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(54) Titre : COMPOSITIONS COMPRENANT DES REGULATEURS D'ARN NON CODANTS INTERMEDIAIRES MODULANT L'EXPRESSION D'ETV6 OU FOXO1 ET LEURS UTILISATIONS

(54) Title: COMPOSITIONS COMPRISING INTERMEDIATE NON-CODING RNA REGULATORS MODULATING THE EXPRESSION OF ETV6 OR FOXO1 AND USES THEREOF

(57) Abrégé/Abstract:
The present invention relates to:compositions for modulating the expression of FOXO1and/or ETV6, the composition comprising one or more intermediate non-coding RNA regulators; attenuating splicing factor expression, the composition comprising an expression modulator of FOXO1 and/or ETV6; attenuating cell senescence and/or re-entry to cell cycle, the composition comprising an expression modulator of FOXO1and/or ETV6 or their downstream targets related to splicing factor expression; or a composition capable of modulating splicing factor expression, the composition comprising one or more compounds able to bind to, or inhibit, FOXO1 and/or ETV6 genes or their downstream targets related to splicing factor expression. Such compositions have therapeutic benefits in the prevention, management, amelioration or treatment of an age-related disease or condition or cancer or also as a research tool/reagent.

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(54) Title: COMPOSITIONS COMPRISING INTERMEDIATE NON-CODING RNA REGULATORS MODULATING THE EXPRESSION OF ETV6 OR FOXO1 AND USES THEREOF

(57) Abstract: The present invention relates to:compositions for modulating the expression of *FOXO1*and/or *ETV6*, the composition comprising one or more intermediate non-coding RNA regulators; attenuating splicing factor expression, the composition comprising an expression modulator of *FOXO1* and/or *ETV6*; attenuating cell senescence and/or re-entry to cell cycle, the composition comprising an expression modulator of *FOXO1*and/or *ETV6* or their downstream targets related to splicing factor expression; or a composition capable of modulating splicing factor expression, the composition comprising one or more compounds able to bind to, or inhibit, *FOXO1* and/or *ETV6* genes or their downstream targets related to splicing factor expression. Such compositions have therapeutic benefits in the prevention, management, amelioration or treatment of an age-related disease or condition or cancer or also as a research tool/reagent.

COMPOSITIONS COMPRISING INTERMEDIATE NON-CODING RNA REGULATORS MODULATING THE EXPRESSION OF ETV6 OR FOXO1 AND USES THEREOF

Technical Field of the Invention

The present invention relates to compositions which modulate expression of *FOXO1* and/or *ETV6* or their targets pertaining to the regulation of splicing factors. Such compositions have therapeutic potential in the prevention, management, amelioration or treatment of an age-related disease or condition or cancer and also as a research tool/reagent.

Background to the Invention

Senescent cells are viable and metabolically active entities that over multiple rounds of cell division have lost the ability to proliferate and have been shown to accumulate during the ageing process in multiple tissues and in multiple species (Faragher *et al.* 2017). Senescent cells release a cocktail of pro-inflammatory cytokines and remodelling proteins termed the senescence-associated secretory phenotype (SASP), which triggers the establishment of senescence in neighbouring cells in a paracrine manner and acts to further stimulate inflammation in surrounding tissues (Salama *et al.* 2014). Mounting evidence suggests that the increased senescent cell load contributes directly to organismal ageing (de Magalhaes 2004) and age-related disease (van Deursen 2014); targeted depletion of senescent cells in transgenic mice improves multiple ageing phenotypes and extends lifespan (Baker *et al.* 2016; Baar *et al.* 2017). Understanding the fundamental biology of cellular senescence, and importantly, the factors that contribute to it, is thus of key importance.

One area that is emerging as a potential driver of cellular senescence and ageing phenotypes is dysregulation of alternative splicing (Deschenes & Chabot 2017; Latorre & Harries 2017). Fine control of gene expression is essential for control of cellular function,

plasticity and cellular identity. Changes in the expression of the regulatory machinery that govern splice site choice are seen in ageing human populations (Harries *et al.* 2011), in aged cells of multiple lineages (Holly *et al.* 2013), and are also linked with lifespan in animal models (Heintz *et al.* 2016; Lee *et al.* 2016). Age-related diseases as Alzheimer's disease, Parkinson's disease or cancer are also characterised by large-scale dysregulation of splicing, highlighting the importance of correct splicing for health throughout the life course (Latorre & Harries 2017). Splicing factors are good candidates for target genes to influence cell senescence, since several are tightly linked with control of proliferation, and some have roles in maintenance of telomere function (Kang *et al.* 2009; Anczukow *et al.* 2012). Loss of regulated alternative splicing in ageing tissues may therefore underpin the deterioration in response to intrinsic and extrinsic cellular stressors that characterises ageing in multiple species (Kourtis & Tavernarakis 2011) and has potential to be a major contributors to increased physiological frailty.

The upstream drivers of age-related dysregulation of splicing remain to be determined. Genes encoding splicing factors are themselves regulated by alternative splicing, and this unsurprisingly represents a strong contributor to their expression (Lareau & Brenner 2015). Regulation of the activity of splicing factors at the protein level is also known to be determined by the action of SRPK protein kinases, and also by PI3K/PTEN/AKT signalling at the level of phosphorylation and subcellular localisation (Blaustein *et al.* 2005; Bullock & Oltean 2017). Previous studies have suggested that some splicing factors may be regulated by alterations in RAF/MEK/ERK signalling (Tarn 2007). The concept of dysregulated cellular signalling during ageing is not a new one. The role of insulin/insulin-like growth factor 1 (IGF1/INS) signalling in ageing is well known and represents the first molecular pathway to be linked to ageing (Cohen & Dillin 2008); many genetic mutations within this pathway have

been shown to extend lifespan (Suh *et al.* 2008). Manipulation of the IGF-1/INS pathway by genetic modification or dietary restriction have also demonstrated the importance of these pathways in extension of human lifespan (van Heemst *et al.* 2005) and has also been associated with longevity in model systems (Slack *et al.* 2015). RAF/MEK/ERK and PI3K/PTEN/AKT signalling intersect just downstream of IGF-1/INS signalling, and are also activated by classical 'ageing' stimuli such as DNA damage, dysregulated growth factors and inflammation (Fontana *et al.* 2012; Lin *et al.* 2013).

Some studies have suggested that the use of MEK or PI3K inhibitors could prevent the induction of cellular senescence and ageing (Demidenko *et al.* 2009; Chappell *et al.* 2011). The NFκB pathway, which regulates the senescence-associated secretory phenotype (SASP) (Salminen *et al.* 2012), is also known to intersect with ERK and AKT signalling (Lin *et al.* 2012), suggesting that inflammatory changes could lie both upstream and downstream of RAF/MEK/ERK signalling. The relationship between these pathways is however not straightforward; there is crosstalk between them and also effects of dose, cell type and context (Rhim *et al.* 2016). Activation of ERK and AKT signalling by classical ageing stimuli such as DNA damage, inflammation or growth factors may therefore induce dysregulation of splicing factor expression and alternative splicing and influence cellular senescence.

It is an object of the present invention to provide a composition which is capable of one or more of the following: attenuating gene expression of *FOXO1* and/or *ETV6;* moderating splicing factor expression; or reducing or reversing cell senescence and/or re-entry to cell cycle. Ideally such a composition could be used as a therapeutic targeting an age-related disease or condition or cancer.

Summary of the Invention

In accordance with an aspect of the present invention, there is provided a composition comprising one or more intermediate non-coding RNA regulators which modulate the expression of ETV6 for use in the prevention, management, amelioration or treatment of an age-related disease or condition or cancer.

The age-related disease or condition or cancer may involve dysregulation of splicing factor expression and/or dysregulation of cellular senescence.

In accordance with a further aspect of the present invention, there is provided a composition comprising one or more intermediate non-coding RNA regulators which modulate the expression of *FOXO1* for use in the prevention, management, amelioration or treatment of an age-related disease or condition or cancer, wherein the age-related disease or condition or cancer involves dysregulation of splicing factor expression and/or dysregulation of cellular senescence.

In accordance with a yet further aspect of the present invention, there is provided a composition for modulating the expression of *FOXO1* and/or *ETV6*, the composition comprising one or more intermediate non-coding RNA regulators.

In accordance with a another aspect of the present invention, there is provided a composition for attenuating splicing factor expression, the composition comprising an expression modulator of *FOXO1* and/or *ETV6* or their downstream targets related to splicing factor regulation.

In accordance with yet a further aspect of the present invention, there is provided a composition for reducing or reversing cell senescence and/or re-entry to cell cycle, the

composition comprising an expression modulator of *FOXO1* and/or *ETV6* or their downstream targets related to splicing factor regulation.

It will be apparent to the skilled addressee that the compositions of the above aspects may comprise a separate modulator of *FOXO1* and a separate modulator of *ETV6*, or their individual or combined target genes. Alternatively, the composition may simply comprise a combined modulator of *FOXO1* and *ETV6 or their target genes*.

The present inventors have advantageously found that modulating the expression of *FOXO1* and/or *ETV6* targets the activity of downstream effectors of splicing and senescence and may therefore represent promising targets for a range of future therapeutics.

The modulation of *FOXO1* and/or *ETV6* or their target genes in the above aspects may be by using a number of types of molecules, such as inhibitors. An inhibitor is any molecule or molecules which limits, prevents or blocks the action or function of *FOXO1* and/or *ETV6* or any downstream effector molecules.

The expression modulator of *FOXO1* and/or *ETV6 may* comprise one or more intermediate non-coding RNA regulators. The one or more intermediate non-coding RNA regulators may comprise two or more intermediate non-coding RNA regulators.

The intermediate non-coding RNA regulators may comprise miRNAs, miRNA mimics or antagomiRs. In certain embodiments, the intermediate non-coding RNA regulators are selected from one or more of the following: MIR142; MIR3124; MIR3188; MIR3196; MIR320E; MIR330; MIR3675; MIR4316; MIR4488; MIR4496; MIR4513; MIR4674; MIR4707; MIR4772; MIR6088; MIR6129; MIR6780A; MIR6797; MIR6803; MIR6810; MIR6842; or MIR7155. More preferably, the intermediate non-coding RNA regulators are selected from

one or more of the following: MIR3124; MIR3675; MIR4496; MIR6780A; MIR6810; MIR6842; or MIR7155.

The biochemical and functional pathways enriched for FOXO1 or ETV6 target genes are exemplified in Table A below. Potentially, disrupting any one of the genes or pathways outlined in Table A may result in the modulation of *FOXO1* and/or *ETV6* expression.

Table A - Biochemical and functional pathways enriched for *FOXO1* or *ETV6* target genes

Term	P-value	Genes
ETV6		
Senescence-Associated Secretory Phenotype (SASP)_R-HSA-2559582	0.0002	IL1A;CDKN1A;HIST1H3A;ANAPC16;H2AFX;UBC;HIST1H2AD;HIST1H2AB
Mitotic Prophase_R-HSA-68875	0.0004	RAB1A;HIST1H3A;PRKCB;NEK6;H2AFX;HIST1H2AD;LPIN1;RAE1;HIST1H2AB
Cellular Senescence_R-HSA-2559583	0.0021	IL1A;CDKN1A;HIST1H3A;ANAPC16;SP1;UBC;H2AFX;HIST1H2AD;HIST1H1A;HIST1H2AB
M Phase_R-HSA-68886	0.0045	RAB1A;ANAPC16;PRKCB;NEK6;H2AFX;HIST1H3A;UBC;HIST1H2AD;KIF2C;CENPP;LPIN1;RAE1;HIST1H2AB
RMTs methylate histone arginines_R-HSA-3214858	0.0024	SMARCC2;HIST1H3A;H2AFX;HIST1H2AD;HIST1H2AB
Activation of HOX genes during differentiation_R-HSA-5619507	0.0027	HIST1H3A;NCOA3;H2AFX;POLR2E;HIST1H2AD;AJUBA;HIST1H2AB
Activation of anterior HOX genes in early embryogenesis_R-HSA-5617472	0.0027	HIST1H3A;NCOA3;H2AFX;POLR2E;HIST1H2AD;AJUBA;HIST1H2AB
RNA Polymerase I Promoter Opening_R-HSA-73728	0.0034	HIST1H3A;H2AFX;HIST1H2AD;HIST1H2AB
Transcriptional regulation by small RNAs_R-HSA-5578749	0.004	HIST1H3A;H2AFX;POLR2E;HIST1H2AD;RAE1;HIST1H2AB
Cell Cycle, Mitotic_R-HSA-69278	0.0091	TOP2A;RAB1A;CDKN1A;ANAPC16;PRKCB;NEK6;H2AFX;HAUS4;HIST1H3A;UBC;CEP72;HIST1H2AD;KIF2C;CENPP;LPIN1;AJUBA;RAE1;HIST1H2AB
FOX01		
Mitotic Prophase_R-HSA-68875	0.0003	RAB1A;HIST1H3A;NEK6;HIST1H2AD;LPIN1;RAE1;HIST1H2AB
Senescence-Associated Secretory Phenotype (SASP)_R-HSA-2559582	0.0003	IL1A;HIST1H3A;ANAPC16;UBC;HIST1H2AD;HIST1H2AB
Cellular Senescence_R-HSA-2559583	0.0008	IL1A;HIST1H3A;ANAPC16;SP1;UBC;HIST1H2AD;HIST1H1A;HIST1H2AB
M Phase_R-HSA-68886	0.0017	RAB1A;HIST1H3A;ANAPC16;NEK6;UBC;HIST1H2AD;KIF2C;LPIN1;RAE1;HIST1H2AB
Cell Cycle, Mitotic_R-HSA-69278	0.0044	TOP2A;RAB1A;ANAPC16;NEK6;HAUS4;HIST1H3A;UBC;HIST1H2AD;KIF2C;LPIN1;AJUBA;RAE1;HIST1H2AB
Transcriptional regulation by small RNAs_R-HSA-5578749	0.0018	HIST1H3A;POLR2E;HIST1H2AD;RAE1;HIST1H2AB
RMTs methylate histone arginines_R-HSA-3214858	0.0021	SMARCC2;HIST1H3A;HIST1H2AD;HIST1H2AB
Transcription-Coupled Nucleotide Excision Repair (TC-NER)_R-HSA-6781827	0.0023	CUL4A;COPS6;ERCC1;UBC;POLR2E
Cellular responses to stress_R-HSA-2262752	0.0054	IL1A;HIST1H3A;ANAPC16;SP1;UBC;HSPA6;HIST1H2AD;LAMTOR4;RAE1;HIST1H1A;HIST1H2AB
Formation of TC-NER Pre-Incision Complex_R-HSA-6781823	0.0039	CUL4A;COPS6;UBC;POLR2E

Cellular plasticity is a key facet of cellular homeostasis requiring correct temporal and spatial patterns of alternative splicing. Splicing factors, which orchestrate this process, demonstrate age-related dysregulation of expression, and are emerging as potential influences on ageing and longevity. The upstream drivers of these alterations are still unclear, but may involve aberrant cellular signalling.

The inventors compared the phosphorylation status of proteins in multiple signalling pathways in early and late passage human primary fibroblasts and determined the responses of 'young' cells to cytokines, known activators of ERK and AKT signalling. They then assessed the impact of chemical inhibition, or targeted knockdown of direct downstream targets of the ERK and AKT pathways, on splicing factor expression, cellular senescence and proliferation kinetics in senescent primary human fibroblasts.

Surprisingly, and unexpectedly, components of both ERK and AKT signalling pathways demonstrated increased activation during cellular ageing. Early passage cells exposed to cytokines also demonstrated alterations in splicing factor expression. Inhibition of AKT and ERK pathways led to upregulation of splicing factor expression, reduction in senescent cell load and reversal of multiple cellular senescence phenotypes in a dose-dependent manner.

