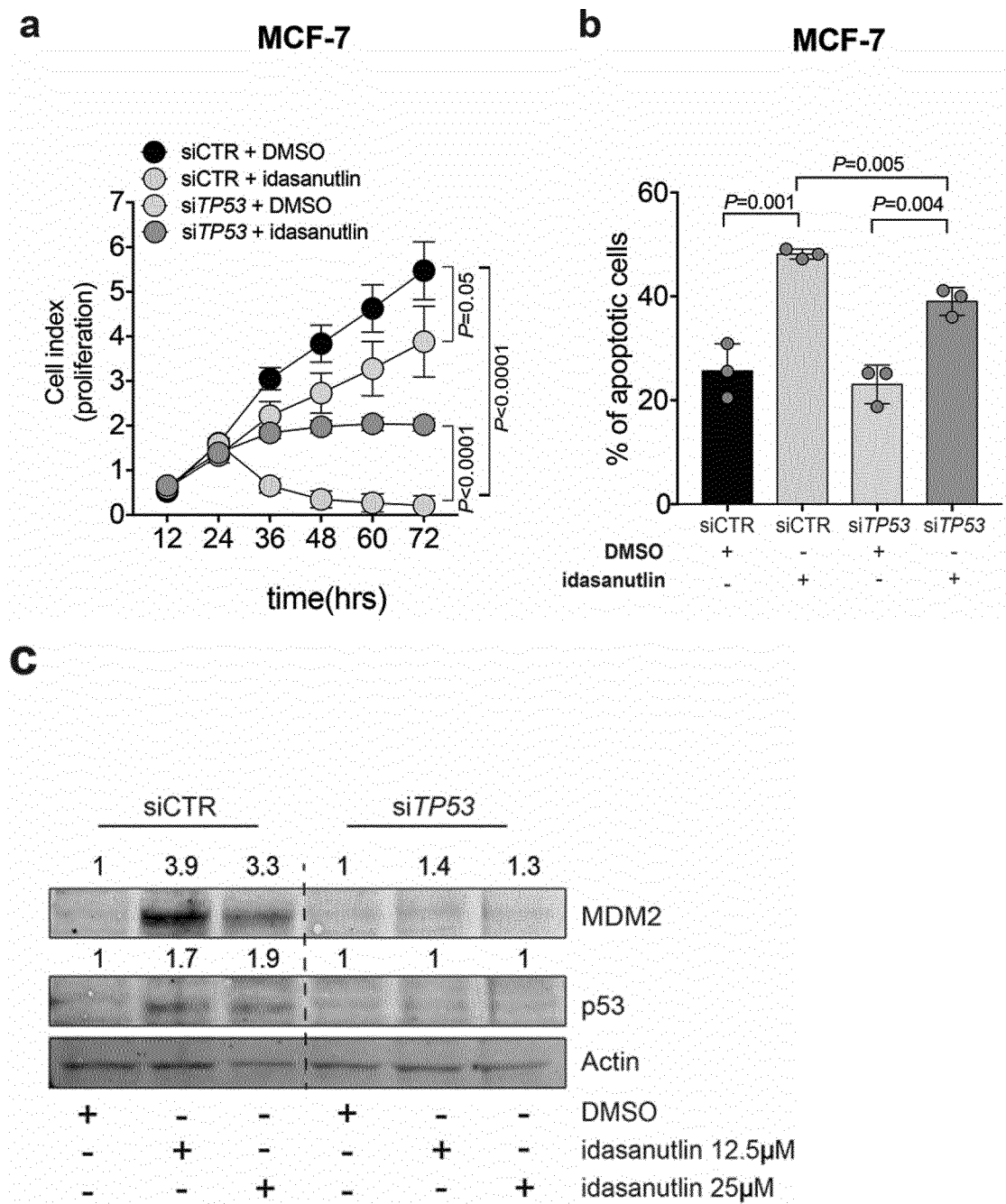

e   **MDA-MB134**

Fig. 9

Fig. 9 (continued)

