SALICYLATE ACTIVATES AMPK AND SYNERGIZES WITH METFORMIN TO REDUCE THE SURVIVAL OF PROSTATE AND LUNG CANCERS *EX VIVO* THROUGH INHIBITION OF *DE NOVO* LIPOGENESIS

By

ANDREW O'BRIEN, B.Sc (H)

A Thesis Submitted to the School of Graduate Studies

In Partial fulfillment of the requirements

For the degree

Masters of Science

McMaster University

Master of Science (2015) McMaster University

Medical Science (Metabolism & Nutrition) Hamilton, Ontario

Title: Salicylate Activates AMPK And Synergizes With Metformin To Reduce The Survival Of Prostate And Lung Cancers *Ex Vivo* Through The Inhibition Of *De Novo* Lipogenesis.

Author: Andrew O'Brien, B.Sc (H) (McMaster University)

Supervisor: Gregory R. Steinberg, PhD

Number of pages: x,71

Abstract

Background: Aspirin, the pro-drug of salicylate, is associated with reduced incidence of death from cancers and is commonly prescribed in combination with metformin in individuals with type 2 diabetes. Salicylate activates the AMP-activated protein kinase (AMPK) via Ser108 of the AMPK β1 subunit, a mechanism that is distinct from metformin, which increases AMP:ATP. Many cancers have high rates of fatty acid synthesis and AMPK inhibits this pathway through phosphorylation of acetyl-CoA carboxylase (ACC). It is unknown if targeting the AMPK-ACC-lipogenic pathway using salicylate and metformin may be effective for inhibiting cancer cell survival.

Results: Salicylate suppresses clonogenic survival of prostate and lung cancer cells at therapeutic concentrations of aspirin. These clinically achievable concentrations of salicylate activated AMPK per the increasing phosphorylation of ACC and suppressing the activity of mTOR effectors kinase p70-S6 kinase and S6; effects that were enhanced with the addition of metformin and blunted in mouse embryonic fibroblasts (MEFS) deficient in AMPK β1. MEF cells deficient in AMPK β1 were more resistant to salicylates inhibitory effect on proliferation. Supplementation of media with fatty acids and mevalonate reverses the suppressive effects on cell survival indicating the inhibition of *de novo* lipogenesis is likely important.

Conclusions: Salicylate increases ACC phosphorylation, reduces phosphorylation of mTOR targets and inhibits *de novo* lipogenesis in prostate and lung cancer cells, with concentrations of salicylate achievable through the ingestion of Aspirin (0.25-1.0mM) these effects are blunted in AMPK β1 deficient cells. Effects on AMPK activity via ACC phosphorylation as well as reductions in mTOR signalling targets and *de novo* lipogenesis are enhanced when used in combination with metformin. Suppressive effects

on prostate and lung cancer cell survival are ameliorated when media is supplemented with mevalonate and fatty acids. Pre-clinical studies evaluating the use of salicylates alone and with metformin to inhibit *de novo* lipogenesis and the growth of prostate and lung cancers are warranted.

Acknowledgements

I would first like to thank my primary supervisor Dr. Greg Steinberg for his mentorship, knowledge and unyielding patience. His accessibility and understanding have created a laboratory culture that is collaborative, positive and productive. I feel grateful to have been able to take part in this. I would also like to thank my co-supervisor Dr. Theos Tsakiridis whose vast knowledge of cancer has been invaluable to the progress of the project. Dr. Tsakiridis expertise in the field of both basic science as well as clinical settings has increased the clinical translatability of the project. Dr. Tsakiridis has made every effort to exemplify the bench-to-bedside ethos and has made a tireless commitment to both his patients as well as his students.

Thank you to Dr. Hal Hirte for being apart of my supervisory committee for his insight and suggestions to improve the overall outcome of the project. Also, I would like to thank Dr. Hitre for sacrificing his time on top of what would be a full schedule.

Thank you to the Cancer Metabolism Team, without you the completion of this project would certainly not be feasible. Specifically, Dr. Vanessa Houde who helped with western blotting in Figure 3 and photo shopping immunoblot images; Linda Villani who contributed to Figure 3 and Lindsay Broadfield who contributed to Figure 4.

I would also like to thank the rest of the Steinberg Lab who have been immensely helpful to the project as well as my progression as an individual- Dr.Rebecca Ford, Dr. Brennan Smith, Dr. James Lally, Dr. Justin Crane, Dr. Emillio Motillo, Katarina Marcinko, Adam Bujak, Eric Desjardins, Alex Green, Emily Day, Rutu Panjabi and Julian Yabut.

Table of Contents

List of Figures

List of Abbreviations

4EBP1	**Eukaryotic Translation Initiation Factor 4E-Binding Protein 1**
ACC	**Acetyl-CoA Carboxylase**
ADP	**Adenosine Diphospahte**
AICAR	**5-Aminoimidazole-4-Carboxamide Ribonucleoside**
AKT/PKB	**AKT/ Protein Kinase B**
AMP	**Adenosine Monophosphate**
AMPK	**5' Adenosine Monophosphate Activated Protein Kinase**
ATP	**Adenosine Triphosphate**
COX1/2	**Cyclooxygenase 1/2**
DMEM	**Dulbeccos Modified Eagles Media**
EDTA	**Ethylenediaminetetraacetic Acid**
EGTA	**Ethylene Glycol Tetraacetic Acid**
EIF4E	**Eukaryotic Translation Initiation Factor 4E**
HCl	**Hydrogen Chloride**
HMG-CoA	**3-Hydroxy-3-Methyl-Glutaryl-CoA Reductase**
IGF1/2	**Insulin-like Growth Factor 1/2**
IKKβ	**Inhibitor of Nuclear Factor Kappa-B Kinase Subunit Beta**
LKB1	**Liver Kinase B1**
M	**Mole**
MEFS	**Mouse Embryonic Fibroblast**
mTORC1	**Mechanistic Target of Rapamycin Complex 1**
mM	**Millimole**
NaCl	**Sodium Cholride**
$Na_4O_7P_2$	**Disodium Phosphonato Phosphate**
Na_3VO_4	**Sodium Orthovanadate**
NF-κB	**Nuclear Factor Kappa-B**
NSAID	**Non-Steriodal Anti-Inflammatory Drug**