The dose of the inhibitor of ERK and/or inhibitor of AKT in the above compositions will preferably be low. During experimentation, the inventors have unexpectedly found low dose chemical inhibition of either ERK or AKT signalling at 1μM for 24 hours resulted in restoration of splicing factor expression to levels consistent with those seen in younger passage cells, reversal of senescence and re-entry to cell cycle for a proportion of the cells tested.

In a further aspect of the present invention, there is provided a composition capable of modulating splicing factor expression, the composition comprising one or more

compounds able to bind to, with, or inhibit, *FOXO1* and/or *ETV6* genes. Preferably, the composition comprises one or more compounds able to bind to, or inhibit, *FOXO1* and *ETV6* genes (or other FOXO or ETS family member genes) or gene products thereof.

The inventors advantageously noted that the targeted knockdown of the genes encoding downstream targets *FOX01* or *ETV6* was sufficient to mimic the observations found in respect of ERK and AKT inhibition.

The compositions of the above aspects may have a number of uses, from laboratory reagents and research tools to medicaments. In respect of laboratory reagents, the compositions may be used as research tools for investigating the effect of reduced gene expression of *FOXO1* and/or *ETV6; or* restoring and/or increasing splicing factor expression; or reducing or reversing cell senescence and/or re-entry to cell cycle. The compositions may also be used as a way of reducing or reversing cell senescence and/or re-entry to cell cycle for cell culture, including stem cell culture for research and therapeutic application. The compositions could be used to increase viable number of passages in cell culture and/or reduce senescent cell populations.

The compositions may comprise inhibitors of ERK or AKT signalling.

The compositions may be in the form of a pharmaceutical preparation.

The compositions may be for use as a medicament.

Advantageously, the results produced by the inventors suggest that age-associated dysregulation of splicing factor expression and cellular senescence may derive in part from altered activity of ERK and AKT signalling, and act through the ETV6 and FOXO1 transcription factors. Targeting the activity of downstream effectors of ERK and AKT may therefore represent promising targets for future therapeutic intervention.

The compositions may be for use in the prevention, management, amelioration or treatment of an age-related disease or condition.

The compositions may be for use in a method of prevention, management, amelioration or treatment of an age-related disease or condition, the method comprising administering an therapeutically effective amount of the composition to a subject in need thereof.

In a related aspect, the invention may comprise the composition, for use in the manufacture of a medicament for the prevention, management, amelioration or treatment of an age-related disease or condition.

The age-related disease or condition may encompass a number of age-related conditions such as Alzheimer's disease, cardiovascular disease, hypertension, arthritis, osteoporosis, type 2 diabetes, cancer, Parkinson's disease, cognitive dysfunction or frailty. The age-related disease or condition may also encompass a number of conditions suffered by younger subjects who are suffering from certain conditions results in premature aging, termed progeroid syndromes – such as Werner syndrome and Hutchinson-Gilford progeria.

The compositions may be for use in the prevention, management, amelioration or treatment of cancer.

The compositions may be for use in a method of prevention, management, amelioration or treatment of cancer, the method comprising administering an therapeutically effective amount of the composition to a subject in need thereof.

In a related aspect, the invention may comprise the composition, for use in the manufacture of a medicament for the prevention, management, amelioration or treatment of cancer.

The compositions may also be for use as a nutraceutical or cosmetic product so as to reduce the effects of aging.

In accordance with a further aspect of the present invention, there is provided a related combination of an inhibitor of ERK and an inhibitor of AKT for:

a) modulating gene expression of *FOXO1* and/or *ETV6* or their target genes;

b) restoring and/or increasing splicing factor expression; or

c) reducing or reversing cell senescence and/or re-entry to cell cycle.

As used herein, the terms "treatment", "treating", “treat” and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect can be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or can be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein, covers any treatment of a disease in a mammal, particularly in a human, and includes: (a) preventing the disease from occurring in a subject which can be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e., arresting or slowing its development; and (c) relieving the disease, i.e., causing regression of the disease.

The term “subject” used herein includes any human or nonhuman animal. The term “nonhuman animal” includes all mammals, such as nonhuman primates, sheep, dogs, cats, cows, horses.

A “therapeutically effective amount” refers to the amount of composition that, when administered to a subject for treating a disease, is sufficient to affect such treatment for the disease. The “therapeutically effective amount” will vary depending on the pharmaceutically

active ingredient used, the disease and its severity and the age, weight, etc., of the subject to be treated.

In general, routes of administration contemplated by the invention include, but are not necessarily limited to, enteral, parenteral, or inhalational routes.

Parenteral routes of administration other than inhalation administration include, but are not necessarily limited to, topical, transdermal, subcutaneous, intramuscular, intraorbital, intracapsular, intraspinal, intrasternal, intrathecal, and intravenous routes, *i.e.*, any route of administration other than through the alimentary canal. Parenteral administration can be carried to effect systemic or local delivery. Where systemic delivery is desired, administration typically involves invasive or systemically absorbed topical or mucosal administration of pharmaceutical preparations. Enteral routes of administration include, but are not necessarily limited to, oral and rectal (*e.g.*, using a suppository) delivery.

Conventional and pharmaceutically acceptable routes of administration include intranasal, intramuscular, intra-tracheal, intrathecal, intracranial, subcutaneous, intradermal, topical, intravenous, intraperitoneal, intra-arterial (for example, via the carotid artery), spinal or brain delivery, rectal, nasal, oral, and other enteral and parenteral routes of administration.

In some embodiments, a composition of the invention, or a combination of the invention, may be administered with one or more other compounds effective for the prevention, management, amelioration or treatment of an age-related disease or condition or cancer.

In an alternative aspect of the present invention, there is provided the use of a composition comprising one or more intermediate non-coding RNA regulators which

modulate the expression of ETV6 for the cosmetic treatment of the effects of ageing. The effects of aging may involve dysregulation of splicing factor expression and/or dysregulation of cellular senescence.

In a yet further aspect of the present invention, there is provided the use of a composition comprising one or more intermediate non-coding RNA regulators which modulate the expression of FOXO1 for the cosmetic treatment of the effects of ageing, where the effects of aging involves dysregulation of splicing factor expression and/or dysregulation of cellular senescence.

The term "cosmetic treatment" is intended to mean any non-medical treatment which may be systemically or topically applied to a human or animal.

The term, "effects of aging" is intended to mean progressive (but not disesase-based) physiological changes in a human or animal that lead to senescence, or a decline of biological functions and its ability to adapt to metabolic stress.

The modulator or modulators of *FOXO1* and/or *ETV6* or their target genes may be artificially generated. That is to say that it is not naturally occurring. The modulator or modulators of *FOXO1* and/or *ETV6* and their target genes may however be a naturally occurring molecule or molecules whose concentration and formulation in a medicament or pharmaceutical preparation or combination enables it to be used for the prevention, management, amelioration or treatment of an age-related disease or condition or cancer, whereas otherwise it would have no or limited efficacy. Whilst the inhibitor or inhibitors may be a naturally occurring molecule or molecules, it will be understood that the concentration and formulation of the molecule or molecules found to be therapeutically effective would not be present in nature at such a concentration or in a formulation with other components.

The inhibitor or inhibitors may comprise an antibody or antibodies or antibody mixture. Such antibody or antibodies may be polyclonal or may be monoclonal. It will be apparent to the skilled addressee how to produce antibodies which would act as inhibitors. Preferably the antibodies will be humanised.

In other embodiments, the inhibitor or inhibitors comprise a peptide or peptide mimetic thereof, or C-terminal amidated peptide thereof.

The terms "peptide" and "peptides" include compounds that have amino acid residues (H-Cα-[side chain]) but which may be joined by peptide (-CO-NH-) or non-peptide linkages.

Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis.

The peptide may be a peptide aptamer. Peptide aptamers typically consist of short, 5-20 amino acid residues long sequences that can bind to a specific target molecule.

There are a number of different approaches to the design and synthesis of peptide composition that do not contain amide bonds. In one approach, one or more amide bonds are replaced in an essentially isoteric manner by a variety of chemical functional groups.

Retro-inverso peptidomimetics, in which the peptide bonds are reversed, can be synthesised by methods known in the art. This approach involves making pseudopeptides containing changes involving the backbone, and not the orientation of side chains. Retro-inverse peptides, which contain NH-CO bonds instead of CO-NH peptide bonds, are more resistant to proteolysis.

The peptide may be linear. Although, it may be advantageous to introduce a cyclic moiety into a peptide-based framework. The cyclic moiety restricts the conformational space of the peptide structure and this may lead to an increased efficacy. An added advantage of this strategy is that the introduction of a cyclic moiety into a peptide may also result in the peptide having a diminished sensitivity to cellular peptidases.

In some embodiments of the invention the peptide may be joined to another moiety. Convenient moieties to which the peptide may be joined include polyethylene glycol (PEG) and peptide sequences, such as TAT and antennapedia which enhance delivery to cells.

In some embodiments, the inhibitor or inhibitors is/are pro-drugs of the peptide. A pro-drug is a compound which is metabolised *in vivo* to produce the molecule, such as a protein. One of skill in the art will be familiar with the preparation of pro-drugs.

The peptide may be a peptide mimetic. A peptide mimetic is an organic compound having similar geometry and polarity to the molecules defined herein, and which has a substantially similar function. A mimetic may be a molecule in which the NH groups of one or more peptide links are replaced by CH_2 groups. A mimetic may be a molecule in which one or more amino acid residues is replaced by an aryl group, such as a napthyl group.

In other embodiments, an inhibitor or inhibitors comprise nucleic acid, such as single stranded DNA or RNA, which is capable of binding to and inhibiting downstream effectors of *FOXO1* and/or *ETV6* or their target genes. It is envisaged that the same targets are also suitable for targeting with peptides and peptide aptamers will also be suitable for targeting with RNA or modified RNA aptamers. Nucleic acids such as single stranded DNAs and RNAs may be provided that bind to and inhibit downstream effectors of *FOXO1* and/or *ETV6* or

their target genes. Typically, the nucleic acids are single stranded and have from 100 to 5000 bases.

In yet other embodiments, an inhibitor or inhibitors comprise a small molecule or small molecules.

It will be apparent to the skilled addressee that if the composition is intended to comprise one or more compounds able to bind to, or modulate *FOXO1* and/or *ETV6* gene expression or gene products thereof.

Features, integers, characteristics, compounds, molecules, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith. All of the features disclosed in this specification (including any accompanying claims, abstract and figures), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive. The invention is not restricted to the details of any foregoing embodiments. The invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including any accompanying claims, abstract and drawings), or to any novel one, or any novel combination, of the steps of any method or process so disclosed.

Detailed Description of the Invention

Embodiments of the invention are described below, by way of example only, with reference to the accompanying figure in which:

Figure 1 shows the differences in proliferative cell fraction between early passage and late passage (senescent) cell cultures. Early and late passage cell populations were subjected to a 24hr label with the S-phase marker BrdU, to selectively stain actively growing cells. *** = p = <0.0001, n = 3 biological replicates, 300 nuclei counted per replicate;

Figure 2 shows age-related changes in protein phosphorylation for targets in the ERK and AKT pathways. **A**. Is a graph showing phosphorylated protein expression of key targets from AKT and ERK pathways were assessed in young (PD=25) and senescent (PD=63) cells. White and grey bars represent young and senescent cell lysates respectively. **B**. Is a schematic diagram showing the genes tested for senescence-related phosphorylation differences in the AKT and ERK pathways. Targets showing significantly different levels of phosphorylation are highlighted in bold underlined text. Targets showing no significant differences in protein phosphorylation are indicated in normal typeface. Statistical significance is indicated by stars with *p<0.05. Error bars represent the standard error of the mean;

Figure 3 shows chemical inhibition of ERK or AKT signalling is associated with rescue from cellular senescence phenotypes. **A**. The proportion of cells staining positive for Senescence associated β-galactosidase (SA-β-Gal) activity following treatment with trametinib and SH-6 at 1 or 10μM was determined by manually counting the percentage of SA-β gal positive cells. N = >300 cells for each sample. **B**. Levels of senescence-associated transcript *CDKN2A*, which encodes the senescence marker p16 was assessed in senescent cells by qRTPCR. Data are expressed relative to stable endogenous control genes *GUSB*, *IDH3B* and *PPIA*, and are given normalised to the levels of the individual transcripts as present in vehicle-only treated control cells. Fold change was calculated for in triplicate for three biological replicates. Statistical significance is indicated by ** p<0.005, *** p<0.0001 (2 way ANOVA). **C**. Protein levels of various pro-inflammatory SASP factors following treatment with

ERK and AKT inhibitors at 1μM and 10μM. **D**. Expression levels of *CDKN1A* transcripts encoding the DNA damage response protein p21. **E**. Is a heat map indicating fold changes in SASP protein expression. Green indicates up-regulation whilst red denotes down-regulation. Only statistically significant changes are presented in the heat map. The colour scale refers to percentage change in expression. Experiments were carried out in duplicate for a total of 6 biological replicates. Error bars represent the standard error of the mean;

Figure 4 shows the effect of ERK or AKT inhibitors on splicing factor expression and senescent cell load under conditions that do not permit cell proliferation. To establish whether apparent 'rejuvenation' of senescent cell cultures was derived from altered proliferation kinetics of non-senescent cells in the culture, or arise from a genuine rescue in response to treatment, selected experiments were repeated under conditions of serum starvation, where cells were prevented from dividing. **A**. Ki67 staining showed cells were non-proliferative, whereas **B**. SA-b-Gal staining revealed rescue was still evident. *** = p = <0.0001, n = 3 biological replicates, 300 nuclei counted per replicate;

Figure 5 shows changes in the phosphorylation status of ERK and AKT signalling proteins, and also proteins in linked signalling pathways in response to low dose ERK or AKT inhibition. White, light grey, light grey hatched, dark grey and dark grey hatched boxes represent controls, low dose trametinib, high dose trametinib, low dose SH-6 and high dose SH-6 respectively. Statistical significance is indicated by stars with *p<0.05. Error bars represent the standard error of the mean. *** = p = <0.0001. Data are derived from 3 biological and 2 technical replicates;

Figure 6 shows inhibition of AKT or ERK pathways affects splicing factor transcript expression and cell proliferation rate. **A**. The change in splicing factor mRNA levels in response to 24hr treatment with ERK or AKT inhibitors at 1 μM and 10μM are given. Green

indicates up-regulated genes, red denotes down-regulated genes. The colour scale refers to fold-change in expression. Only statistically significant changes are presented in the heat map **B**. Proliferation index was assessed for treated cells as assessed by Ki67 immunofluorescence (>400 nuclei counted per sample). **C**. Cell counts following treatment with 1μM and 10μM ERK or AKT inhibitors. **D**. Telomere length as quantified by qPCR relative to the 36B4 endogenous control and normalised to telomere length in vehicle-only control. **E.** Apoptotic index in senescent cells treated with inhibitors as determined by TUNEL assay. Data are derived from duplicate testing of 3 biological replicates. Statistical significance is indicated by ** $p<0.005$, *** $p<0.0001$. Error bars represent the standard error of the mean;