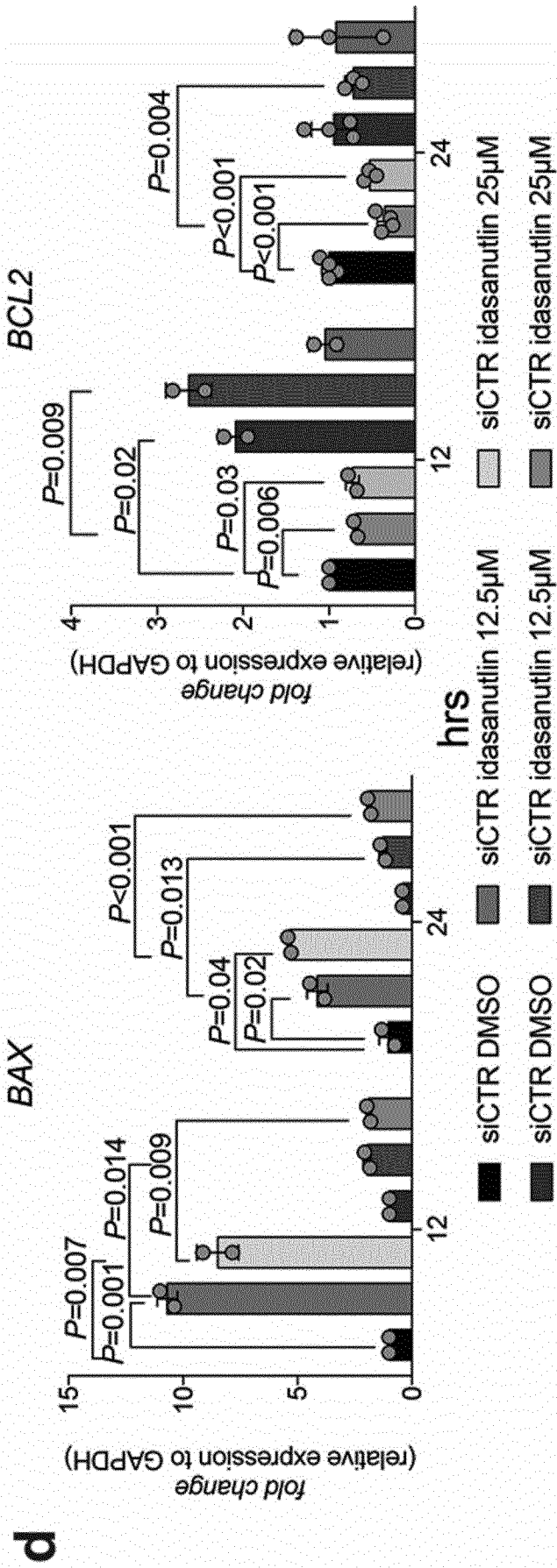