OCT1	**Organic Cation Transporter-1**
p70S6K	**Protein 70 S6 Kinase**
PAGE	**Polyacrylamide Gel Electrophoresis**
PBS	**Phosphate Buffered Saline**
PI3K	**Phosphatidylinositide 3-Kinase**
PTEN	**Phosphatase and Tensin Homolog**
PVDF	**Polyvinylidene Fluoride**
Raptor	**Regulatory associated protein of mTOR**
SDS	**Sodium Dodecyl Sulfate**
Ser	**Serine**
TAK1	**Transforming Growth Factor β activated kinase 1**
TCA	**Tricarboxylic Acid Cycle**
Thr	**Threonine**
TRAMP	**Transgenic Adenocarcinoma of Mouse Prostate**
TSC2	**Tuberous Sclerosis 2**

1.0 Introduction:

1.1 General Introduction:

Adenocarcinoma of the prostate is a commonly diagnosed cancer among men in the developed world[1] and the second leading cause of death for men in the world[2]. Prevention of prostate cancer remains a critical issue as approximately 903,500 new cancer cases will be diagnosed worldwide annually and of the diagnosed cases 258,400 will lead to death[3]. Interestingly, the highest mortality rates from prostate cancer appear to be in the developed world including Oceania, Europe and North America[3]. Researchers have suggested that the global variation is due to the convergence of several factors including detection, treatment, lifestyle and genetic factors[4].

Similarly, adenocarcinoma of the lung remains a major public health concern as 1.6 million cases were diagnosed worldwide and 1.4 million individuals diagnosed died due to lung cancer[3]. The major driver of lung cancer incidence is tobacco usage in a region, of particular importance is smoking tobacco accounting for 80% of lung cancer incidence in males and 50% of incidence in females[5,6].

Moving forward developing cost effective preventative strategies aimed at reducing the occurrence of cancer will be imperative. Preventative strategies are further hastened as a result of high global mortality and incidences of these cancers; moreover these factors will likely be compounded in the future[7]. Specifically the combination of smoking and an aging population will put tremendous stress on already scarce medical

resources[8]. To this end the repurposing of well tolerated and approved drugs for cancer use may be vital for future chemopreventative strategies.

Although these cancer sites can have a multitude of causes some researchers have suggested that lifestyle may play a role in the development of cancer, specifically, obesity[9]. Obesity is now considered to be a global epidemic as 300 million individuals worldwide are now considered to be obese[10] by clinical standards, where body-mass index is >30 kg/m^2. Given the global prevalence of obesity, the pathological links between cancer aetiology and obesity are now more apparent than ever. Strikingly, in 10% of all cancer deaths in non-smokers obesity is a contributing factor[9]. While smoking is the strongest predictor of lung cancer incidence amongst never smokers, it should be noted that in some studies there is a strong correlation between BMI and lung cancer incidence[11]. Most research seems to suggest that cancer progression and obesity is related to a number of metabolic alterations induced by obesity, among these changes can be insulin resistance and deregulated lipid metabolism[12].

It is now known that people who are obese have some degree of insulin resistance, although they may not be clinically diagnosed as diabetic[13]. Interestingly, those who are diagnosed with type 2 diabetes have a higher incidence of cancers of the breast and colon[14,15]. Concurrently, the prognosis of type 2 diabetics with prostate cancer is significantly worse than their non-diabetic counter parts[16]. This would seem to suggest that classical pathologies associated with diabetes including high circulating insulin or insulin like growth factors contributes to cancer development. Although, the role of insulin-like growth factor binding proteins has yet to be fully characterized[12]. However,

the convergence of population based epidemiological studies, clinical and basic science research strongly suggest that insulin and insulin-like growth factors (IGFs) drive cancer progression[17]. In prostate cancer high plasma IGF-1 levels have been shown to be an important predictor of prostate cancer incidence [18]. Similarly, in lung cancer it has been shown that IGF-2 is a critical mediator of cancer proliferation in human cell lines as well as in transgenic mice overexpressing the IGF-2 peptide[19]. Taken together both IGF-1/2 are important cellular mitogenic drivers as their signalling cascades activate the PI3K-mTORC1 pathway, which is constitutively active in many cancers[20], via AKT/PKB phosphorylation[21].

The higher incidence of population-based obesity has correlated with an increase in the rate of cancer of the colon, breast, kidney, oesophagus, pancreas, gall bladder amongst other common cancer sites[22]. Increased activty in the enzyme fatty acid synthase, which uses acetyl-CoA, malonyl-CoA and NADPH to build long-chain fatty acids[23], has been shown to be associated with a poorer prognosis in patients with prostate cancer [24,25]. Also an increase in fatty acid synthase activity is evidence of clinically aggressive lung cancer[26]. These data would indicate that deregulation of lipid metabolism is crucial in the development of cancer. In a cancer context, overexpressing the enzymes associated with lipogenesis is important as cancer cells dividing quickly have a rudimentary need for basic macromolecules for the development of a phospholipid membrane[27]. Increased lipogenesis in cancer can also be driven by constitutively active upstream growth factors and growth factor receptor signalling cascades especially the PI3K-AKT/PKB pathway[28,29].

The above evidence would appear to highlight an incontrovertible link between obesity including insulin resistance as well dyslipidemia and cancer. Targeting enzymes that ameliorate the above pathologies present a potentially attractive avenue for prevention and treatment of the suite of metabolic features associated with cancer. Leveraging the molecular action of 5' adenosine monophosphate activated protein kinase (AMPK) to inhibit cancer progression would be may be an attractive approach as the enzyme is intimately involved in the regulation of bodily insulin/IGF levels[30,31] as well as lipid metabolism[32]. Interestingly, obese individuals who are insulin resistant tend to have lower basal expression levels of AMPK[33], although AMPK activity can still be induced pharmacologically.