Figure 7 shows the cellular and molecular effects of targeted knockdown of *ETV6* and *FOXO1* genes. **A**. Levels of splicing factor expression following *FOX01*, *ETV6* or *ETV6/FOX01* gene knockdown. Green indicates up-regulated genes, red denotes down-regulated genes. The colour scale refers to fold-change in expression. Only statistically significant changes are presented in the heat map **B.** Senescent cell load as indicated by SA-β-Gal following *FOX01, ETV6* and *ETV6/FOX01* gene knockdown. n>300 cells for each sample. **C.** Senescent cell load as indicated by *CDKN2A* gene expression following *FOX01, ETV6* and *ETV6/FOX01* gene knockdown. Data are expressed relative to stable endogenous control genes *GUSB*, *IDH3B* and *PPIA*, and normalised to the levels of the individual transcripts in vehicle only controls. **D**. Proliferation index was assessed following *FOX01, ETV6* and *ETV6/FOX01* gene knockdown by Ki67 immunofluorescence (>400 nuclei counted per sample). **E**. The effect of *FOX01* or *ETV6* gene knockdown on reciprocal expression of *ETV6* and *FOXO1* genes respectively. Data are expressed relative to stable endogenous control genes *GUSB*, *IDH3B* and *PPIA*, and normalised to the levels of the individual transcripts in control. Data are derived from

duplicate testing of 3 biological replicates. Statistical significance is indicated by *p<0.05, ** p<0.005, *** p<0.0001. Error bars represent the standard error of the mean;

Figure 8 shows the effect of FOXO1 and/or ETV6 knockdown achieved by a second methodology, siRNA. The effects of *ETV6* or *FOXO1* gene knockdown were confirmed by siRNA against the genes in question. Levels of knockdown were determined to be 43% for ETV6 and 65% for FOXO1. The effects of gene knockdown on splicing factor expression (**A**), *ETV6* and *FOXO1* expression (**B**); the identity of the gene manipulated is given on the X axis. The first 4 bars refer to effects on *FOXO1* gene expression and the latter to effects on *ETV6* expression. Effects on senescent cell load (**C**) and *CDKN2A* expression (**D**) were also examined. Only statistically significant changes are presented in the heat map *** = p = <0.0001, n = 3 biological replicates. Data are from 3 independent biological replicates;

Figure 9 shows the effect of ERK or AKT inhibition on FOXO1 and ETV6 transcript expression and subcellular localisation. A. The effects of ERK and AKT inhibition on ETV6 and FOXO1 expression; The treatment is indicated on the X axis. The first 5 bars refer to effects on FOXO1 expression, the second 5 bars refer to effects on ETV6 expression. Data are from 3 independent biological replicates each with 3 technical replicates. B. The effects of ERK and AKT inhibition on ETV6 and FOXO1 protein subcellular localisation; the treatment is indicated on the X axis. The first 5 bars refer to effects on ETV6 localisation, the second 5 bars refer to effects on FOXO1 localisation. *** = p = <0.0001. Data are from at least 50 cells from each of 3 independent biological replicates; and

Figure 10 is a schematic diagram showing the indirect control of senescence as elucidated by the experiments conducted by the inventors.

Example

The aims of the experiments in the example were to study the effect of manipulation of ERK and AKT signalling pathways by chemical inhibition or targeted gene knockdown on splicing factor expression and cellular senescence and proliferation kinetics in late passage human primary fibroblasts.

1. Increased phosphorylation of target proteins in the MEK/ERK and PI3K/AKT pathways in senescent cells

Cell cultures were considered senescent when the population doubling time had slowed to <0.5 cell divisions per week. At this point, ~3% of cells stained positive for the S-phase marker BrdU, which indicates actively replicating DNA (figure 1). Late passage primary human dermal fibroblasts were found to have reached senescence at PD = 63. Earlier passage cells were at PD = 25. We noted increased phosphorylation was noted for AKT, CREB, ERK, GSK3α, MEK, and MSK2 signalling proteins in late passage fibroblasts, whereas no increase in phosphorylation of GSK3β, HPS27, JNK, MKK3, MKK6, mTOR, p38, p58, p70SK6 or RSK1 proteins was apparent in these cells (figure 2A). Several of these targets lie in the AKT or ERK signalling pathways (figure 2B).

2. Inhibition of MEK/ERK and PI3K/AKT pathways rescue cellular senescence phenotypes

We assessed the effects of targeted inhibition of AKT or MEK/ERK in late passage human primary fibroblasts using specific inhibitors. Treatment with SH-6 (AKT) or Trametinib (MEK/ERK) at both 1μM and 10μM resulted in a specific reduction in AKT or ERK protein phosphorylation for SH-6 and trametinib respectively, at both high and low dose (figure 3A). This was accompanied by a small but robust decrease in the senescent cell population as indicated by reduced SA-β-Gal staining ($p = <0.0001$; figure 3B) and a decrease in the

expression of both p16 and p21 (figure 3C and figure 3D). Reduction in levels of several secreted SASP proteins were also noted in cells treated with both low and high dose trametinib and SH-6 (table 1 below; figure 3E).

Table 1: The changes in SASP protein expression in response to MEK/ERK or AKT inhibition

	Control	Trametinib 1uM	Trametinib 10uM	SH-6 1uM	SH-6 10uM
GM-CSF	100	**24.65*** (1.76)**	**9.85*** (0.99)**	**47.63** (2.27)**	129.67 (10.16)
IL10	100	**34.37*** (11.05)**	86.01 (29.30)	**52.94** (5.07)**	97.78 (25.08)
IL1b	100	**156.70** (26.51)**	**50.35** (4.12)**	115.68 (21.8)	84.22 (9.52)
IL2	100	**50.72** (17.36)**	**14.39*** (2.56)**	**52.78* (11.25)**	**58.26* (12.62)**
IL6	100	77.75 (2.63)	**33.57*** (3.57)**	83.47 (0.93)	**66.18* (11.87)**
IL8	100	**44.42** (2.43)**	**5.33*** (0.51)**	**48.21** (0.88)**	88.68 (5.41)
TNFa	100	104.61 (8.05)	118.65 (6.92)	109.81 (6.83)	**164.04** (15.86)**
INF-g	100	95.08 (9.81)	106.33 (6.39)	96.65 (1.11)	117.02 (6.79)
IL12p70	100	69.64 (4.40)	77.78 (3.02)	67.45 (3.14)	100.47 (10.97)

(Protein levels of pro-inflammatory components of the senescence associated secretory phenotype (SASP) were determined using Mesoscale ELISA platform in the culture medium from senescent cells treated with the ERK inhibitor trametinib or the AKT inhibitor SH-6 at 1 μM or 10μM for 24hrs. The results are expressed as a percentage of the control value (vehicle only) of 3 independent experiments. Standard error of the mean (SEM) is given in parentheses. Statistical significance is indicated by stars with *$p<0.05$, **$p<0.005$ and ***$p<0.001$ compared with the corresponding control value.)

To establish whether the drop in the senescent cell fraction was due to active 'rescue', or merely due to increased proliferation of the growth arrested cells in the culture, we repeated the treatments under conditions of serum starvation, which inhibits cell proliferation. An approximately 20% reduction of SA-β-Gal positive cells was also noted in serum starved cells, indicating that the effect was not derived merely from altered culture

division kinetics (figure 4). This is in accordance with our previous findings (Latorre *et al.* 2017).

3. Inhibition of MEK/ERK and P13K/AKT alters target protein phosphorylation status both upstream and down-stream of ERK and AKT.

To investigate potential downstream targets which might be influencing cellular senescence, we assessed the effect of ERK and AKT inhibition on downstream signalling targets. As expected, changes to the phosphorylation levels of ERK and AKT in response to both high and low dose pathway inhibition were observed, along with changes to the phosphorylation status of GSK3α, GSK3β, p70SK6 and CREB downstream targets. Down regulation of GSK3β was evident for both high and low concentrations of trametinib, but high dose SH-6 only, whereas GSK3α phosphorylation demonstrated decreased levels at low dose and increased levels at high dose for both trametinib and SH-6 alone, although results were only statistically significant for high dose SH6 and low dose trametinib (figure 5A). This pattern of antagonistic action was also apparent for upstream kinases including p38. Several other upstream targets including p53, MKK3, MKK6, and MEK demonstrated differences in phosphorylation status after treatment, with effects being most commonly at high dose (figure 5B). Altered p53 phosphorylation is consistent with cells being restored to a more proliferative and less senescent state by treatment with AKT or ERK inhibitors (Pise-Masison *et al.* 1998).

4. ERK and AKT pathways influence splicing factor expression

Dysregulated splicing factor expression have been implicated in cellular senescence and ageing in human populations and cells (Harries *et al.* 2011; Holly *et al.* 2013) and in animal models (Heintz *et al.* 2016; Lee *et al.* 2016). Furthermore, restoration of splicing factor

expression using small molecules has been associated with rescue of cellular senescence in our previous work (Latorre *et al.* 2017). We therefore assessed the expression of an a priori list of 20 splicing regulator genes previously demonstrated to show senescence-related changes in late passage human fibroblasts treated with low (1μM) and high (10μM) dose AKT and ERK inhibitors (SH-6 and trametinib respectively). Treatment with both trametinib and SH-6 at low dose (1μM) was associated with upregulation of multiple splicing factors (figure 6A; table 2 below).

Table 2: Changes in splicing factor expression in response to low (1μM) and high (10μM) dose MEK/ERK or AKT inhibitors.

	Control	Trametinib 1uM	Trametinib 10uM	SH-6 1uM	SH-6 10uM
AKAP17A	1.00 (0.02)	**2.01*** (0.21)**	**0.74** (0.04)**	**1.38*** (0.07)**	**0.51*** (0.05)**
HNRNPA0	1.00 (0.01)	**2.38*** (0.16)**	1.11 (0.15)	**1.27*** (0.05)**	**0.64*** (0.03)**
HNRNPA1	1.00 (0.01)	**1.52* (0.34)**	**0.49*** (0.09)**	**1.17*** (0.04)**	1.01 (0.09)
HNRNPA2B1	1.00 (0.01)	**1.53* (0.25)**	**0.70*** (0.02)**	0.98 (0.06)	**0.88* (0.07)**
HNRNPH3	1.00 (0.01)	**1.89*** (0.17)**	0.95 (0.03)	**1.20** (0.07)**	**0.88* (0.05)**
HNRNPK	1.00 (0.02)	**1.68*** (0.13)**	1.02 (0.06)	**1.14** (0.04)**	0.99 (0.07)
HNRNPM	1.00 (0.01)	**1.46* (0.22)**	**0.69*** (0.05)**	1.05 (0.05)	**0.68** (0.01)**
HNRNPUL2	1.00 (0.01)	**1.63*** (0.14)**	0.94 (0.05)	1.06 (0.06)	1.04 (0.07)
IMP3	1.00 (0.01)	**1.77*** (0.26)**	0.97 (0.03)	0.99 (0.02)	0.93 (0.05)
LSM14A	1.00 (0.01)	**1.61** (0.26)**	1.18 (0.07)	**1.10* (0.04)**	0.89 (0.08)
LSM2	1.00 (0.04)	0.85 (0.14)	0.82 (0.10)	0.93 (0.02)	**1.43* (0.28)**
PNISR	1.00 (0.02)	**1.63** (0.21)**	1.11 (0.03)	0.99 (0.02)	0.99 (0.13)
HNRNPD	1.00 (0.03)	**1.79* (0.38)**	**0.75** (0.07)**	**1.21*** (0.15)**	0.95 (0.13)
SF3B1	1.00 (0.02)	**1.93*** (0.14)**	0.96 (0.07)	**1.29*** (0.03)**	**0.86* (0.07)**
SRSF1	1.00 (0.01)	**1.69* (0.37)**	**0.68** (0.14)**	**1.19*** (0.03)**	**0.83** (0.05)**
SRSF2	1.00 (0.02)	**2.07** (0.42)**	**0.86* (0.04)**	**1.16* (0.08)**	0.93 (0.20)
SRSF3	1.00 (0.03)	**1.64*** (0.10)**	**0.72*** (0.02)**	**1.17*** (0.04)**	0.92 (0.05)
SRSF6	1.00 (0.02)	**2.68*** (0.47)**	**0.69*** (0.03)**	**1.49*** (0.07)**	**0.88* (0.05)**
TRA2B	1.00 (0.02)	**1.66* (0.33)**	**0.73** (0.11)**	1.09 (0.07)	0.94 (0.13)
SRSF7	1.00 (0.01)	**2.38*** (0.32)**	**0.57*** (0.02)**	**1.26** (0.07)**	**0.71*** (0.04)**

(Changes to mRNA levels in senescent primary human fibroblasts in response to treatment with ERK inhibitors (trametinib) or AKT inhibitors (SH-6) at 1 μM or 10μM for 24hrs. Data are derived from duplicate testing of 3 biological replicates. Standard error of the mean (SEM) is

given in parentheses. Statistical significance is indicated by stars with *p<0.05, **p<0.005 and ***p<0.0001 compared with the corresponding control value.)

Effects were more marked for MEK/ERK inhibited cells than for AKT inhibited cells. Surprisingly, high dose inhibitors (10μM) demonstrated an antagonistic effect on splicing factor expression (figure 6A; table 2). This dose-dependent response was also noted for proliferation kinetics (figure 6B,C). Low dose trametinib led to a 31% increase in proliferative index, whereas low dose SH-6 resulted in a 27% increase in Ki67 staining (p = <0.0001 and <0.0001 respectively). High dose treatment with either inhibitor resulted in no reactivation of proliferation (figure 6B, C). These data demonstrate a clear link between re-entry to cell cycle and splicing factor expression. No rescue of telomeres was apparent after any treatment (figure 7D) and no increase in apoptotic index was noted with any treatment (figure 7E).

5. ETV6 and FOXO1 are regulators of splicing factor expression and cell senescence phenotypes.

ERK and AKT signalling have multiple downstream effector pathways, with significant evidence of crosstalk and autoregulation (Rhim *et al.* 2016). In order to clarify mechanism, it was necessary to identify the downstream effector genes to give a cleaner assessment of phenotype upon manipulation. Targeted deletion of the *Foxo* and *Aop* genes have been reported to be associated with increased lifespan in *D. Melanogaster* (Slack *et al.* 2015), and the closest human homologues of these are *FOXO1* and *ETV6* (Jousset *et al.* 1997; Kramer *et al.* 2003). Targeted gene knockdown with morpholino oligonucleotides revealed that down-regulation of either *FOXO1* or *ETV6* in late passage human primary fibroblasts resulted in a similar rescue of splicing factor gene expression to that seen with AKT or ERK inhibition, although this time the results were more marked for *FOXO1* (figure 7A). Changes in splicing factor expression were accompanied by an approximate 18% and 40% reduction in senescent

cell load as measured by SA-β-Gal staining for *ETV6* and *FOXO1* knockdown respectively (p = <0.0001 and 0.0001; figure 7B) and a corresponding 24% and 43% reduction in the expression of *CDKN2A* expression was also noted (p = <0.05 and <0.005 for *ETV6* and *FOXO1* respectively; figure 7C). This represents a more marked change than that noted with inhibition of either ERK or AKT signalling as a whole. Again, as we noted for splicing factor expression, effects were strongest in cells where *FOXO1* expression had been manipulated. Cell proliferation was also restored in late passage fibroblasts in which expression of *ETV6* or *FOXO1* had been abrogated. *ETV6* knockdown led to a 36% increase in proliferative index, whereas abrogation of *FOX01* resulted in a 19% increase (p = <0.0001 and <0.005 respectively; figure 4D). The effects of *ETV6* or *FOXO1* gene knockdown was also confirmed at the level of splicing factor expression, cell senescence and *CDKN2A* expression by the use of siRNAs targeted against the genes in question (figure 8).