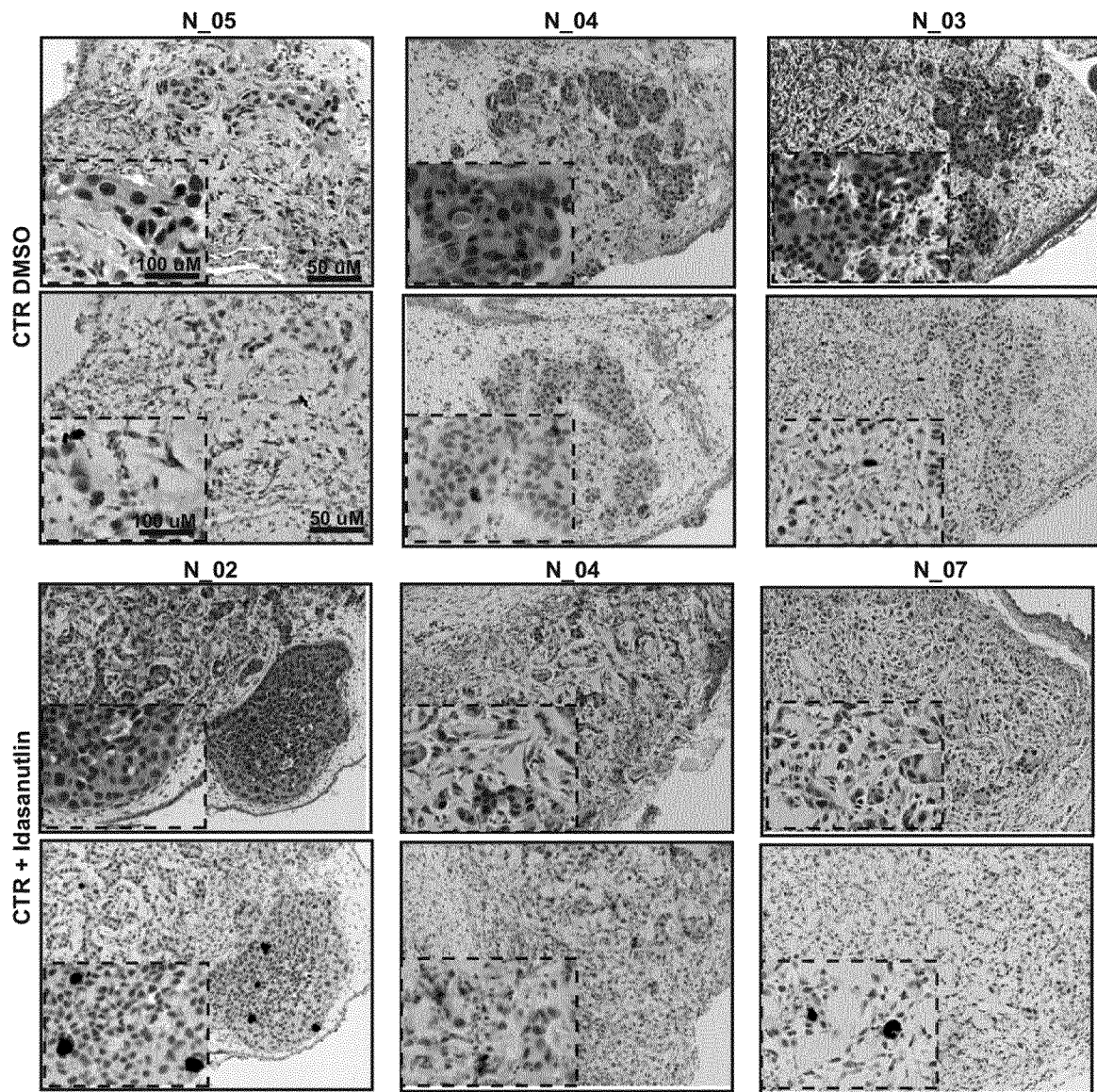