1.2 AMPK

5' adenosine monophosphate activated protein kinase (AMPK) is an evolutionarily conserved central regulator of cell energy status and becomes activated under low nutrient conditions[34], or when there is high cellular AMP:ATP[35]. Structurally, AMPK is heterotrimeric protein consisting of an α catalytic, β structural and γ regulatory subunits respectively[36] (for review see [37]). The α and β subunits consist of two isoforms while the γ subunit consists of three; therefore there can be twelve different expression profiles of AMPK in tissues[36]. This evolutionary redundancy underscores the importance of the enzyme, as organisms need to respond rapidly to an ever-changing environment to maintain homeostasis.

Classically, AMPK activation is mediated through the phosphorylation on amino acid threonine 172 on the α subunit, by upstream kinase liver kinase B1 (LKB1) in

response to environmental signals of low nutrients such as fasting or exercise[38,39]. Importantly, those with a rare mutation in the gene region coding for LKB1 known as Peutz-Jagers Syndrome have a strong predisposition to the development of both benign and malignant tumors[40,41]. The above would suggest that the LKB1-AMPK signaling axis is physiologically tumor suppressive. In a similar fashion the phosphorylation of Thr172 on AMPK is also regulated by upstream kinase calmodulin-dependent kinase kinase-β via an increase in intracellular calcium level[42,43]. More recently, another upstream kinase has been postulated to activate AMPK via a mechanism that is independent of both LKB1 and CaMKK-β, TGF-β-activates kinase -1 (TAK1). Importantly, AMPK activation by TAK1 is thought to mediate specific anticancer effects through induction of autophagy and inhibition of mTORC1[44] in hepatocellular carcinoma. AMPK can also be negatively regulated via phosphorylation on Ser487 on the α1 subunit as regulated by AKT/PKB activity[45]. AKT/PKB activation can be an important mediator of cancer progression and its hyper activation has recently been shown to restrain the LKB1-AMPK axis that would normally inhibit cell proliferation and growth[46].

Critically, AMPK is also allosterically regulated by compounds like A-769662 developed by Abbot Laboratories, as well as salicylate that have been shown to activate AMPK directly via a mechanism involving amino acid serine 108 on the β1 subunit of the AMPK complex[47]. Similarily, 5-Aminoimidazole-4-Carboxamide Ribonucleoside (AICAR) also activates AMPK allosterically, but, this is done through the γ subunit as it is metabolized into zinc monophosphate (ZMP) and acts as an adenosine monophosphate (AMP) mimetic[48]. Finally, AMPKs major allosteric regulator is AMP, which binds to the

γ subunit and increase the phosphorylation status of Thr172[49]. Although, other hypotheses have been proposed about the importance of ADPs ability to activate AMPK, this remains controversial.[50]

For cancer development AMPK appears to play an important role to restrain cell growth, proliferation and tumor burden via covalent activation by LKB1[46,51]. Importantly, AMPK has been shown to inhibit prostate cancer cell growth and proliferation in several studies[52,53], conversely AMPK knockouts promote cell growth in prostate cancer cells[54]. Some groups have noted the particular importance of the targeting the lipogenic pathway using AMPK activators to inhibit prostate cancer development[55]. Comparable inhibition of growth and proliferation have been obtained in lung cancer cell using AMPK activators metformin as well as ionizing radiotherapy[56,57], although some groups have also suggested that the LKB1 status of the lung cancer is also important when determining to use AMPK activators therapeutically[58]. The evidence above would underscore AMPKs ability to act as a tumor suppressor at multiple cancer sites specifically both prostate and lung by phosphorylating a number of different metabolic targets at the serine/threonine residue to try and induce a state of energetic homeostasis and inhibit physiologic anabolic processes like protein translation as well as the *de novo* synthesis of lipids[37].

1.3 mTORC1

In the past decade AMPK has become a widely studied target for anti-cancer therapies due to its inhibitory effects on the mechanistic target of rapamycin complex one or mTORC1 pathway, which is commonly mutated and constitutively active in many

adenocarcinomas[20]. mTORC1 is a point of cellular convergence, where by the mTORC1 protein complex must integrate metabolic cues to drive cell proliferation and survival or if conditions are unfavorable to cell division acquiesce accordingly[59], low cellular energy status will activate AMPK. AMPK will inhibit mTORC1 through phosphorylating and inhibiting Raptor[60]. Raptor (regulatory associated protein of mTOR) is used to recruit effector targets 4EBP1 and p70S6K to mTORC1[60-62]. AMPK also activates Tuberous Sclerosis Complex 2 (TSC2) [63] via phosphorylation, which inhibits Rheb-GTP an upstream activator of mTORC1[64]. The decrease in mTORC1 activity leads to a decrease in major downstream kinase p70-S6K, which is important in mRNA biogenesis as well as translation initiation and elongation[59], and p70-S6K downstream target S6 that enhances mRNA translation[65].

Consistent with the activation of AMPK, salicylate was shown to reduce the activity mTORC1/S6 kinase in HCT116 colon cancer cells[66-68]; however higher concentrations of salicylate are thought to be independent of AMPK activation[69]. Importantly, targeting of the mTORC1 pathway has been the subject of much research for both prostate as well as lung cancer, it is thought that overexpression of the AKT/PKB-mTORC1-S6 kinase pathway can lead to the development of prostatic neoplasms[70]. Given this, researchers have used the drug RAD001, a specific mTORC1 inhibitor developed by Novartis, in a PTEN deficient murine model of prostate cancer to abrogate tumor development[71]. Similarly, in lung cancer mTORC1 is known to be commonly mutated and specifically 90% of adenocarcinomas, and clinically represents poorer prognosis for patients[72,73]. The activation of mTORC1 and inhibition of 4EBP1 helps to over express

eukaryotic initiation factor 4E (EIF4E), which functions to initiate cap-dependent translation[74] as well act as oncogene during transformation. Oncogenic transformation of a cell has been described classically as unlimited replicative ability, evading apoptosis, self sufficiency in growth signals, insensitivity to anti growth signals, sustained angiogenesis and the ability to metastasize and invade neighboring tissues[75]. Importantly, the effects of aspirin/salicylate on AMPK and mTORC1 signaling in prostate and lung cancer cells have not yet been characterized.