6. Significant cross-regulation exists between AKT and ERK signalling pathways

Cross-regulatory relationships were evident between both ERK and AKT signalling pathways, and also between *ETV6* and *FOXO1* themselves; dual knockdown of both *ETV6* and *FOXO1* genes caused abrogation of rescue from multiple senescence phenotypes (figure 7B-D). Part of the co-regulation may partly be at the level of transcriptional interaction. *ETV6* gene knockdown affected both *ETV6*, and *FOXO1* gene expression, whereas *FOXO1* gene knockdown was associated with down-regulation of *FOXO1* levels alone (figure 8B). A similar cross-regulation is noted in the response of *FOXO1* and *ETV6* gene expression to ERK or AKT inhibitors. Treatment of senescent cells with low dose trametinib yielded induction of both *ETV6* and *FOXO1* expression at the mRNA level, whilst treatment with low dose SH-6 caused upregulation of *FOXO1* alone (figure 9A). This may represent a compensatory counter-regulation at the level of mRNA expression although this remains to be established. High

doses of SH-6 caused a significant down-regulation of both *FOXO1* and *ETV6* expression, whereas higher doses of trametinib were associated with altered *FOXO1* expression only (figure 9A). Trametinib or SH-6 treatment also caused some small but significant changes in the subcellular localisation of ETV6 and FOXO1 proteins. Low dose trametinib was associated with more nuclear retention of ETV6 protein, but less nuclear FOXO1 protein. High dose trametinib was associated with less nuclear FOXO1 protein alone. Low dose SH-6 was associated with significantly lower ETV6 nuclear retention but FOXO1 subcellular localisation was unaffected. High dose SH-6 caused reductions in nuclear localisation of both ETV6 and FOXO1 proteins (figure 9B). These data indicate that interplay between FOXO1 and ETV6 occurs at the level of both transcription and protein localisation, as well as cross regulation at the level of protein activity as presented here and in previously published data (Rhim *et al.* 2016). Cross regulation is typical of these signalling pathways and merits future exploration.

7. Splicing factors are indirect targets of FOXO1 and ETV6.

The target genes of FOXO1 and ETV6 were compared by analysis of publically-available Chromatin immunoprecipitation (ChIP) datasets from human cell types. 419 genes were identified that were targets of ETV6 and 242 which were targets of FOX01. All 242 targets of FOX01 were also targets of ETV6. Two splicing factors (HNRNPF and HNRNPLL) were direct targets of ETV6, but most splicing factors were not directly targeted, suggesting that their regulation by these proteins is indirect. FOXO1 and ETV6 target genes comprised several molecular functions, but surprisingly, almost a quarter of genes (58/242) targeted by both ETV6 and FOXO1 comprised non-coding RNA regulators (miRNAs, snoRNAs, lncRNAs), transcription factors or cell signalling proteins (Table 3 below).

Table 3 - Common target genes of FOXO1 and ETV6 identified from publically-available ChIP datasets

Gene	Function
ACRBP	Acrosome binding protein
ADIPOQ-AS1	ncRNA
AGFG2	Nucleocytoplasmic transport of RNAs and proteins
AGTRAP	Negative regulation of angiotensin
AJUBA	CDK phosphorylation
ANAPC16	involved in mitosis
ANKRD52	regulatory subunit of protein phosphatase 6
AP4B1	Targets proteins to lysosomes
AP4B1-AS1	ncRNA
APOA1BP	Interacts with apolipoprotein A-I
ARHGAP25	Cell signalling
ARL8B	Potential role in lysosome motility
ASCC1	Transcriptional coactivator
ATF3	Transcription factor
BHLHA15	Transcriptional regulator
BIN3	Intracellular transport
BRD3	Ser/Thr kinase
BZRAP1-AS1	ncRNA
C16orf70	unknown function
C2orf42	unknown function
C4BPA	Activation of complement cascade
CCDC153	unknown function
CD27	TNFα family submember
CD27-AS1	ncRNA
CD4	Interaction with MHC
CD44	Cell:Cell interactions
CD79A	Ig-alpha component of B-cell antigen
CEACAM3	Related to innate immune system
CENPBD1P1	Pseudogene
CLCN6	Ion channel protein
CLTC	component of intracellular organelles
COPS6	Signalling protein
CST6	Cysteine protease inhibitor
CTCFL	Transcription regulator
CUL4A	DNA damage response
CYB561A3	Oxidoreductase activity
DAK	dihydroxyacetone kinases
DCLRE1B	DNA repair
DCTPP1	dCTP pyrophosphatase
DMTF1	Regulation of cell cycle
DPAGT1	Glycoprotein biosynthesis
DRAP1	Regulation of transcriptional initiation

EGR3	Transcription factor
ELMSAN1	Chromatin binding
EML2	Cell division
EMR2	G coupled protein receptor
EPS15	Signalling protein
ERCC1	DNA repair
ERVK13-1	ncRNA
EZH1	Gene regulation
FAM63A	Deubiquitinase
FIZ1	Transcriptional regulator
FLJ42969	Unknown function
FMNL1	Cell division
G6PC	Glycolytic enzyme
GADD45B	Cell Cycle Regulator
GAL3ST4	Galactosidase
GNAI2	Signalling protein
GPI	Glucose-6-Phosphate isomerase
GPIHBP1	Lipolytic processing
GPR162	Signalling protein
GRTP1	Signalling protein
GTF2E2	Transcription factor
HAUS4	Cell Cycle
HCST	Signalling protein
HDC	Histidine decarboxyllase
HIST1H1A	Histone protein
HIST1H2AB	Histone protein
HIST1H2AD	Histone protein
HIST1H2BF	Histone protein
HIST1H3A	Histone protein
HIST1H3D	Histone protein
HIST1H3E	Histone protein
HIST1H4B	Histone protein
HMBS	Hydroxymethylbilane Synthase
HSPA6	Molecular chaparone
IKZF4	Transcription factor
IL19	cytokine
IL1A	cytokine
INPP5B	Signalling protein
IRF1	Transcription factor
IRF2BPL	Transcription factor
KCNH6	Ion channel
KIAA1522	Unknown function
KIAA1683	Unknown function
KIF2C	Cell Division

KISS1R	Signalling protein
KLK11	Serine protease
LAMTOR4	Signalling protein
LBHD1	Unknown function
LIME1	Signalling protein
LIMS1	Signalling protein
LINC00114	long non-coding RNA
LINC00671	long non-coding RNA
LINC00881	long non-coding RNA
LINC01348	long non-coding RNA
LINC01573	long non-coding RNA
LOC100129148	Unknown function
LOC100288152	Unknown function
LOC100419583	Unknown function
LOC100507144	Unknown function
LOC101926911	Unknown function
LOC283575	Unknown function
LPAR5	Signalling protein
LPIN1	Triglyceride synthesis
LSP1	Actin binding protein
LTBR	Cell signalling
LYG2	Lysozyme
MALAT1	long non-coding RNA
MAPRE3	Cell Division
MB21D1	Signalling protein
MEFV	Immunomodulator
MIR142	microRNA
MILR1	Signalling protein
MIR3124	microRNA
MIR3188	microRNA
MIR3196	microRNA
MIR320E	microRNA
MIR330	microRNA
MIR3675	microRNA
MIR4316	microRNA
MIR4488	microRNA
MIR4496	microRNA
MIR4513	microRNA
MIR4674	microRNA
MIR4707	microRNA
MIR4772	microRNA
MIR6088	microRNA
MIR6129	microRNA
MIR6780A	microRNA

MIR6797	microRNA
MIR6803	microRNA
MIR6810	microRNA
MIR6842	microRNA
MIR7155	microRNA
MOSPD3	Signalling protein
MPZL1	Signalling protein
MTHFR	Enzyme
MTRNR2L3	Unknown function
MYL12A	Myosin light chain
NEK6	Signalling protein
NFKBID	NFKB binding protein
NTRK1	Signalling protein
OPA3	Mitochondrial membrane protein
OR2V1	Signalling protein
PCID2	RNA export
PCOLCE	Collagen metabolism
PCOLCE-AS1	ncRNA
PHKG2	Signalling protein
PIGV	Signalling protein
PIM1	Signalling protein
PLEKHG2	Signalling protein
PLEKHG4	Signalling protein
PLEKHM3	Signalling protein
PLK3	Signalling protein
PNKD	Enzyme
POLR2E	DNA polymerase II subunit E
PP7080	Unknown function
PPM1N	Protein phosphatase
PPP1R35	Protein phosphatase
PRUNE	phosphoesterase
PTPN23	Protein phosphatase
PTPN6	Protein phosphatase
PTTG1IP	Transcription factor
RAB1A	GTPase
RABAC1	GTPase
RAE1	RNA export
RAMP2	Signalling protein
RAMP2-AS1	ncRNA
RCSD1	Actin filament binding
RHPN1	Signalling protein
RHPN1-AS1	ncRNA
RILPL2	Signalling protein
RNF144A	Transcription factor

RNF144A-AS1	ncRNA
RNU1-1	Core U1 spliceosome subunit
RNU1-2	Core U1 spliceosome subunit
RNU1-27P	Core U1 spliceosome subunit
RNU1-28P	Core U1 spliceosome subunit
RNU1-3	Core U1 spliceosome subunit
RNU1-4	Core U1 spliceosome subunit
RNU5D-1	Core U1 spliceosome subunit
RNU5F-1	Core U5 spliceosome subunit
RNVU1-18	Core U1 spliceosome subunit
RPS26	Ribosomal protein
RTN2	Vesicle transport
SAA4	Folate metabolism
SCNN1A	ion channel
SEPHS2	Selenophosphate Synthetase
SH3BP5	Signalling protein
SH3BP5L	Unknown function
SHE	Unknown function
SIGLEC8	Signalling protein
SLC27A5	Solute carrier
SLC7A7	Solute carrier
SLFN12L	Unknown function
SMARCC2	Chromatin regulator
SNHG12	snoRNA
SNHG15	snoRNA
SNORA16A	snoRNA
SNORA17	snoRNA
SNORA44	snoRNA
SNORA9	snoRNA
SNORD10	snoRNA
SNORD12	snoRNA
SNORD12C	snoRNA
SNORD9	snoRNA
SNX8	phosphatidylinositol binding
SORBS1	Signalling protein
SORT1	Protein trafficking
SP1	Transcription factor
SPIB	Transcription factor
ST6GAL1	glycosyltransferase
STX6	Protein trafficking
TAPBPL	Antigen processing
TCOF1	Ribosome biogenesis
TDRD10	Nucleotide binding
TMEM138	Signalling protein

TMEM180	Signalling protein
TMEM265	Signalling protein
TNFAIP8L2	Innate immunity
TNFAIP8L2-SCNM1	Unknown function
TOP1MT	DNA topoisomerase
TOP2A	DNA topoisomerase
TRIM69	Transcription factor
UBALD2	Unknown function
UBC	ubiquitination
UQCC3	ubiquitination
USP3	ubiquitination
USP8	ubiquitination
VASP	Actin binding protein
VEGFB	Angiogenesis factor
VPS33B	Organelle formation
VTRNA1-2	ncRNA
ZDHHC18	Transcription factor
ZFAS1	ncRNA
ZNF131	Transcription factor
ZNF234	Transcription factor
ZNF512B	Transcription factor
ZNF688	Transcription factor
ZNF706	Transcription factor
ZNF785	Transcription factor

Also interesting was the observation that in 4 cases, a co-ordinate module of coding RNA and its cognate non-coding RNA regulator were both targeted by FOXO1 and ETV6 (*AP4B1* and *AP4B1-AS1*; *CD27* and *CD27-AS1; PCOLCE* and *PCOLCE-AS1; RAMP2* and *RAMP2-AS1*). Gene set enrichment analysis (GSEA) suggested that FOXO1 and ETV6 targeted genes were clustered into pathways involved in senescence processes. The top 4 most associated pathways for targets of both genes were “senescence related secretory phenotype (SASP)” ($p = 0.0002$ for ETV6 and 0.0003 for FOXO1), “Mitotic prophase” ($p = 0.0004$ for ETV6 and 0.0003 for FOXO1), “Cellular senescence “ ($p = 0.0021$ for ETV6 and 0.0001 for FOXO1) and “M-Phase” ($p= 0.0045$ for ETV6 and 0.00017 for FOXO1) (see Table 4 below).

Table 4 - GSEA pathways analysis of FOXO1 and ETV6 target genes

ETV6		
Term	***P value***	***Combined score***
Senescence-Associated Secretory Phenotype (SASP)	*0.0002*	*17.09*
Mitotic prophase	*0.0004*	*16.13*
Cellular Senescence	*0.0021*	*13.75*
M phase	*0.0045*	*12.73*
RMTs methylate histone arginines	*0.0024*	*12.18*
HOX gene activation in differentiation	*0.0027*	*11.49*
HOX gene activation in hindbrain	*0.0027*	*11.44*
RNA Polymerase I Promoter Opening	*0.0034*	*10.95*
Transcriptional regulation by small RNAs	*0.0040*	*10.84*
Cell Cycle, Mitotic	*0.0091*	*10.76*
FOXO1		
Term	***P value***	***Combined score***
Mitotic Prophase	*0.0003*	*16.67*
Senescence-Associated Secretory Phenotype (SASP)	*0.0003*	*15.96*
*Cellular Senescence_*	*0.0001*	*15.94*
*M Phase_*	*0.0017*	*15.46*
Cell Cycle, Mitotic	*0.0044*	*13.02*
Transcriptional regulation by small RNAs	*0.0018*	*12.66*
RMTs methylate histone arginines	*0.0021*	*12.26*
Transcription-Coupled Nucleotide Excision Repair (TC-NER)	*0.0023*	*12.24*
*Cellular responses to stress_*	*0.0054*	*11.78*
Formation of TC-NER Pre-Incision Complex	*0.0039*	*10.70*

DISCUSSION

Receptor tyrosine kinases integrate multiple signals from the interior and exterior of cells, and communicate this information to the cellular regulatory machinery. Our data suggest that proteins in ERK and AKT signalling pathways show higher levels of phosphorylation in late passage cells, and show significant cross- and auto-regulation. We propose that a major downstream consequence of this may be dysregulation of splicing factor expression in late passage cells, mediated primarily through altered activity and cross reactivity of the FOX01 and ETV6 transcription factors, and that these changes are linked to senescence phenotypes in this system. Low dose chemical inhibition of either ERK or AKT signalling, or reduction of the expression of *FOXO1* or *ETV6* genes in late passage human

primary fibroblasts resulted in restoration of splicing factor expression to levels consistent with those seen in younger passage cells, reversal of senescence and re-entry to cell cycle for a proportion of the cells tested.

ERK and AKT signalling pathways can be activated by classical ageing stimuli such as DNA damage, dysregulated nutrient signalling and the chronic inflammation of ageing (Fontana *et al.* 2012; Lin *et al.* 2013). The NF-kβ pathway, a major contributor to the senescence-associated secretory phenotype (SASP), is also known to be activated by both ERK and AKT signalling (Lin *et al.* 2012), raising the possibility of a vicious cycle of positive feedback. Dysregulation of normal splicing processes is a key feature of many age-related diseases such as Alzheimer's disease, Parkinson's disease and cancer (Latorre & Harries 2017). Altered splicing regulation is itself associated with ageing in human populations (Harries *et al.* 2011), with cellular senescence in in vitro models (Holly *et al.* 2013) and with longevity in mouse models (Lee *et al.* 2016). Several studies have suggested that splicing regulation may be on the causal pathway to ageing, since targeted disruption of specific splicing factors is able to moderate lifespan in invertebrate models (Heintz *et al.* 2016). The inventors recent work suggests that features of cellular senescence can be reversed by small molecule restoration of splicing factor levels (Latorre *et al.* 2017). Recent thinking suggests that changes in the decision-making processes surrounding precisely which isoforms are expressed from genes may contribute directly to ageing and age-related phenotypes (Deschenes & Chabot 2017).