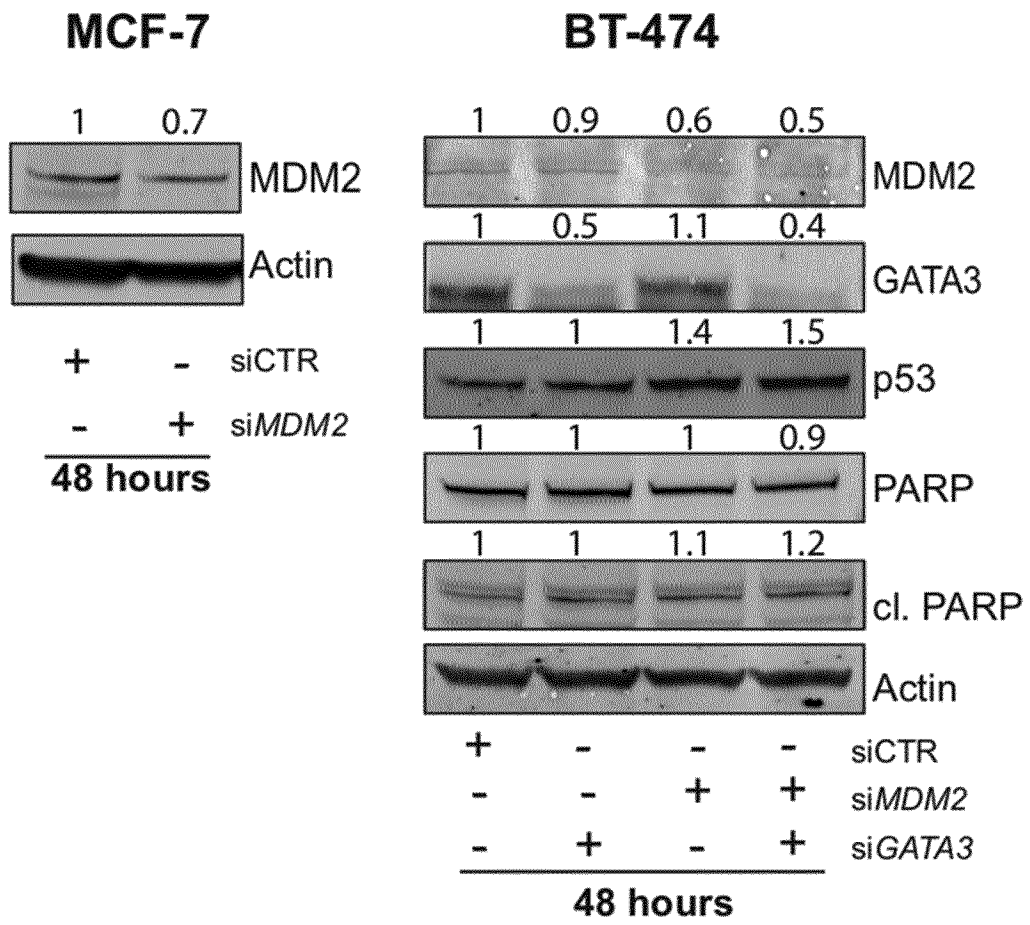Fig. 10