1.4 Lipid Synthesis

De novo synthesis of lipids are essential for rapid cell growth and is elevated in many tumours suggesting that inhibition of this pathway may prevent proliferation[23]. AMPK is a negative regulator of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA) and acetyl-CoA-carboxylase (ACC), the rate limiting enzymes for cholesterol and free fatty acid biosynthesis, respectively[32]. We have recently demonstrated that in the liver metformin and the direct AMPK β1 activator A769662 inhibits *de novo* lipogenesis through AMPKβ1 dependent phosphorylation and inhibition of ACC[76]. Salicylate also inhibits *de novo* lipogenesis in the liver[77] but whether this effect is dependent on AMPK β1 has not yet been fully established, although very recent work in bone marrow derived macrophages has started to characterize the β1 subunits importance[78]. In prostate cancer AMPK expression is vital for inhibiting prostate cancer cell proliferation [54] an effect that may be related to the inhibition of *de novo* lipogenesis [52,53]. Similarly, lung cancer cells have been shown to have an increased expression of ATP citrate lysase[79,80], which is a critical enzyme in *de novo* lipogenesis as it generates cystolic acetyl-CoA. Importantly,

over expression of ATP citrate lysase has been correlated with a poorer prognosis in patients with lung cancer[80]. This will be the first study to look at specific AMPK mediated inhibition of *de novo* lipogenesis in lung cancer cells. Targeting the *de novo* lipogenic pathway may provide effective clinical leverage to blunt cancer progression in certain contexts[81], whether salicylate and metformin reduce lipogenesis in cancer cells and whether this is important for inhibition of proliferation/survival is not known.

1.5 Aspirin

Daily aspirin ingestion reduces the risk of death from adenocarcinomas of the colon, lung and prostate[82,83]. While the anti-tumour effects of aspirin are widely believed to involve its anti-inflammatory properties, most studies have found that other non-steroidal anti-inflammatory (NSAIDs) [84-86] or antiplatelet[82] therapies do not have the same anti-cancer properties indicating that other mechanisms may be important.

Upon ingestion aspirin is rapidly broken down into salicylate by carboxyesterases in the liver, which enhances its relative effective circulating concentration and half-life[87]. Salicylate is a plant-derived anti-infection hormone[88] that has been used for medicinal purposes for over 200 years, originally being crudely synthesized from the bark of the willow tree[89]. Vane et al characterized aspirins classical mechanism of disease amelioration in 1971, showing that aspirin inhibited prostaglandin production via a reduction in cyclooxygenase activity[90]. But, further studies have shown that salicylate, which unlike aspirin lacks an acetyl group and is considered to be less effective at inhibiting cyclooxygenase (COX) activity, is still able to reduce inflammation in patients with rheumatoid arthritis[91]. Other mechanisms for salicylates anti-inflammatory

properties have been suggested, including the inhibition of IKKβ, as salicylate binds at the same site as ATP inactivating the protein[92]. Mechanistically, IKKβ is a critical driver of the pro-inflammatory NF-κB signalling cascade as it phosphorylates and degrades IKβ allowing the NF-κB protein complex to translocate to the nucleus[92]. However, the evidence for this hypothesis is weak when trying to scale to physiological concentrations of salicylate[92,93]. Given the above evidence there appears to be another major target to act as a conduit for salicylates therapeutic actions.

Critically, salicylate/aspirin have been shown to have an effect on both prostate as well as lung cancer, having been confirmed from population based evidence and corroborated by basic science studies. Broadly speaking, there has been a wealth of epidemiological studies that highlight an inverse correlation between aspirin use and a lower incidence of prostate cancer in men[83,84,94-97]. Some research has attributed the anti-cancer mechanism of aspirin use specifically to its anti-inflammatory properties[98]. Prostate cancer can be triggered in part by chronic inflammation and overexpression of pro inflammatory markers[99-101], but, interestingly studies have noted that aspirin use and not use of other NSAIDs is associated with reduced prostate cancer risk[83,84,94], particularly prostate cancer that is aggressive and metastatic[95-97]. This information leads to the intriguing possibility that other enzymes are mediating the anti-cancer effect of aspirin in prostate cancer.

Similar results have been obtained with lung cancer as aspirin has been correlated with a decrease in disease incidence[102]. Specifically, aspirin has had a noted effect for the reduction of non-small cell lung carcinoma[103,104]. Again, researchers have postulated

aspirin ability to reduce lung cancer incidence is due to the reduction in the COX-2, a pro-inflammatory enzyme that converts arachidonic acid to prostaglandins. Interestingly, COX-2 overexpression is found in 70% of lung adenocarcinomas[105,106], however, like prostate cancer other NSAIDs like ibuprofen and acetaminophen which are able to reduce COX-2 expression are noted as not having the same anti-cancer effects of aspirin[47,107,108]. The importance of salicylate induced AMPK activation via β1 expression in mediating salicylates anti-cancer properties on prostate and lung cancer is not known.