Altered cellular signalling is a key hallmark of ageing. The action of pathways such as mTOR and IGF-1 signalling are well known and well defined (Cohen & Dillin 2008). ERK and AKT signalling have both previously been implicated in ageing and senescence phenotypes (Demidenko *et al.* 2009; Chappell *et al.* 2011), and modification of these pathways is also

associated with lifespan extension and ageing phenotypes in animal models (Slack *et al.* 2015). Both ERK and AKT have been previously associated with regulation of splicing factor activity (Shin & Manley 2004; Tarn 2007), and AKT is known to have a role in the regulation of splicing factor genes at the level of kinase activation (Blaustein *et al.* 2005). However, study of the precise underlying mechanisms by which these pathways may regulate splicing factor expression and influence and ageing phenotypes is fraught with difficulty since they demonstrate definite tissue, dose and context effects, and there is also significant crosstalk between them (Rhim *et al.* 2016), as the inventors have demonstrated. This implies that results may not be consistent with different doses of inhibitor, and effects seen in one cell type may not necessarily hold true in another. This was evident in our data, where we noted definite effects of dose. At low dose, we saw increased expression of some splicing factors, rescue of senescence phenotypes and re-entry into cell cycle, whereas at higher dose, decreases in splicing factor expression and no increase in proliferative index were noted. This suggests that in case of high dose activation, other feedback loops within AKT or ERK signalling pathways that lead to inhibition of splicing factor expression and proliferation may be activated. Again, this may arise from feedback loops within AKT or ERK signalling pathways leading to inhibition of splicing factor expression and proliferation which may be activated at higher concentrations. This is in itself not unusual, since antagonistic effects in terms of dose or tissue response are not uncommon in cellular signalling pathways (Pardo *et al.* 2003; Wang *et al.* 2017). Indeed, our data indicate the presence of a number of cross-regulatory and auto-regulatory feedback loops, which may indicate that splicing factor expression is tightly controlled within fairly narrow expression limits by homeostatic mechanisms, which would be in keeping with their know role in control of proliferation and the combinatorial, dose-

responsive nature by which they regulate alternative splicing (Kang *et al.* 2009; Anczukow *et al.* 2012; Fu & Ares 2014).

A better understanding of the precise relationship between altered ERK and AKT signalling, splicing factor expression and cellular senescence requires finer molecular dissection. Our data suggest that may of the features of AKT and ERK activation on splicing factor expression and cell senescence phenotypes can be replicated by targeted disruption of two key downstream effectors of ERK or AKT signalling; *FOXO1* and *ETV6*. The *FOXO1* and *ETV6* genes both encode transcription factors, the closest drosophila homologues of which (*Foxo* and *Aop*) have been reported to contribute to the effects of ERK and AKT activation on lifespan in *D. melanogaster* (Slack *et al.* 2015). FOXO proteins have a long history of involvement in ageing pathways; they are well-known players in longevity in nematodes, files, and mammals (Salih & Brunet 2008), but to our knowledge have never been linked previously to the regulation of splicing factors.

Targeted knockout of either gene resulted in both increased splicing factor expression and rescue from senescence. Again, the relationship between these genes may not be synergistic, since simultaneous knockdown of both genes results not in an additive or multiplicative effect on splicing factor expression and senescent cell load, but to a complete abrogation of effect. This may partially be explained by a reciprocal regulation of *FOXO1* and *ETV6* genes at the level of transcription and protein sub-cellular localisation; knockdown of *FOXO1* results in increased *ETV6* expression and moderation of either signalling pathway by specific inhibitors induces effects on the nuclear localisation of both genes. Several splicing factors have evolutionally-conserved FOXO binding motifs in their promoter regions (Webb *et al.* 2016), and FOXO1 protein has been reported to co-localise with the nuclear speckles within the cell where splicing occurs (Arai *et al.* 2015). ETV6 is a member of the ETS family of

transcription factors, and is perhaps a less obvious candidate for a longevity gene, although it has a well-known role in control of cellular proliferation and in haematopoietic cancer (Hock & Shimamura 2017). Like FOXO1, ETV6 has similarly not previously been reported as a regulator of splicing factor expression, but other members of the wider *ETS* family of genes, which have very similar binding sites, have been reported to have such activity (Kajita *et al.* 2013). Both *FOXO1* and *ETV6* have been reported to have activity as tumour suppressor genes (Dansen & Burgering 2008; Rasighaemi & Ward 2017), so a role in negatively regulating the expression of genes required for cellular proliferation is not unexpected.

The ChIP analyses indicate that although FOXO1 and ETV6 may directly regulate some splicing factors (HNRNPF, HNRNPL), most regulators of splicing factor expression, but rather act through a series of intermediates, almost 25% of which are non-coding RNAs. Splicing processes are implicated directly, since 9 of the common targets are components of the U1 or U5 spliceosomal complex. There is surprising overlap between the genes regulated by FOXO1 and ETV6, indicating that the regulation may be co-ordinate or competitive. We assume that the regulation may also include the action of other FOXOs and other ETS family members. The role of these genes in mediating senescence processes is also suggested by our GSEA results. FOXO1 and ETV6 targets cluster in pathways fundamental to senescence ("senescence-associated secretory phenotype (SASP)", "cellular senescence, "M-Phase", "mitotic cell cycle). Thus our data provide evidence that FOXO1 and ETV6 may co-ordinately regulate a module of mediator genes that are involved in the regulation of splicing factors and influence their relationship with senescence.

Although our data suggest that *ETV6* and *FOXO1* may have activity as novel regulators of splicing factor expression, it does not rule out the contribution of other genes in these networks. Indeed, although the overall picture is similar, some splicing factor genes behave differently when

challenged with inhibition of the whole pathway compared with specific inactivation of *FOXO1* or *ETV6* (e.g. *SRSF3, SRSF6* for ERK signalling and *AKAP17A, LSM2* and *LSM14A* for AKT signalling). This strongly suggests the presence of other regulators. It is also clear that only a subset of cells are rescued, since the whole cell population does not revert. Even 'clonal' cultures of senescent cells are heterogeneous, containing growth-arrested, but non-senescent cells and senescent cells, depending on the degree of paracrine inhibition. The percentage of senescent cells at growth arrest can range from approximately 40% to over 80%, even in different cell lines from the same tissue type.

In conclusion, we present here evidence that activation of ERK and/or AKT signalling by age-related phenomena may lead to a cascade of events culminating in altered activity of FOXO1 and ETV6 transcription factors. We demonstrate here for the first time that ETV6 and FOXO1 represent novel regulators of splicing factor expression, and that age-related alterations in their activity could lead dysregulated splicing and ultimately cellular senescence. Inhibitors of AKT and ERK signalling pathways are already in use as therapeutic agents for cancer, raising the interesting possibility that genes in these pathways may represent targets for early intervention for healthier ageing in the future.

EXPERIMENTAL PROCEDURES

Culture of human primary fibroblasts

Normal human dermal fibroblasts derived from neonatal foreskin from a single donor (NHDF; Promocell, Heidelburg, Germany) were cultured in fibroblast growth medium (C-23020, Promocell, Heidelburg, Germany) supplemented with 2% FBS, growth factors (recombinant fibroblast growth factor and recombinant human insulin), 100U/ml penicillin, and 100ug/ml streptomycin in a seeding density of 6×10^3 cell/cm^2. Early passage cells at population doubling (PD) = 25 (85% growth fraction and 7% senescent fraction) or late

passage cells at PD = 63 (33% growth fraction and 58% senescent fraction) were maintained in culture for 10 days prior to subsequent experiments. Inhibitors of MEK/ERK (trametinib) and AKT (SH-6) were added at 1μM or 10μM for 24h, based on previous work in the literature (Krech *et al.* 2010; Slack *et al.* 2015). Vehicle-only (DMSO) controls were included for each experiment. Where serum starvation was required to differentiate senescence rescue from altered proliferation kinetics, cells were maintained in DMEM (Sigma Aldrich, Dorset, UK) supplemented with 0.1% of serum and 1% penicillin and streptomycin in the absence of fibroblast-specific supplement, for 24 h prior to treatment.

Assessment of phosphorylation changes in key signalling pathways during cellular senescence

Early and late passage human primary fibroblasts cells were plated in 25cm^2 flasks at a density of 6 x 10^3 cells/cm^2 and grown for 10 days. Phosphorylation status for proteins in key signalling pathways thought to be activated in senescence was then assessed in cell lysates from early passage (PD = 25) or late passage (PD = 63) primary human fibroblast cells using the human MAPK phosphorylation antibody array (ab211061, Abcam, Bristol, UK) according to the manufacturer's instructions. Phosphorylation sites tested were: AKT (pS473), CREB (pS133), ERK1 (pT202/Y204), ERK2 (pT185/Y187), GSK3a (pS21), GSKb (pS9), HSP27 (pS82), JNK (pT183), MEK (pS217/221), MKK3 (pS189), MKK6 (pS207), MSK2 (pS360), mTOR (pS2448), p38 (pT180/Y182), p53 (pS15), P70S6K (pT421/S424), RSK1 (pS380). In brief, membranes were blocked with blocking buffer for 30 min at room temperature and incubated with 1ml of cell lysate overnight at 4°C. After washing, detection antibody cocktail was added and incubated for 2 hours, followed by a 2-hours incubation with HRP-anti-rabbit IgG at room temperature. Membranes were incubated with detection buffer and results were documented on a chemiluminescence imaging system (LI-COR biosciences, Nebraska USA).

Signal intensity was quantified using Image Studio software V5.2 (LI-COR biosciences, Nebraska USA). Results were normalised to total cellular protein content and expressed relative to the positive control.

Assessment of cellular senescence

Late passage primary human fibroblasts at PD = 63 were seeded in 3 biological replicates of 6 x 10^4 cells per well in 6-well plates. Cells were treated with the MEK/ERK inhibitor trametinib or the AKT inhibitor SH-6 at 1μM and 10μM, or with a combination of the two inhibitors at 1μM each for 24hrs. Cell senescence was then assessed using the biochemical senescence marker SA β-Gal, tested in triplicate using a commercial kit (Sigma Aldrich, UK); according to manufacturer's instructions, with a minimum of 400 cells assessed per replicate. Senescence was also quantified in molecular terms by assessing *CDKN2A* (p16) gene expression and by changes in cell morphology typical of senescence as in our previous work (Holly *et al.* 2013). *CDKN2A* (p16) was measured by qRTPCR relative to *GUSB, PPIA* and *GADPH* endogenous control genes, on the QuantStudio 12K Flex platform (Applied Biosystems, Foster City, USA). PCR reactions contained 2.5 μl TaqMan Universal Mastermix (no AMPerase) (Applied Biosystems, Foster City, USA), 0.25 μM probe and 0.5 μl cDNA reverse transcribed as above in a total volume of 5 μl. PCR conditions were a single cycle of 95 ºC for 10 minutes followed by 40 cycles of 95 ºC for 15 seconds and 60ºC for 1 minute. Quantitative RTPCR assay accession numbers for p16 and p21 are given in table 5 below.

Table 5 - Sequences of qRTPCR assays, siRNAs and morpholinos used in this study

Gene name	Probe	Transcript accession
CDKN2A	N/A	Hs00923894_m1
p21 (CDKN1A)	N/A	Hs00355782_m1
FOXO1	N/A	Hs00231106_m1
ETV6	N/A	Hs00231101_m1

Splicing factors	N/A	
AKAP17A	N/A	Hs00946624_m1
HNRNPA0	N/A	Hs00246543_s1
HNRNPA1	N/A	Hs01656228_s1
HNRNPA2B1	N/A	Hs00242600_m1
HNRNPH3	N/A	Hs01032113_g1
HNRNPK	N/A	Hs00829140_s1
HNRNPM	N/A	Hs00246018_m1
HNRNPUL2	N/A	Hs00859848_m1
IMP3	N/A	Hs00251000_s1
LSM14A	N/A	Hs00385941_m1
LSM2	N/A	Hs01061967_g1
PNISR	N/A	Hs00369090_m1
HNRNPD	N/A	Hs01086914_g1
SF3B1	N/A	Hs00202782_m1
SRSF1	N/A	Hs00199471_m1
SRSF2	N/A	Hs00427515_g1
SRSF3	N/A	Hs00751507_s1
SRSF6	N/A	Hs00607200_g1
TRA2B	N/A	Hs00907493_m1
SRSF7	N/A	Hs00196708_m1
siRNA		
FOXO1	ID: s5257	NM_002015.3
ETV6	ID: s533885	NM_001987.4
morpholino		
FOXO1	TCCCCCAGCCGCAGGAGAGCCAAGA (SEQ ID No. 1)	
ETV6	CATGTCTCACAGCGAGAGAGATCAG (SEQ ID No. 2)	

Quantification of treatment-related changes to SASP protein expression

Late passage primary human fibroblast cells at PD = 63 were seeded at 6 x 10^4 cells per well in a 6 well plate, cultured for 10 days and then treated with at 1μM or 10μM trametinib (MEK/ERK inhibitor) or SH-6 (AKT inhibitor) for 24 hours. Cell supernatants were then harvested and stored at -80 °C. Levels of GMCSF, IFNγ, IL1β, IL2, IL6, IL8, IL10, IL-12p70,

and TNFα SASP components were measured in cell supernatants from treated and vehicle-only control cells using the K15007B MesoScale Discovery multiplex ELISA immunoassay (MSD, Rockville, USA) in 4 biological replicates. Proteins were quantified relative to a standard curve using a Sector Imager SI-6000 according to the manufacturer's instructions.

Assessment of splicing factor expression in late passage primary human fibroblasts treated with ERK or AKT inhibition

Late passage primary human fibroblasts at PD = 63 were seeded at 6 x 10^4 cells per well as 3 biological replicates in 6-well plates, allowed to grow for 10 days and then treated with the ERK inhibitor trametinib or the AKT inhibitor SH-6 at 1μM or 10μM for 24hrs. Vehicle (DMSO) only controls were also included under the same growth conditions. The expression levels of 20 splicing factor transcripts previously associated with age, replicative senescence or lifespan in our previous work (Harries *et al.* 2011; Holly *et al.* 2013; Lee *et al.* 2016) were then assessed by qRTPCR. Accession numbers for splicing factor assays are given in Table 5. RNA was extracted using TRI reagent ® (Life Technologies, Foster City USA) according to the manufacturer's instructions. Total RNA (100 ng) was reverse transcribed in 20 μl reactions using the Superscript III VILO kit (Life Technologies). Transcript expression was then quantified in duplicate for each biological replicate using TaqMan Low Density Array (TLDA) on QuantStudio 12K Flex (Applied Biosystems, Foster City, USA). Cycling conditions were 1 cycle each of 50 °C for 2 min, 94.5 °C for 10 min and then 40 cycles of 97 °C for 30 s and 57.9 °C for 1 min. The reaction mixes included 50 μl TaqMan Fast Universal PCR Mastermix (Life Technologies), 30 μl dH_2O and 20 μl cDNA template. 100 μl reaction mixture was dispensed into the TLDA card chamber and centrifuged twice for 1 min at 1000 rpm. Transcript expression was assessed by the Comparative Ct approach, relative to the *IDH3B*, *GUSB* and

PPIA endogenous control genes and normalised to their expression in RNA from untreated late passage cells.

Assessment of cell proliferation

The proliferative capacity of treated cells was assessed by Ki67 and BrdU staining and by cell counts. Late passage primary human fibroblasts at PD = 63 were seeded at 1 x 10^4 cells per well in 3 biological replicates in 24-well plates, allowed to grow for 10 days and then assayed for cell proliferation by ki67 staining. For assessment of cells in S phase, late passage primary human fibroblasts at PD = 63 were seeded at 1 x 104 cells per coverslip as 3 biological replicates in 24-well plates, allowed to grow for 10 days and then were incubated with 5-Bromo-2'-deoxy-uridine (BrdU) for 24 hrs. BrdU incorporation was determined by the 5-Bromo-2'-deoxy-uridine labeling and detection kit I following instructions of the manufacturer (Roche Molecular Biochemicals). BrdU positive cells were visualized and counted by fluorescence microscopy. Cell counts were carried out manually in 3 biological replicates in treated and vehicle-only cultures following trypsinisation and suspension of cells and are presented as mean (+/-SEM). Following treatment, **c**ells were fixed for 10 min with 4% PFA and permeabilized with 0.025 % Triton and 10 % serum in PBS for 1 hour. Ki-67 staining was carried out by rabbit monoclonal antibody (ab16667, Abcam, UK) at a 1:400 dilution and samples were incubated overnight at 4 °C, followed by FITC-conjugated secondary goat anti-rabbit antibody (1:400) for 1 hour, and nuclei were counterstained with DAPI. Coverslips were mounted on slides in DAKO fluorescence mounting medium (S3023; Dako, Santa Clara, USA). The proliferation index was determined by counting the percentage of Ki67 positive cells from at least 400 nuclei from each biological replicate at 400× magnification under a Leica D4000 fluorescence microscope.