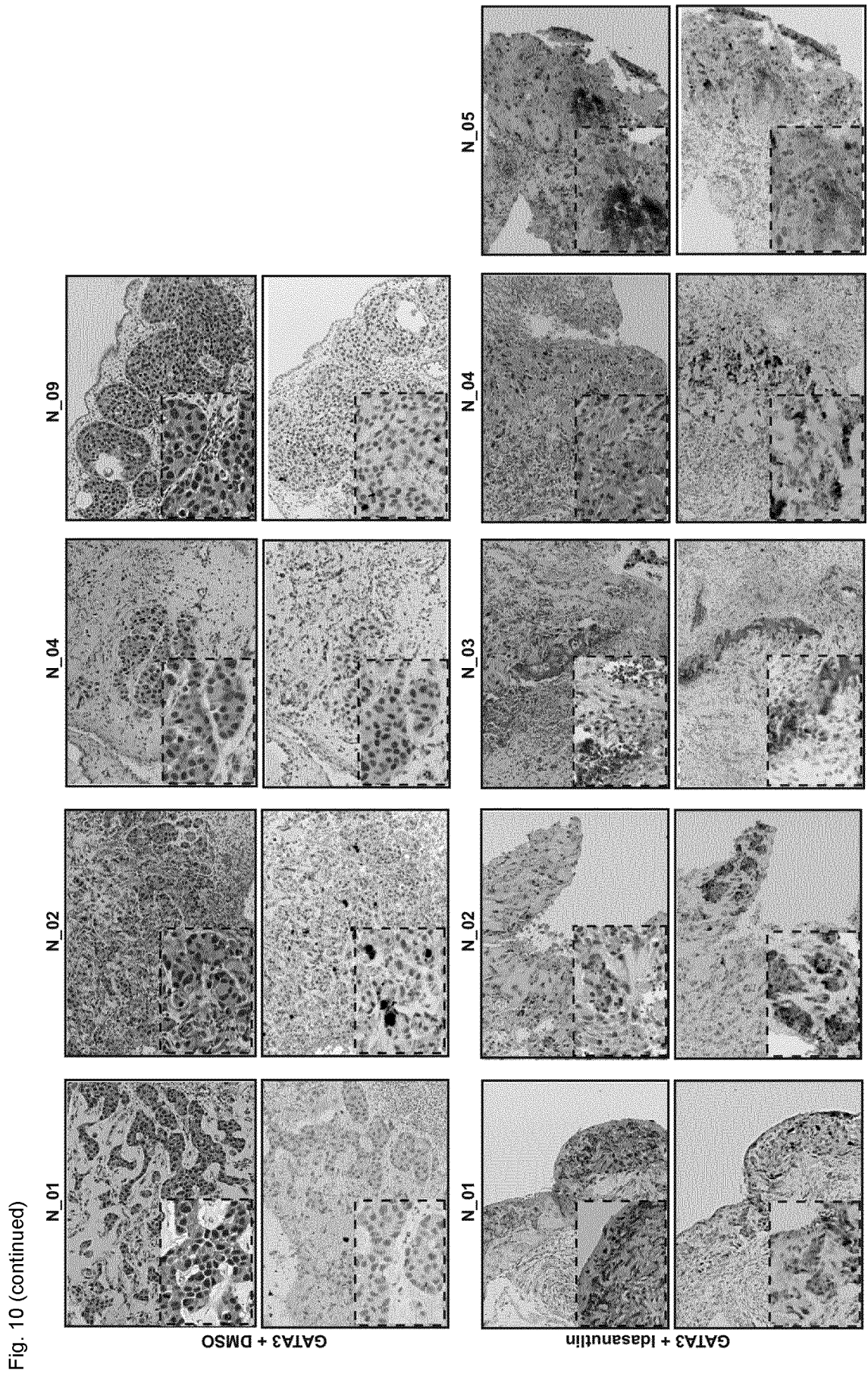
Fig. 10 (continued)