1.6 Metformin

Like aspirin, metformin is a widely prescribed therapeutic, typically used as a first line treatment for type-two diabetes. There are, however, several clinical trials looking at the efficacy of metformin's antineoplastic ability[109]. Metformin use for its part has been correlated with a life-long reduction in cancer development [110-117]. Despite extensive research over the last decade and multiple potential targets, the exact mechanism by which metformin may have antineoplastic activity is still not known, but, many have postulated that it may be due to metformins ability to induce cellular energetic stress[118]. Metformins ability to disrupt cellular energy homeostasis is critical in a type-two diabetes context as it inhibits gluconeogenesis and ameliorates the classic pathologies associated with type-two diabetes hyperinsulinemia, hyperglycemia as well as hepatic *de novo* lipogenesis[76,109,119,120]. In an oncology setting the lowering of circulating insulin may also be critical, as high circulating insulin levels are correlated with poorer patient outcomes[121]. Insulin and IGFs activate the PI3K-mTOR signalling pathway, which is a crucial molecular junction that drives cell proliferation and survival[109]. If metformin can

buffer against high circulating insulin levels this may attenuate some of the proliferative action of the PI3K-mTOR pathway[109]. Many factors will determine if a cancer type will be sensitive to the metabolic modulation induced by metformin. Since it is known that metformin enters the cell via an OCT1 dependant manner, expression of the transporter protein is a necessary component, when considering metformin therapeutically[118]. Other groups have reported that LKB1 status of the cancer is also important. As cells lacking a functional LKB1 protein cannot effectively adjust their energy status and thus under go apoptosis[58]. This study, however, used metformins more potent biguanide cousin phenformin[58]. Critically, similar results using cells with knocked down LKB1 have been duplicated using metformin[122].

Clinically, there is recent evidence that metformin use is associated with a lower incidence of prostate cancer. A study from Denmark has suggested that after controlling for diabetic severity and other lifestyle factors, metformin use was associated with a decreased risk of prostate cancer compared to no-use or other oral hyperglycaemia medications[123], a reduction of prostate cancer that has been noted previously by other studies[124-126]. The population-based evidence has also been corroborated by both *in vivo* and *in vitro* studies that show metformin is able to reduce cancer progression[127-129]. Interestingly metformin has been combined with other anti-cancer agents to lower toxicity and enhance the efficacy of traditional modalities[57,130-132]. Again, similar results have been obtained for metformins ability to reduce the incidence of lung cancer at the population-based levels as some epidemiologists have noted diabetics who use metformin have a lower incidence of lung cancer, although the preventative effects of metformin

appear to be most efficacious in subpopulations of never smokers[133-135]. Metformin use has been associated with longer survival times of diabetic patients with advanced staged lung cancer[136]. The hypothesis-generating population studies have been reinforced by a number of *in vivo* and *in vitro* pre-clinical studies that have underscored metformins anti-cancer effect both when used alone[137-139] as well as in combination with chemo and radiotherapy[56,140-144].

The exact mechanism for metformins anti-neoplastic ability is currently unknown, although there is intriguing evidence suggesting that cancers with mutations that encode for complex I of the mitochondria are also particularly sensitive to the effects of metformin[145]. In concurrence to the emerging evidence, more conventional molecular models suggest that the effects of metformin may be linked to the inhibition of mitochondrial respiration[146] an idea consistent with the well-established action of metformin to activate AMPK by reducing the adenylate charge of the cell[147,148]. Importantly, this mechanism of AMPK activation is entirely distinct from the direct actions of salicylate on the AMPK β1 subunit[47]. The activation of AMPK with metformin may be an attractive approach to abrogate some of the Warburg effect. The Warburg effect describes metabolic perturbations in a cancer cell where by the cell will have tremendously high glycolytic rates and preferentially produce lactate even in the presence of oxygen, yielding lower net ATP but conferring biomass and biosynthetic advantages[149,150].

However, a major caveat to many of the preclinical studies which have utilized metformin to inhibit cancer growth is that mM concentrations have been used when

maximum clinical concentrations are 50-100 μM[118]. Although, an intensive investigation of metformin in cancer therapy is underway, strategies aimed at reducing the dose of metformin required for clinical effectiveness are important to maximize the anti-neoplastic activity of this well tolerated therapeutic. Combination therapy of metformin with salicylate may be a promising avenue to achieve this.

In the current study we demonstrate that salicylate dose dependently reduces the clonogenic survival of adenocarcinomas with differing sensitivities (prostate>lung). In prostate and lung cancers reductions in clonogenic survival occur at serum salicylate concentrations achievable through intake of aspirin, and are associated with increases in ACC phosphorylation, reductions in the phosphorylation of downstream targets of mTOR and the suppression of *de novo* lipogenesis. Importantly, MEFS expressing the wildtype AMPK β1 subunit are more sensitive to the inhibition of proliferation using salicylate relative to β1 knockouts; concurrently salicylates effects on ACC and p70S6K are dependent on the AMPK β1 subunit. Salicylate induced inhibition of clonogenic survival in prostate and lung cancer is prevented by supplementing the media with fatty acids and/or mevalonate indicating that the suppression of *de novo* lipogenesis may be vital for reducing cell survival. Importantly, the effects of salicylate on cell survival, ACC, mTORC1 and *de novo* lipogenesis are enhanced when used in combination with metformin. These data suggest that salicylate (aspirin) and metformin may work collaboratively to reduce the survival of prostate and lung cancers through activation of AMPK and the inhibition of *de novo* lipogenesis.

2.0 Hypothesis and Objectives:

2.1 Hypothesis:

The Steinberg Lab in collaboration with others have recently shown that salicylate, the active component in aspirin, was found to activate AMPK via a β1 allosteric mechanism. It is also known that there is a statistically significant decrease in cancer incidence among users of aspirin relative to other NSAIDs and the type two-diabetes medication metformin relative to non-users. 1. We hypothesize that the treatment of prostate and lung cancer cells with salicylate will decrease the survival of the cancer cells via inhibition of *de novo* lipogenesis. Also, that there will be an increase in AMPK signalling target ACC using salicylate and decrease mTORC1 signalling kinase p70S6K and its downstream target S6. Salicylates activity will be most effectively mediated with wildtype expression of the β1 subunit of AMPK. 2. Finally, we hypothesize that salicylate and metformin will work collaboratively to decrease cellular *de novo* lipogenesis; enhance the phosphorylation of AMPK target ACC and conversely a decrease in the phosphorylation status of mTORC1 target p70S6K and its effector S6.