Assessment of apoptosis using TUNEL assay

Terminal DNA breakpoints in situ 3 - hydroxy end labeling (TUNEL) assay was to quantify levels of apoptosis in NHDF cells. Late passage primary human fibroblasts at PD = 63 were seeded at 1 x 10^4 cells per well as 3 biological replicates in 24-well plates, allowed to grow for 10 days and a TUNEL assay was performed with Click-iT® TUNEL Alexa Fluor® 488 Imaging Assay kit (Thermofisher, UK) following the manufacturer's instructions. Negative (vehicle only) and positive (DNase1) controls were also performed. The apoptotic index was determined by counting the percentage of positive cells from at least 400 nuclei from each biological replicate at 400× magnification.

***ETV6* and *FOXO1* gene knockdown**

Previous work in invertebrate systems has identified the *Aop* and *Foxo* genes, involved in ERK and AKT signalling respectively, as determinants of lifespan in *D. melanogaster* (Slack *et al.* 2015). The closest human homologues of these genes are *FOX01* (Kramer *et al.* 2003) and *ETV6/TEL* (Jousset *et al.* 1997). We assessed the effect of knockdown of *FOXO1* or *ETV6* gene expression on cellular senescence and splicing factor expression in late passage primary human fibroblasts. Late passage cells at PD = 63 were seeded at 6 x 10^4 cells per well into 6-well plates were cultured for 10 days. Antisense oligonucleotides (morpholinos, MOs) were designed to the 5' untranslated region of the FOXO1 or ETV6 genes, in the vicinity of the initiation codon (Gene Tools LLC, Philomath, USA). Morpholino oligonucleotides (10μM) were introduced into the cells by endo-porter delivery according to the manufacturer's instructions. A fluorescein-conjugated scrambled negative control morpholino was also included as a negative control and to monitor delivery of constructs. Transfection efficiency was assessed by microscopy. Splicing factor expression and cellular senescence were then determined as described above. Results were confirmed by another

method of gene knockdown; siRNA. For this work, late passage primary human fibroblasts at PD = 63 were seeded in 3 biological replicates of 6 x 10^4 cells per well in 6-well plates and transfections were carried out using 15nM *FOXO1*, *ETV6* siRNA or 15 nM control siRNA (Themofisher) using Lipofectamine RNAiMAX reagent (Invitrogen, Paisley, UK) for 48 hr. siRNA and morpholino sequences are given in Table 5.

Assessment of FOXO1 and ETV6 subcellular localization

The subcellular localization of ETV6 and FOXO1 proteins were assessed by immunofluorescence. Late passage primary human fibroblasts at PD = 63 were seeded at 1 x 104 cells per coverslip as 3 biological replicates in 24-well plates, allowed to grow for 10 days and then assayed for subcellular localisation. Following treatment with 1μM trametinib or SH-6, cells were fixed for 10 min with 4% PFA and permeabilized with 0.025 % Triton and 10 % serum in PBS for 1 hour. Anti-rabbit ETV6 (ab64909) and anti-rabbit FOXO1 from (Abcam, UK) at a 1:1000 and 1:100 dilution respectively and samples were incubated overnight at 4 °C, followed by FITC-conjugated secondary goat anti-rabbit antibody (1:400) for 1 hour, and nuclei were counterstained with DAPI. Coverslips were mounted on slides in DAKO fluorescence mounting medium (S3023; Dako, Santa Clara, USA). The nuclear localization was determined by counting the percentage of nuclear staining from at least 50 cells from each biological replicate at 400× magnification under a Leica D4000 fluorescence microscope.

Assessment of telomere length

DNA was extracted from 2x10^5 late passage primary human fibroblasts at PD= 63 which had been plated in 3 biological replicates and then treated with 1μM of either the ERK inhibitor trametinib or the AKT inhibitor SH-6 for 24hrs, using the PureLink® Genomic DNA Mini Kit (Invitrogen™/Thermo Fisher, MA, USA) according to the manufacturer's instructions.

DNA quality and concentration was checked by Nanodrop spectrophotometry (NanoDrop/Thermo Fisher, MA, USA). Relative telomere length was determined using a modified qPCR protocol (O'Callaghan & Fenech 2011). PCR reactions contained 1μl EvaGreen (Solis Biodyne, Tartu, Estonia), 2μM each primer and 25ng DNA in a total volume of 5 μl in a 384 well plate. The quantitative real time polymerase chain reaction telomere assay was run on the StepOne Plus, cycling conditions were: a single cycle of 95 ºC for 15 minutes followed by 45 cycles of 95 ºC for 10 seconds, 60ºC for 30 seconds and 72ºC for 1 minute. The average relative telomere length was calculated as the ratio of telomere repeat copy number to a single copy number gene (*36B4*) and normalised to telomere length in untreated cells.

Statistical analysis

Unless otherwise indicated, differences between treated and vehicle-only control cultures were assessed for statistical significance by two way ANOVA analysis. Statistical analysis was carried out with the computer-assisted Prism GraphPad Program (Prism version 5.00, GraphPad Software, San Diego, CA).

Chromatin immunoprecipitation (ChIP) and Gene Set enrichment analysis

Input data for this work was 4 publically available human ChIP datasets for ETV6 and 3 for FOXO1. ETV6 datasets comprised 2 sets derived from K562 cells (GSE91511 and GSE95877), and 2 datasets derived from GM12878 cells (GSE91904 and GSE96274). FOXO1 datasets comprised datasets from human endometrial stromal cells (GSE69542), human pre-leukaemia B cells (GSE80773) and normal human B cells (GSE68349). These datasets were imported into Cistrome Project software (www.cistrome.org) for identification of FOXO1 and ETV6 target genes using the BETA (Binding and Expression Target Analysis) minus application using default parameters. This software detects transcription factor binding sites in input data

based on regulatory potential score, following filtering of peaks with less than 5 fold signal to background ratio. GSEA pathway analysis was then carried out using the Enrichr program using the 2016 reactome interface.

The forgoing embodiments are not intended to limit the scope of the protection afforded by the claims, but rather to describe examples of how the invention may be put into practice.

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Claims

1. A composition comprising one or more intermediate non-coding RNA regulators which modulate the expression of *ETV6* for use in the prevention, management, amelioration or treatment of an age-related disease or condition or cancer.
2. The composition of claim 1, wherein the age-related disease or condition or cancer involves dysregulation of splicing factor expression and/or dysregulation of cellular senescence.
3. A composition comprising one or more intermediate non-coding RNA regulators which modulate the expression of *FOXO1* for use in the prevention, management, amelioration or treatment of an age-related disease or condition or cancer, wherein the age-related disease or condition or cancer involves dysregulation of splicing factor expression and/or dysregulation of cellular senescence.
4. The composition of any one of claims 1-3, wherein the age-related disease or condition is selected from Alzheimer's disease, cardiovascular disease, hypertension, osteoporosis, type 2 diabetes, cancer, Parkinson's disease, cognitive dysfunction, frailty or progeroid syndromes.
5. The composition of any one of claims 1-4, wherein the composition comprises two or more of the intermediate non-coding RNA regulators.
6. The composition of any one of claims 1-5, wherein the intermediate non-coding RNA regulators comprise miRNAs, miRNA mimics or antagomiRs.
7. The composition of claim 6, wherein the intermediate non-coding RNA regulators comprises two or more miRNAs, miRNA mimics or antagomiRs capable of binding to two or more different complementary and antisense miRNA sequences.

8. The composition of any one of claims 5-7, wherein the intermediate non-coding RNA regulators are selected from one or more of the following: MIR142; MIR3124; MIR3188; MIR3196; MIR320E; MIR330; MIR3675; MIR4316; MIR4488; MIR4496; MIR4513; MIR4674; MIR4707; MIR4772; MIR6088; MIR6129; MIR6780A; MIR6797; MIR6803; MIR6810; MIR6842; or MIR7155.

9. The composition of claim 8, wherein the intermediate non-coding RNA regulators are selected from one or more of the following: MIR3124; MIR3675; MIR4496; MIR6780A; MIR6810; MIR6842; or MIR7155.

10. A method of increasing splicing factor expression, reducing cellular senescence and/or promoting re-entry to the cell cycle in a cell culture comprising administering to the cell culture a composition comprising intermediate non-coding RNA regulators which modulate the expression of *FOXO1* and/or *ETV6*.

11. The method of claim 8, wherein the composition comprises two or more of the intermediate non-coding RNA regulators.

12. The method of claim 8 or claim 9, wherein the intermediate non-coding RNA regulators comprise miRNAs, miRNA mimics or antagomiRs.

13. The method of any one of claims 8-10, wherein the expression modulator of *FOXO1* and/or *ETV6* comprises two or more miRNAs, miRNA mimics or antagomiRs capable of binding to two or more different complementary and antisense miRNA sequences.

14. Use of a composition comprising one or more intermediate non-coding RNA regulators which modulate the expression of *ETV6* for the cosmetic treatment of the effects of ageing.

15. Use according to claim 14, wherein the effects of aging involves dysregulation of splicing factor expression and/or dysregulation of cellular senescence.

16. Use of a composition comprising one or more intermediate non-coding RNA regulators which modulate the expression of *FOXO1* for the cosmetic treatment of the effects of ageing, where the effects of aging involves dysregulation of splicing factor expression and/or dysregulation of cellular senescence.

17. Use according to any one of claims 14 to 16, wherein the composition comprises two or more of the intermediate non-coding RNA regulators.

18. Use according to any one of claims 14-17, wherein the intermediate non-coding RNA regulators comprise miRNAs, miRNA mimics or antagomiRs.

19. Use according to claim 18, wherein the expression modulator of *FOXO1* or *ETV6* comprises two or more miRNAs, miRNA mimics or antagomiRs capable of binding to two or more different complementary and antisense miRNA sequences.

20. Use according to any one of claims 14-19, wherein the intermediate non-coding RNA regulators are selected from one or more of the following: MIR142; MIR3124; MIR3188; MIR3196; MIR320E; MIR330; MIR3675; MIR4316; MIR4488; MIR4496; MIR4513; MIR4674; MIR4707; MIR4772; MIR6088; MIR6129; MIR6780A; MIR6797; MIR6803; MIR6810; MIR6842; or MIR7155.

21. Use according to claim 20, wherein the intermediate non-coding RNA regulators are selected from one or more of the following: MIR3124; MIR3675; MIR4496; MIR6780A; MIR6810; MIR6842; or MIR7155.

22. Use of a composition comprising one or more intermediate non-coding RNA regulators which modulate the expression of *FOXO1* and/or *ETV6* as a research tool

for restoring and/or increasing splicing factor expression; or reducing or reversing cell senescence and/or re-entry to cell cycle.

23. Use of a composition comprising one or more intermediate non-coding RNA regulators which modulate the expression of *FOXO1* and/or *ETV6* for cell culture.

24. Use according to claim 23, wherein the composition is for increasing viable number of passages in cell culture and/or reduce senescent cell populations.

Figure 1

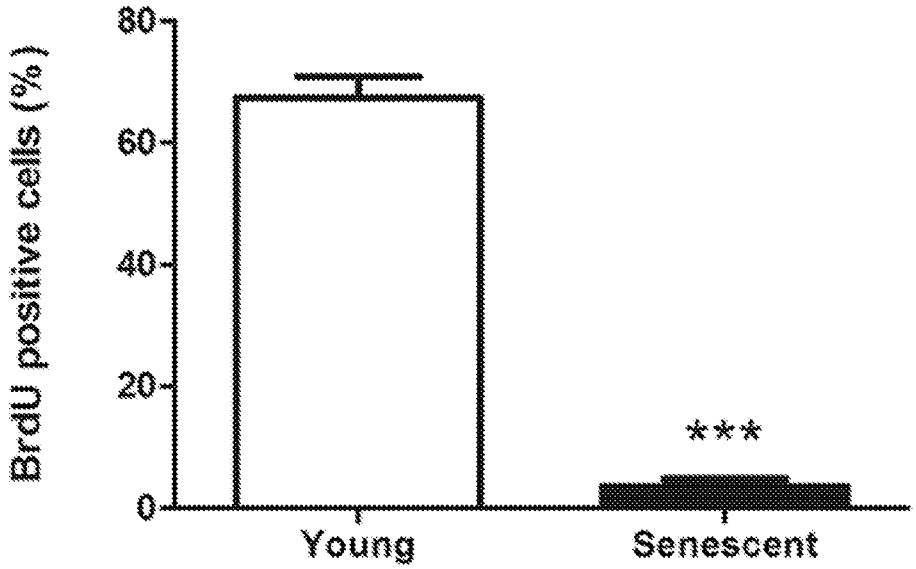
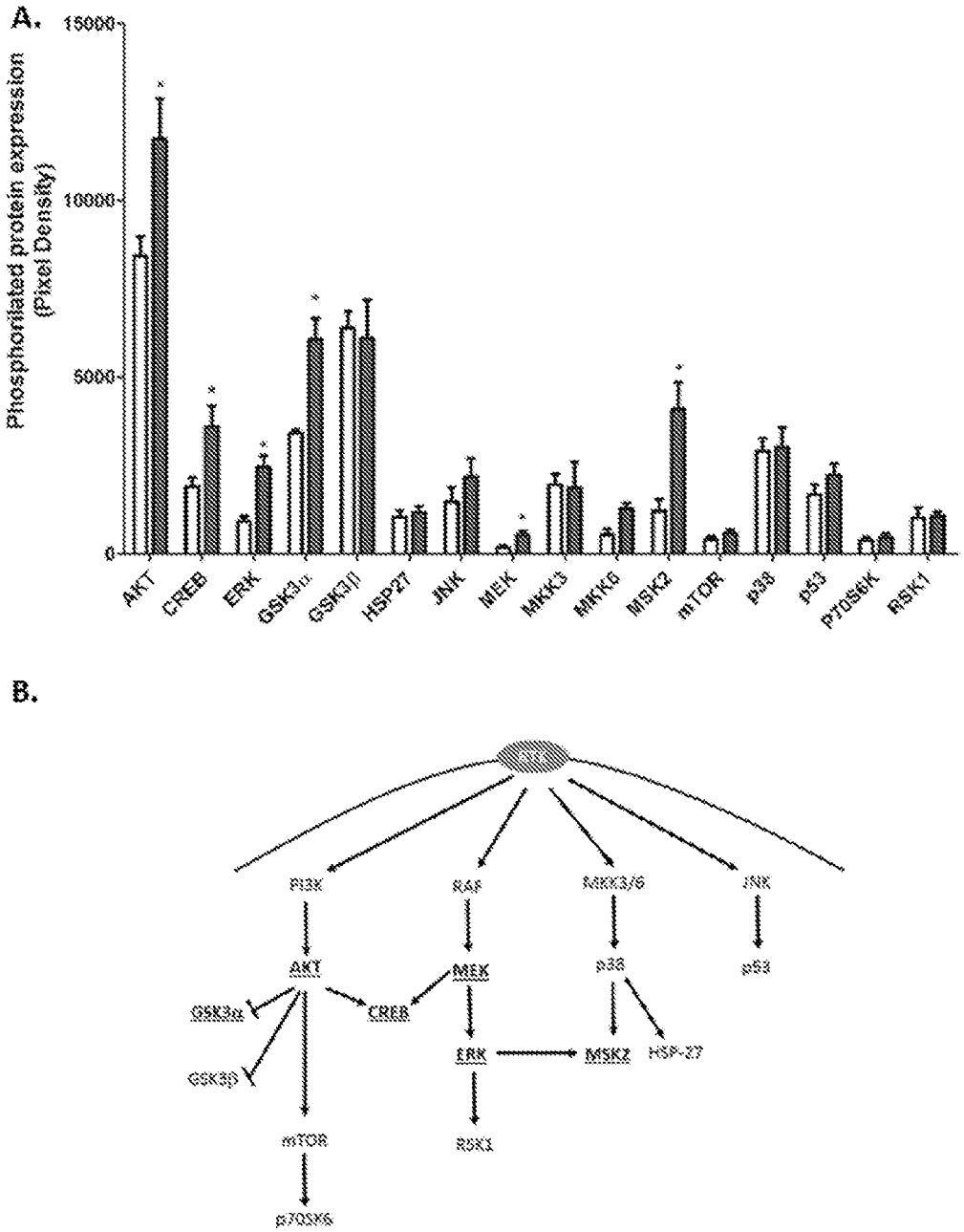