Fig. 11
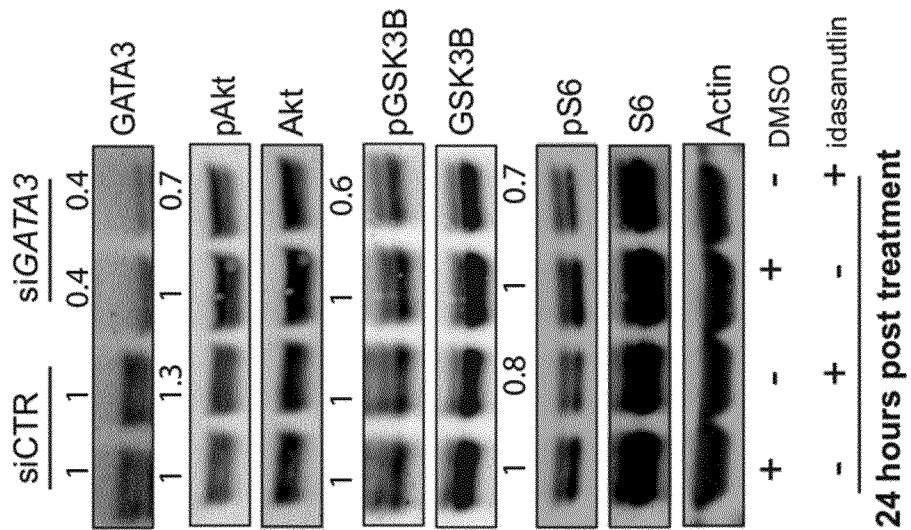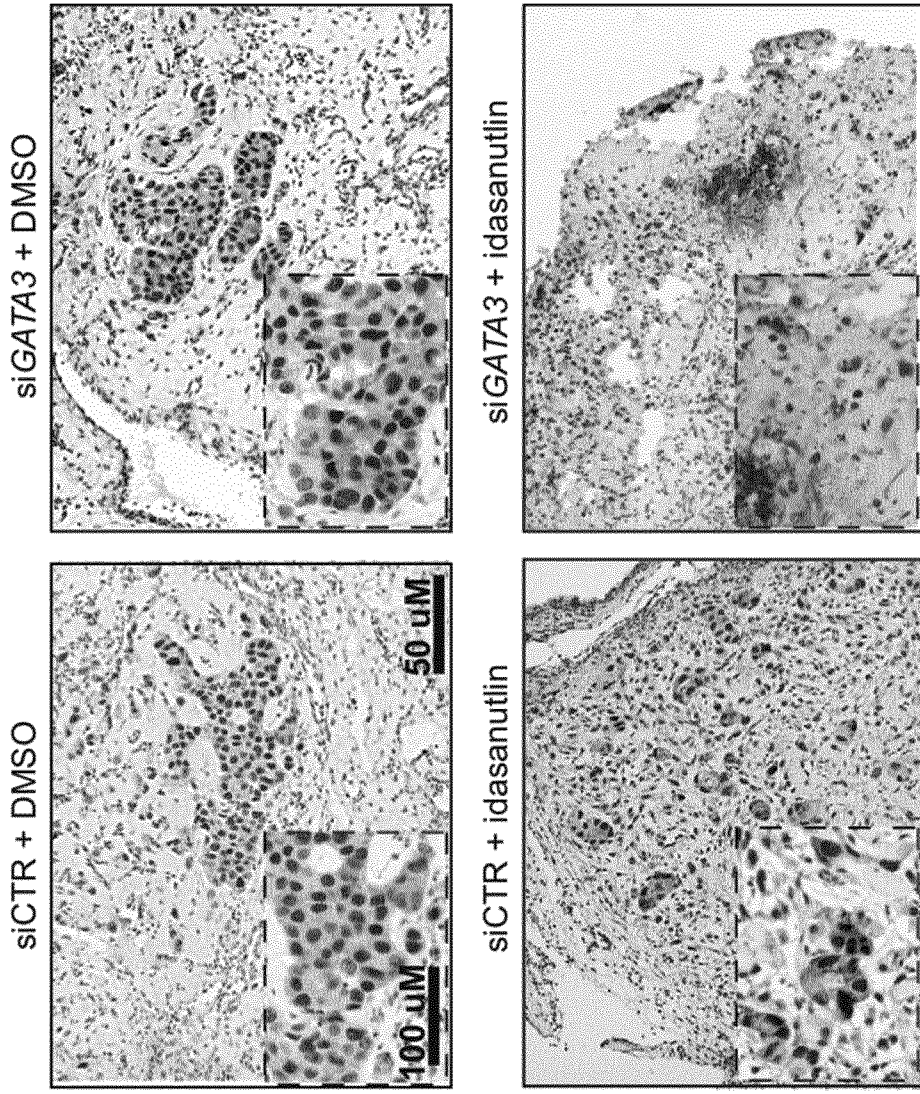
Fig. 11 (continued)