2.2 Objectives:

1.1 Investigate the effect of salicylate on the survival of prostate and lung cancer cell lines.

1.2 Examine the effect of salicylate on AMPK and mTORC1 signalling events.

1.3 Delineate the importance of β1 expression in mediating the effects of salicylate.

1.4 Determine the effect of salicylate on cellular *de novo* lipogenesis.

1.5 Delineate the importance of exogenous lipids on cellular proliferation.

2.1 Examine the combined effect of salicylate and metformin on AMPK and mTORC1 signalling targets.

2.2 Investigate if salicylate and metformin work co-operatively to reduce *de novo* lipogenesis.

2.3 Rationale:

Long-term aspirin intake has been associated with a reduction in cancer incidence of common cancer epithelial sites- especially of the prostate and lungs[82,83]. Likewise, type 2 diabetic patients who are prescribed metformin for blood glucose control also have a lower incidence of cancer[116]. Although, the reduction of common cancers has been noted, the exact mechanisms by which the reductions in cancer incidence have yet to be fully characterized. It is known that AMPK is potently activated by salicylate, aspirins active component, as well as metformin. Thus, AMPK activity is potentially a critical driver of some of the anti-cancer properties of both salicylate[108] as well as metformin [118]. Importantly, salicylate activation of AMPK is thought to be completely different from that of metformin, as the former allosterically activates AMPK[47] while the latter operates indirectly by changing the adenylate charge of the cell[148]. This will be the first study to report the effect of salicylate, as well as low dose metformin in combination with salicylate on common cancer sites of the prostate and lung. This study will investigate how AMPK activity mediates the effect of the drugs. Finally, this study will delineate the importance of *de novo* lipogenesis to cancer cell survival; of which AMPK is an

important mediator[32,76]; and crucially cancer cells are known to have an increased rate of lipogenesis [23].

3.0 Methods and Materials:

3.1 Cell lines and treatments Lung (A549, H1299), and prostate (PC3, 22RV-1) cancer cells were obtained from the American Type Culture Collection (ATCC: Manassa, VA). Mouse embryonic fibroblasts (MEFS) from wildtype and AMPKβ1 knockout (KO) mice were generated from primary MEFS through spontaneous immortalization using sequential passaging. Where immortalization was considered to be when the cells showed an ability to maintain a constant or rising growth rate as well as uniform morphology, which occurred typically at passages 20-24. MEFS were grown in DMEM medium (Gibco: Mississauga, ON) supplemented with 1% antibiotic-antimycotic and 10% FBS. Lung and prostate cancer cells were grown using RPMI medium supplemented with 1% antibiotic-antimycotic and 10% FBS**.** All cells were maintained at 37^{o}C and were treated with the indicated concentrations of salicylate (Sigma: Toronto, ON) or metformin (Sigma: Toronto, ON) for 48h unless stated otherwise.

3.2 Cell Proliferation Assay Approximately 1,000 (MEF AMPK β1 wildtype) or 500 (MEF AMPK β1KO) were seeded into individual wells of a 96-well plate in triplicate and maintained with the indicated dose of salicylate. After 72 hours cells were fixed for 10 minutes in 10% buffered formalin and washed; the cells were then stained for 10 minutes with 0.5% (w/v) crystal violet in 25% methanol and washed. The crystal violet stain was solubilized in 0.5mM sodium diphosphate for 10 minutes. The absorbance values of the solubilized stained DNA were quantified using spectrophotometer set at 570nm (MBI, Dorval, PQ, Canada).

3.3 Clonogenic Assay Cells were subjected to clonogenic assays as described [151]. In brief, approximately 500 cells were seeded into individual wells of a 12-well plate in triplicate and maintained at the indicated doses of salicylate. After 7 days cells the cells were fixed with 0.5% (w/v) crystal violet in 50% methanol for 10 minutes and viable colonies (>50 cells) were counted manually.

3.4 Immunoblotting Cells were washed with PBS and lysed in ice-cold lysis buffer (20mM Tris HCl, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% triton-X100, 2.5mM $Na_4O_7P_2$, 1mM Na_3VO_4, and 1 Roche inhibitor cocktail). 4x Laemmli-SDS-sample buffer was then added and the samples were subsequently boiled for ten minutes at 95^{o}C. Ten (22RV-1) or thirty (H1299/MEFS) µg of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membrane or nitrocellulose as indicated and incubated with primary antibodies (1:1,000 dilution) for sixteen hours at 4^{o}C and horseradish peroxidase (HRP)-conjugated secondary antibodies (1: 5,000 dilution) at room temperature for one hour (Cell Signalling, Mississauga, ON). The membranes were washed using Tris-buffered saline with 1% Tween 20 for thirty minutes and finally visualized using Bio-Rad Clarity Enhanced Chemiluminescence for approximately one minute.

3.5 Densitometry of immunoblots was performed using Image J software (McMaster University Biophotonics Lab, Hamilton, ON). Densitometry values are expressed as percent of control.

3.6 Lipogenesis Assay Cells were treated with the indicated drugs for 48h prior to the addition of radiolabelled ^{3}H-sodium acetate (10µCi/mL, Perkins Elmer) and unlabelled

sodium acetate (0.5mM, Sigma, ON) for 4h. This assay is able to measure the rate of lipogenesis as the acetate once in the cell is converted by acetyl-CoA synthase to acetyl-CoA, once converted to acetyl-CoA the labelled acetate can enter either the fatty acid synthesis or cholesterol biosynthesis pathway as acetyl-CoA is a critical substrate in both pathways .The cells were then washed with PBS, manually scraped and collected into 1.5mL tubes, and lipid extraction with chloroform:methanol (2:1) was performed. In brief, 750µL was added to the tubes and vortex for 15 seconds. The tubes were then put into a -20^{o}C freezer for 10 minutes, after, which 250µL of choloform was added to the tubes, which were vortexed for 15 seconds. Finally, 250µL of ddH_20 was added and the solution was vortexed for 15 seconds. The tube was the centrifuged at 4^{o}C for 10 minutes at 3,000 rpm. From that, 100µL of the bottom organic phase was taken and put into a scintillation tube with 5mL of scintillation fluid and read in a scintillation counter, all as previously described[76].