Figure 2


A.
Phosphorilated protein expression (Pixel Density)
15000
10000
5000
0
AKT
CREB
ERK
GSK3α
GSK3β
HSP27
JNK
MEK
MKK3
MKK6
MSK2
mTOR
p38
p53
P70S6K
RSK1
B.
PI3K
RAF
MKK3/6
JNK
AKT
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HSP-27
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RSK1
p70S6K

Figure 3

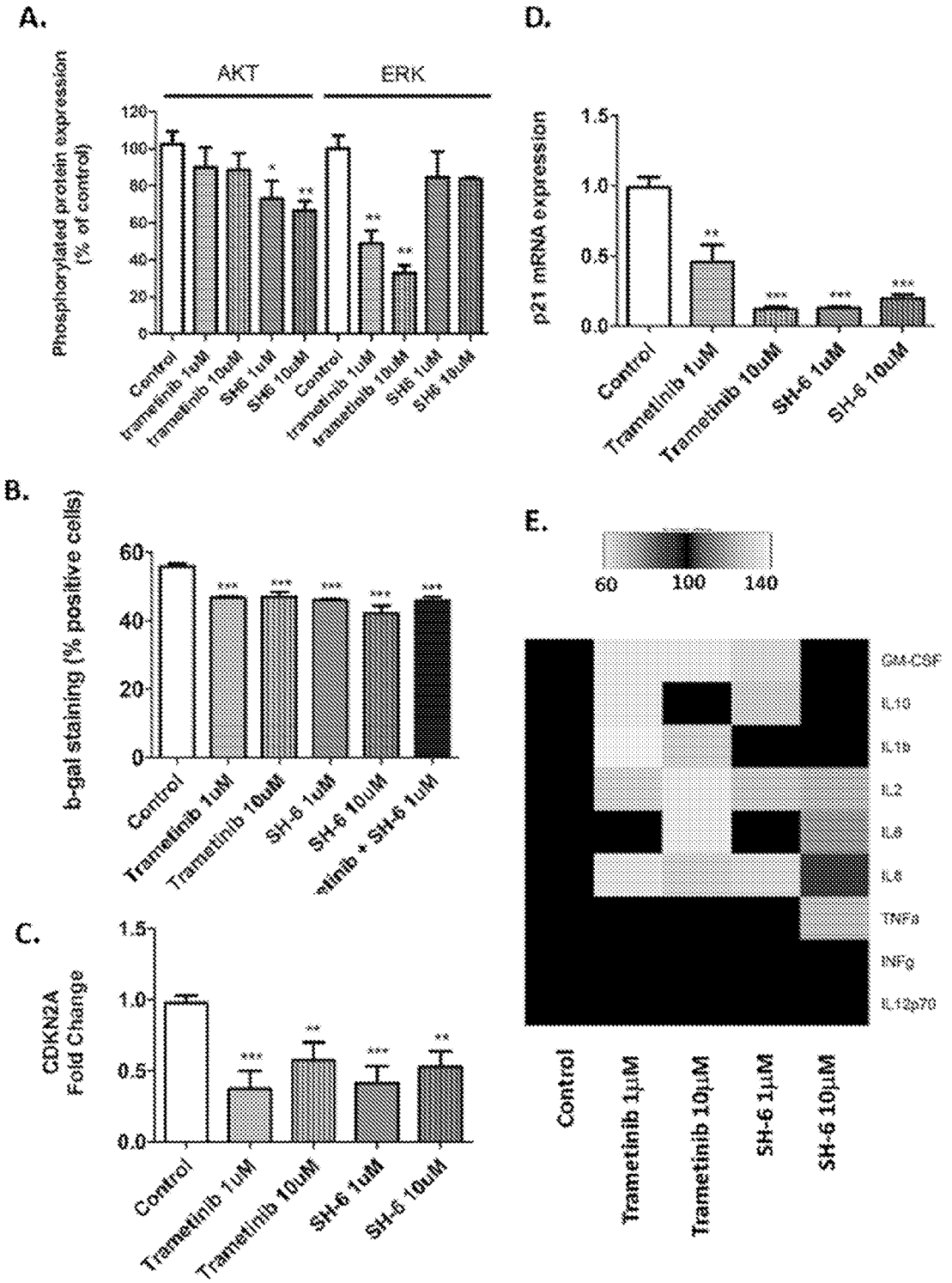

A.
AKT
ERK
Phosphorylated protein expression (% of control)
Control
Trametinib 1uM
Trametinib 10uM
SH6 1uM
SH6 10uM
D.
p21 mRNA expression
Control
Trametinib 1uM
Trametinib 10uM
SH-6 1uM
SH-6 10uM
B.
b-gal staining (% positive cells)
Control
Trametinib 1uM
Trametinib 10uM
SH-6 1uM
SH-6 10uM
E.
60
100
140
GM-CSF
IL10
IL1b
IL2
IL6
IL8
TNFa
INFg
IL12p70
Control
Trametinib 1µM
Trametinib 10µM
SH-6 1µM
SH-6 10µM
C.
CDKN2A Fold Change
Control
Trametinib 1uM
Trametinib 10uM
SH-6 1uM
SH-6 10uM

Figure 4

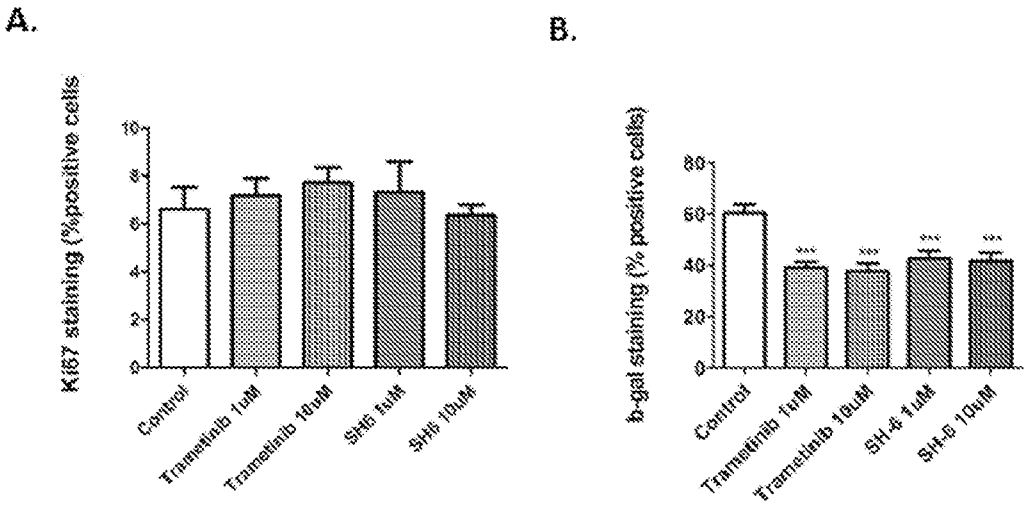

A.
Ki67 staining (%positive cells
Control
Trametinib 1uM
Trametinib 10uM
SH6 1uM
SH6 10uM
B.
b-gal staining (% positive cells)
Control
Trametinib 1uM
Trametinib 10uM
SH-6 1uM
SH-6 10uM

Figure 5

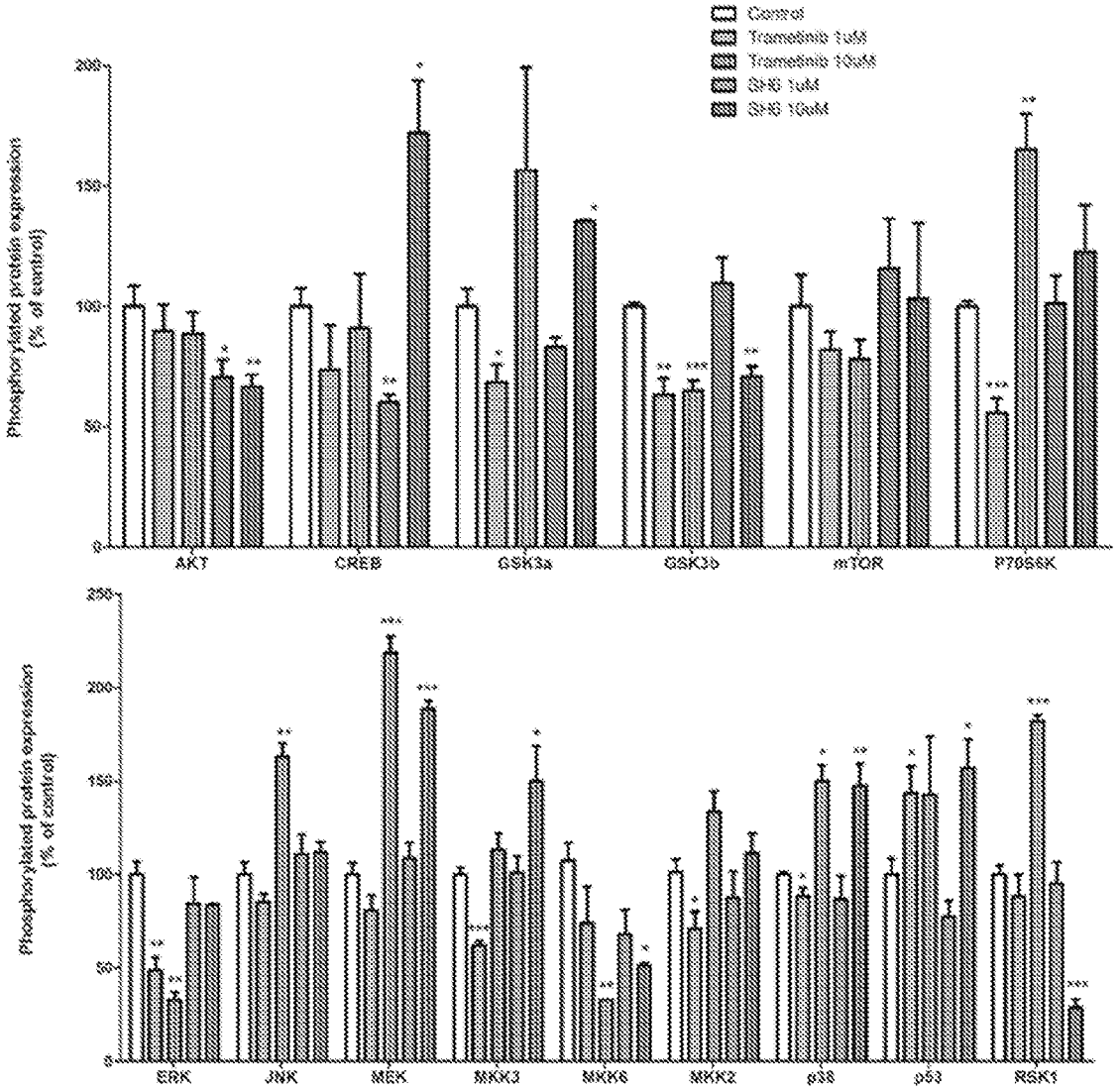

Control
Trametinib 1uM
Trametinib 10uM
SHG 1uM
SHG 10uM
Phosphorylated protein expression (% of control)
AKT
CREB
GSK3a
GSK3b
mTOR
P70S6K
ERK
JNK
MEK
MKK3
MKK6
MKK2
p38
p53
RSK1

Figure 6

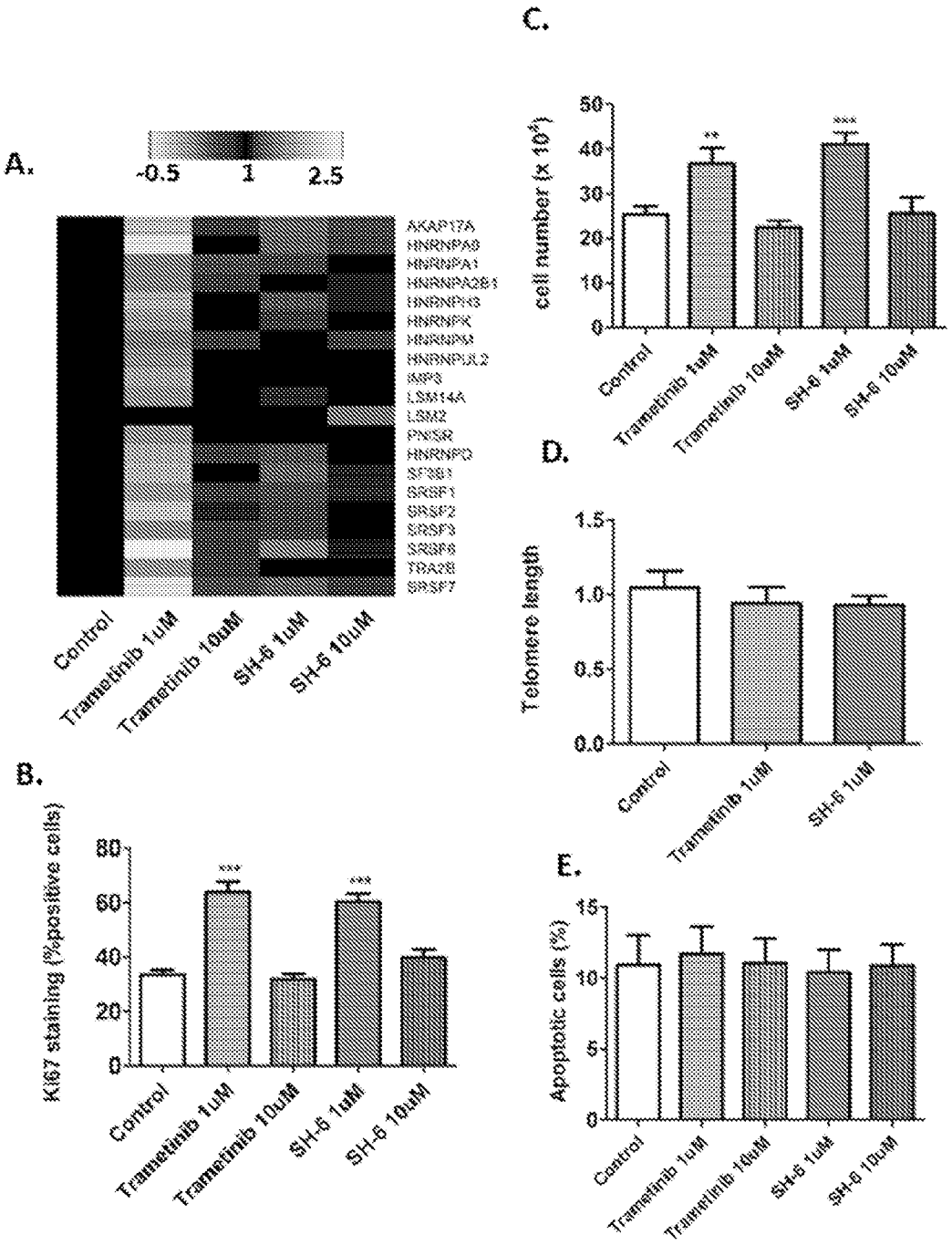

A.
-0.5
1
2.5
AKAP17A
HNRNPA0
HNRNPA1
HNRNPA2B1
HNRNPH3
HNRNPK
HNRNPM
HNRNPUL2
IMP3
LSM14A
LSM2
PNISR
HNRNPD
SF3B1
SRSF1
SRSF2
SRSF3
SRSF6
TRA2B
SRSF7
Control
Trametinib 1uM
Trametinib 10uM
SH-6 1uM
SH-6 10uM
B.
Ki67 staining (%positive cells)
C.
cell number (x 10⁴)
D.
Telomere length
E.
Apoptotic cells (%)