Fig. 12
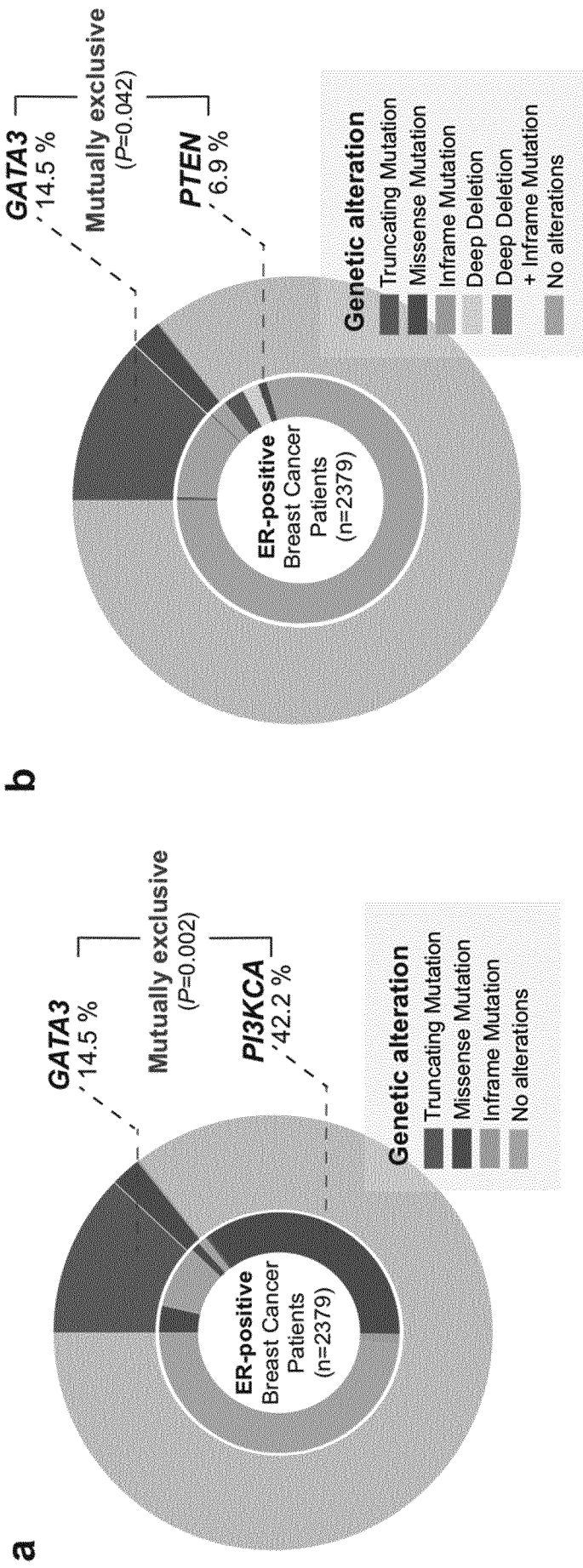
Fig. 13

a

GATA3 low vs GATA3 high

Pathway	Gene ranks	NES	pval	padj
Inflammatory response		2.23	1.1e-03	4.8e-03
Interferon gamma response		2.23	1.1e-03	4.8e-03
TNFA signalling via NFkB		2.05	1.1e-03	4.8e-03
IL6-JAK-STAT3 signalling		1.97	1.2e-03	4.8e-03
Interferon alpha response		1.68	2.4e-03	7.5e-03
Apoptosis		1.57	2.2e-03	7.5e-03
P53 pathway		1.47	2.2e-03	7.5e-03
UV response down		1.44	1.1e-02	3.0e-02
PI3K-Akt-mTOR signalling		1.43	1.7e-02	3.7e-02
KRAS signalling down		1.42	1.7e-02	3.7e-02
MTORC1 signalling		1.37	9.7e-03	2.7e-02
Estrogen response late		-1.46	1.3e-02	3.1e-02
Estrogen response early		-2.01	1.3e-02	3.1e-02

0   5000   10000   15000

HALLMARK_MTORC1_SIGNALING

enrichment score

rank

HALLMARK_PI3K_AKT_MTOR_SIGNALING

enrichment score

rank

Fig. 13 (continued)

b

GATA3-*mutant* vs GATA3-*wild type*

Pathway	Gene ranks	NES	pval	padj
MTORC1 signalling		1.37	1.4e-02	5.5e-02
Estrogen response early		1.73	2.9e-03	1.9e-02
MYC targets V1		1.75	2.8e-03	1.9e-02
Unfolded protein response		1.75	2.5e-03	1.9e-02
Oxidative phosphorylation		1.45	5.7e-03	2.4e-02
Inflammatory response		−1.86	1.5e-03	1.9e-02
KRAS signalling down		−1.65	1.6e-03	1.9e-02
IL6-JAK-STAT3 signalling		−1.72	1.7e-03	1.9e-02
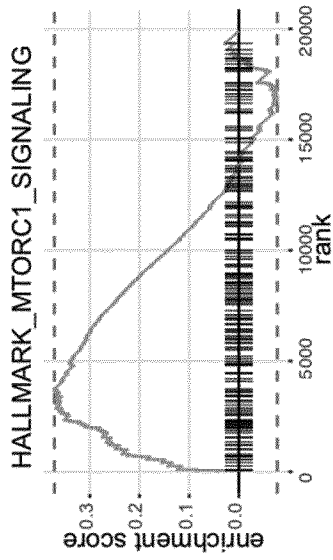
0   5000   10000   15000

HALLMARK_MTORC1_SIGNALING

rank

enrichment score

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/574
ADD.