3.7 Statistical Analysis Unless otherwise indicated results are expressed as mean with standard error (SE). Statistical analyses were performed using a two-way or one-way analysis of variance (ANOVA) when appropriate. For between treatments analysis Fisher-LSD post-hoc test was used; for genotype analysis Sidak multiple comparison test was utilized. Significance was set at * = $P<0.05$ using GraphPad Prism 6 software (La Jolla, CA). For colonogenic and lipogenesis assays IC_{50} values were calculated using a non-linear regression model (with normalized slope) in GraphPad Prism 6 (La Jolla, CA). Drug synergy was calculated using the combination index-isobologram (CI) theorem and CompuSyn software[152].

4.0 Results:

4.1 Salicylate dose dependently inhibits clonogenic survival

Salicylate inhibited the survival of prostate (Figure 1A) and H1299 lung cancer cells (Figure 1B) by greater than 50% at concentrations of less than 1.0 mM, which are observed in serum following the intake of 325mg regular strength aspirin[92,153], these concentrations are higher than what would be expected with the ingestion of 81mg baby aspirin which is 5-25μmol/L[153,154]. We hypothesized that the inhibition of clonogenic survival in prostate and lung cancers involved activation of AMPK so we examined phosphorylation of AMPK α (Thr172) and its downstream substrate ACC (Ser79). ACC is considered the most sensitive measure of cellular AMPK activity as it takes into account both the allosteric and covalent regulation of AMPK[49]. In 22RV-1 prostate cancer cells only 5.0 mM salicylate increased Thr172 AMPK phosphorylation. In contrast, salicylate increased the phosphorylation of Ser79 ACC starting at concentrations as low as 1 mM (Figure 2A-2C). The lack of phosphorylation of AMPK but increases in ACC phosphorylation at concentrations <5.0 mM is consistent with direct activation of AMPK via allosteric mechanisms involving the β1-subunit and independent of energy charge [47,108]. Salicylate dose dependently reduced mTOR, as measured by phosphorylation of its downstream kinase p70S6K and its effector S6 (Figure 2A, D and E). It should be noted that 3-5mM salicylate also could effect the total protein expression of AMPK, ACC, p70S6K and S6 relative to β-actin (Table 1), these changes in protein level are probably occurring due to the well defined toxicity profile of salicylate[155]. In H1299 lung cancer cells salicylate did not alter Thr172 phosphorylation of AMPK

(Figure 3A and B) but did increase the phosphorylation of ACC (Figure 3A and C). It also dose dependently lowered p70S6K (Figure 3A and D) and S6 (Figure 3A and E) phosphorylation.

Importantly, the expression of AMPK β1 appears to be critical for most effectively mediating salicylates anti proliferative action (Figure 4). Concurrently, salicylate dose dependently increased AMPK and ACC phosphorylation in wildtype (WT) but not AMPK β1 null MEFS (Figure 5A-C). At salicylate concentrations between 0.25 and 1.0mM the suppression of p70S6K occurred via an AMPK β1-dependent pathway (Figure 5A, D). In contrast salicylate suppressed the phosphorylation of S6 in both WT and AMPK β1 null MEFS (Figure 5A, E). These data indicate that at clinically relevant concentrations salicylate increases the phosphorylation of ACC and inhibits the phosphorylation of p70S6K through an AMPK β1 dependent pathway.

4.2 Salicylate inhibits *de novo* lipogenesis and clonogenic survival in prostate and lung cancer via an AMPK β1 dependent pathway

AMPK phosphorylation of ACC is the rate-limiting step controlling *de novo* fatty acid synthesis[76]. Elevated rates of *de novo* lipogenesis are observed in prostate and lung cancers[23] therefore we hypothesized that salicylate may inhibit cell survival through the regulation of this pathway. Consistent with changes in ACC phosphorylation salicylate dose dependently inhibited *de novo* fatty acid synthesis in both 22RV-1 prostate (Figure 6A) and H1299 lung (Figure 6B) cancer cells. The inhibition of *de novo* fatty acid synthesis correlated strongly with clonogenic survival in both cell lines suggesting a possible interrelationship (Figure 6C). In MEFS the salicylate-induced inhibition of *de*

novo lipogenesis was dependent on the expression of the AMPK β1 subunit at salicylate concentrations less than 3mM (Figure 6D). At higher concentrations the inhibition of lipogenesis was independent of AMPK β1 consistent with previous reports in hepatocytes indicating that this effect is independent of ACC activity and is likely due to salicylate-induced uncoupling and activation of the citric acid cycle, which in turn starves ACC of substrate[156]. To directly test whether salicylate-induced inhibition of lipogenesis was important for reducing clonogenic survival, we supplemented the media with mevalonate and/or oleate at concentrations previously demonstrated to alleviate the effects of a direct AMPK activator on prostate cancer cell proliferation[52]. In 22RV-1 prostate cancer cells supplementation with mevalonate largely prevented the inhibitory effects of salicylate on clonogenic survival (Figure 6E). Supplementation of the media with oleate had more modest effects that were not additive with mevalonate (Figure 6E). In H1299 lung cancer cells mevalonate was without effect but oleate supplementation completely prevented salicylate-induced suppression of clonogenic survival (Figure 6F). These data indicate that suppression of *de novo* cholesterol and fatty acid biosynthesis is likely an important mechanism contributing to the reduced survival of 22RV-1 prostate and H1299 lung cancer cells. Clinically many factors will contribute to the efficacy of salicylate, including if the patient is in the fed or fasted state, in a fasted state their will be a higher concentration of free fatty acids which may blunt some of the effectiveness of salicylate to reduce lipogenesis.