Figure 7

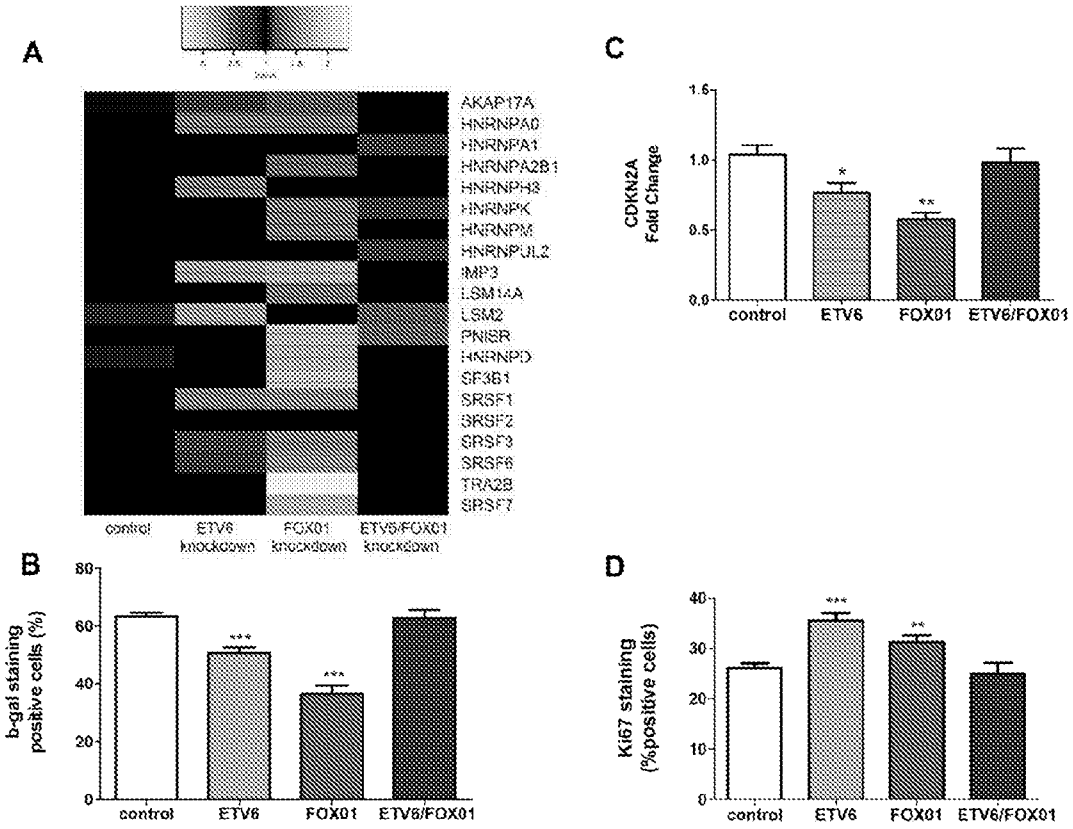

A
AKAP17A
HNRNPA0
HNRNPA1
HNRNPA2B1
HNRNPH3
HNRNPK
HNRNPM
HNRNPUL2
IMP3
LSM14A
LSM2
PNISR
HNRNPD
SF3B1
SRSF1
SRSF2
SRSF3
SRSF6
TRA2B
SPSF7
control
ETV6 knockdown
FOX01 knockdown
ETV6/FOX01 knockdown
B
b-gal staining positive cells (%)
control
ETV6
FOX01
ETV6/FOX01
C
CDKN2A Fold Change
control
ETV6
FOX01
ETV6/FOX01
D
Ki67 staining (%positive cells)
control
ETV6
FOX01
ETV6/FOX01

Figure 8

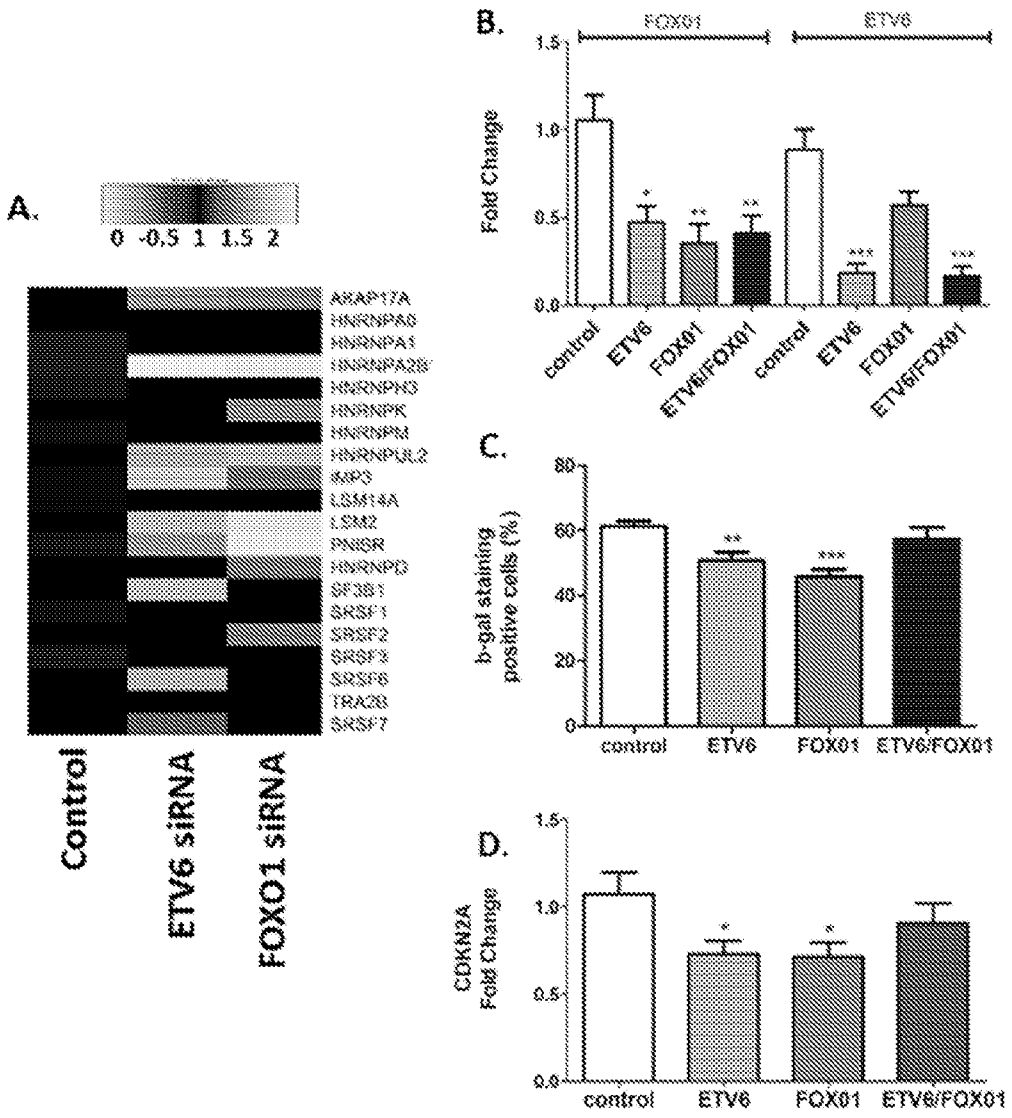

A.
0 -0.5 1 1.5 2
ARAP1TA
HNRNPA0
HNRNPA1
HNRNPA2B
HNRNPH3
HNRNPK
HNRNPM
HNRNPUL2
IMP3
LSM14A
LSM2
PNISR
HNRNPD
SF3B1
SRSF1
SRSF2
SRSF3
SRSF6
TRA2B
SRSF7
Control
ETV6 siRNA
FOXO1 siRNA
B.
FOX01
ETV6
Fold Change
control
ETV6
FOX01
ETV6/FOX01
C.
b-gal staining positive cells (%)
D.
CDKN2A Fold Change

Figure 9

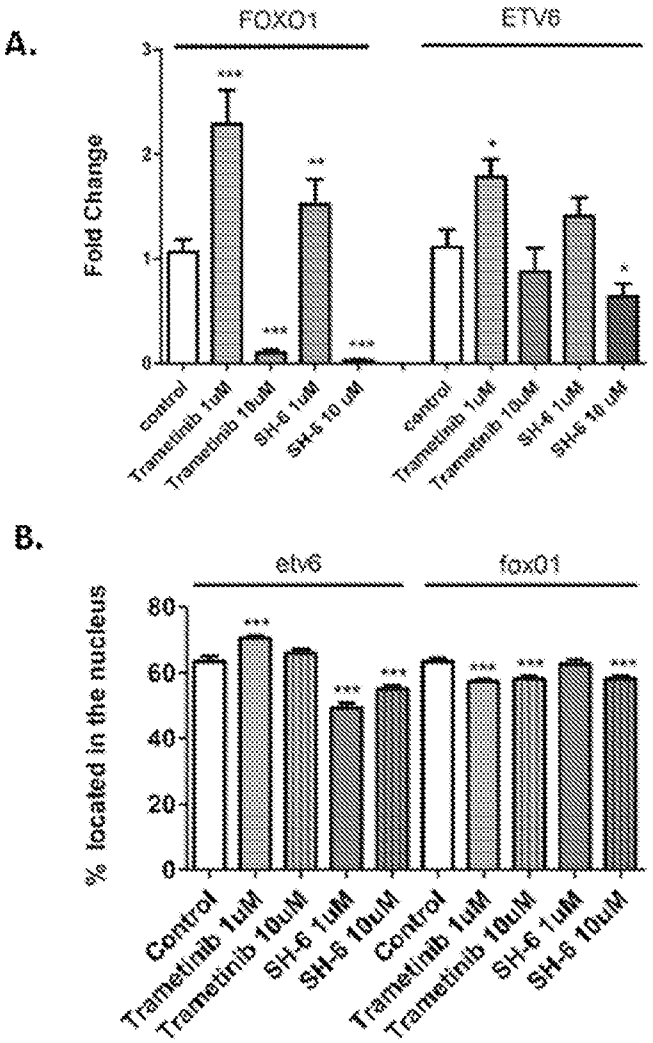
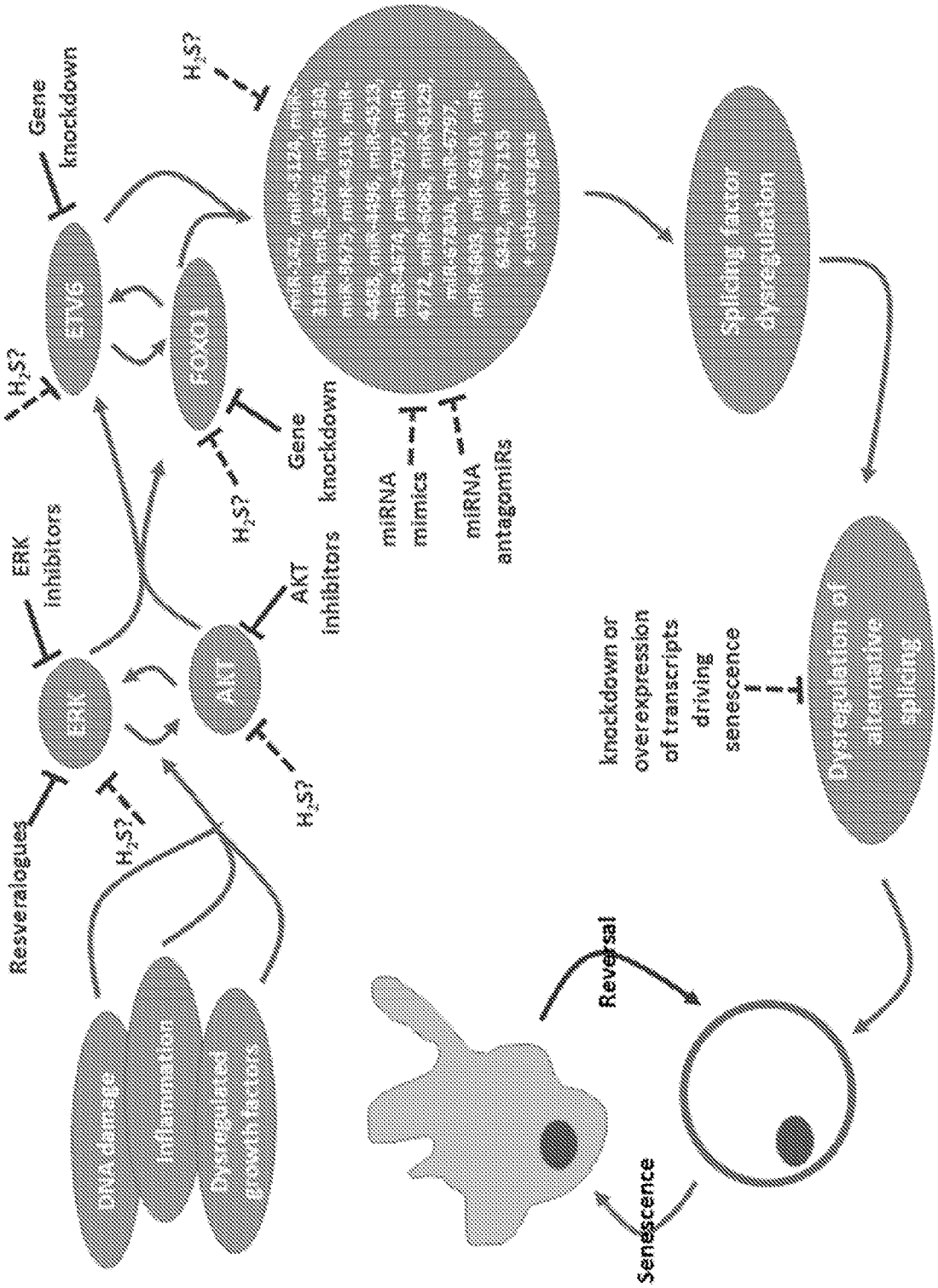

A.
FOXO1
ETV6
Fold Change
control
Trametinib 1uM
Trametinib 10uM
SH-6 1uM
SH-6 10 uM
B.
etv6
fox01
% located in the nucleus
Control
Trametinib 1uM
Trametinib 10uM
SH-6 1uM
SH-6 10uM

Figure 10


Resveratrogues
ERK inhibitors
AKT inhibitors
Gene knockdown
miRNA mimics
miRNA antagomiRs
H_2S?
ERK
AKT
FOXO1
ETV6
DNA damage
Inflammation
Dysregulated proliferation
Reversal
Senescence
Splicing factor dysregulation
Dysregulation of alternative splicing
knockdown or overexpression of transcripts driving senescence