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)
G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SÉBSTIEN JEAY ET AL: "A distinct p53 target gene set predicts for response to the selective p53-HDM2 inhibitor NVP-CGM097", ELIFE, 12 May 2015 (2015-05-12), pages 1-23, XP055736377,	1,4,7-11
Y	abstract; figures 2-6; table 1 -----	2,3,5,6
X	WO 2014/020502 A2 (NOVARTIS AG [CH]; GAULIS SWANN [CH]; JEAY SEBASTIEN [CH]) 6 February 2014 (2014-02-06)	1,4,7, 9-11
Y	claim 1 ----- -/--	2,3,5,6

[X]	Further documents are listed in the continuation of Box C.		[X]	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 application or patent 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	Date of mailing of the international search report
1 September 2021	08/09/2021

Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Wiesner, Martina

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	C. SADDLER ET AL: "Comprehensive biomarker and genomic analysis identifies p53 status as the major determinant of response to MDM2 inhibitors in chronic lymphocytic leukemia", BLOOD, vol. 111, no. 3, 25 October 2007 (2007-10-25), pages 1584-1593, XP055139885, ISSN: 0006-4971, DOI: 10.1182/blood-2007-09-112698	1,4,7, 9-11
Y	abstract	2,3,5,6
X	WO 2016/056673 A1 (DAIICHI SANKYO CO LTD [JP]) 14 April 2016 (2016-04-14)	1,4,7, 9-11
Y	claim 1	2,3,5,6
A	CHAPEAU EMILIE A ET AL: "Resistance mechanisms to TP53-MDM2 inhibition identified by in vivo piggyBac transposon mutagenesis screen in an Arf(-/-) mouse model", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, vol. 114, no. 12, 21 March 2017 (2017-03-21), pages 3151-3156, XP002779205, ISSN: 0027-8424 the whole document	1-11
A	ANNE Y. SAIKI ET AL: "Identifying the determinants of response to MDM2 inhibition", ONCOTARGET, vol. 6, no. 10, 10 April 2015 (2015-04-10) , pages 7701-7712, XP055736359, DOI: 10.18632/oncotarget.3116 the whole document	1-11
A	SI WENZHE ET AL: "Dysfunction of the Reciprocal Feedback Loop between GATA3- and ZEB2-Nucleated Repression Programs Contributes to Breast Cancer Metastasis", CANCER CELL, CELL PRESS, US, vol. 27, no. 6, 28 May 2015 (2015-05-28), pages 822-836, XP029166208, ISSN: 1535-6108, DOI: 10.1016/J.CCELL.2015.04.011 the whole document	1-11

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6

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NAM K. YOON ET AL: "Higher levels of GATA3 predict better survival in women with breast cancer", HUMAN PATHOLOGY., vol. 41, no. 12, 1 December 2010 (2010-12-01), pages 1794-1801, XP055736202, US ISSN: 0046-8177, DOI: 10.1016/j.humpath.2010.06.010 the whole document -----	1-11
A	CIOCCA V ET AL: "The significance of GATA3 expression in breast cancer: a 10-year follow-up study", HUMAN PATHOLOGY, SAUNDERS, PHILADELPHIA, PA, US, vol. 40, no. 4, 1 April 2009 (2009-04-01), pages 489-495, XP025983245, ISSN: 0046-8177, DOI: 10.1016/J.HUMPATH.2008.09.010 [retrieved on 2008-12-11] the whole document -----	1-11

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Box No. I   Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 2014020502	A2	06-02-2014	CN	104520714 A	15-04-2015
			DK	2880447 T3	12-08-2019
			EP	2880447 A2	10-06-2015
			ES	2742285 T3	13-02-2020
			HU	E045880 T2	28-01-2020
			JP	2015526078 A	10-09-2015
			PL	2880447 T3	31-10-2019
			PT	2880447 T	02-08-2019
			SI	2880447 T1	30-09-2019
			US	2014038986 A1	06-02-2014
			US	2015159222 A1	11-06-2015
			US	2016289770 A1	06-10-2016
			WO	2014020502 A2	06-02-2014
WO 2016056673	A1	14-04-2016	EP	3204514 A1	16-08-2017
			JP	2017532959 A	09-11-2017
			US	2017283885 A1	05-10-2017
			WO	2016056673 A1	14-04-2016