4.3 Metformin and salicylate synergistically inhibit cancer cell proliferation

Many type 2 diabetics who take metformin for blood glucose control are also prescribed between 81-325mg of aspirin for cardioprotection[157]. To examine the interaction of these two commonly used medications we performed dose responses with salicylate, metformin or both therapies and found that the IC_{50} for clonogenic survival was dramatically reduced in all cell types when metformin and salicylate were used in combination, A549 cells showed particular sensitivity to metformin as has been previously described[122] (Figure 7, Table 2). Further analysis of the IC_{50} curves using CompuSyn based software indicated that in prostate (PC3 and 22RV-1) and lung (A549 and H1299) cancer cells the effects of metformin and salicylate were either synergistic or additive (Table 2). We subsequently examined the effects of clinically achievable concentrations of metformin (100μM) with or without 1mM salicylate on AMPK signalling and lipogenesis in 22RV-1 (Figure 8) and H1299 (Figure 9) cells. In 22RV-1 prostate cancer cells metformin treatment did not affect the phosphorylation of AMPK or downstream substrates (ACC, p70S6K or S6) (Figure 8A-E). Consistent with previous results salicylate at 1mM had modest effects on ACC, p70 and S6 phosphorylation but when combined with metformin these effects became more dramatic (Figure 8A-E). Similar results were observed in H1299 lung cancer cells (Figure 9A-E). Mirroring changes in ACC phosphorylation, metformin alone did not affect lipogenesis but when combined with salicylate it enhanced the inhibition of lipogenesis in both 22RV-1 (Figure 10A) and H1299 (Figure 10B) cells. Enhanced ACC phosphorylation and suppression of lipogenesis was noted with combination therapy in both 22RV-1 (Figure 10A) and H1299 (Figure 10B) cells; an effect which was dependent on the presence of the AMPK β1

subunit in MEFS (Figure 10C). Importantly, the supplementation of the media with oleate and/or mevalonate blunted the suppressive effects of salicylate and metformin co-treatment on clonogenic survival in both 22RV-1 prostate (Figure 10D) and H1299 lung (Figure 10E) cancer cells. The clinical concentrations of free fatty acids and sterols tends to be determined by what nutritional state the individual is in. Specifically fasting increases the serum concentration of free fatty acids and sterols [158], which may blunt the effectiveness of salicylate and metformin to inhibit clonogenic survival.

5.0 Discussion:

An important role for aspirin in suppressing colon cancer has been highly documented[68,86]. Reduced incidence of adenocarcinomas of the lung, prostate and breast have also been noted in numerous studies (for detailed meta-analysis and review see [159]). We find that the clonogenic survival of prostate and lung cancer cells is impaired with salicylate treatment of cells that are consistent with the ingestion of regular strength aspirin. Dose dependent reductions in clonogenic survival in prostate and lung cancer cells were associated with activating AMPK and subsequent inhibitory phosphorylation of Ser79 on ACC. Using mice with a targeted knock-in mutation we have recently demonstrated that phosphorylation at this site is essential for mediating the anti-lipogenic effects of AMPK[76]. In addition salicylate dose dependently reduced the phosphorylation of downstream mTORC1 target kinase p70S6K (Thr389) and its downstream target S6 (Ser240/244). Taken together these results are consistent with previous findings that salicylate activates AMPK and increases ACC phosphorylation[47] while reducing mTORC1 activity[68]. However, some have reported that very high doses of salicylate are independent of AMPK activation[69] . The expression of the β1 subunit of AMPK appears to be important for most effectively mediating the anti proliferative action of salicylate. To be sure, however, these data indicate that MEF cells may not be as dependent on lipogenesis for proliferation. This discrepancy may be a function of the *in vitro* environment; as previous studies have reported that cells cultured in high glucose media (DMEM 25mmol/L of glucose) are more resistant to the anti-proliferative action of AMPK activators[160,161]. Specific phosphorylation of ACC and S6 kinase targets within

the therapeutic window of salicylate (up to 1mM) requires β1 expression, however, at higher concentrations the inhibition of mTORC1 occurs independently of AMPK β1. These AMPK-independent effects on mTORC1 activity at high salicylate concentrations are consistent with a very recent study in MEFS lacking AMPK α subunits[69].

It has been known for over 5 decades that salicylate suppresses liver lipid synthesis but the mechanism mediating this effect was not established[77]. Given the essential requirement for *de novo* lipogenesis in rapidly dividing cells and the elevated rates of *de novo* lipogenesis observed in many prostate and lung cancers[26,162-164], we examined the effects of salicylate on this pathway. We show for the first time that salicylate dose dependently inhibits lipogenesis in prostate and lung cancers. Linear regression analysis indicated the highly correlative nature of clonogenic survival and lipogenesis across both cells types suggesting an important role for this process in limiting cell survival. Importantly, we demonstrated that the expression of the AMPK β1 subunit is vital for the inhibition of lipogenesis at concentrations <3mM. The finding that higher concentrations of salicylate inhibited *de novo* lipogenesis independently of AMPK β1 is consistent with previous observations that high concentrations of salicylate can uncouple mitochondrial respiration[47] resulting in increased TCA cycle flux which reduces acetyl-CoA, starving ACC of substrate[156]; an effect which occurs irrespective of AMPK phosphorylation of ACC. Importantly, we find that when the cancer cells were supplemented with oleate and/or mevalonate, the inhibitory effects of salicylate on cell survival were severely limited indicating a vital need for lipogenesis in controlling cell survival in rapidly replicating cells. Previous studies have indicated the presence of a

trans-methylglutaconate shunt in many tissues including the brain, spinal cord, skin and aorta. It is postulated that mevalonate is diverted through a shunt to 3-hydroxy-3-methylglutaryl- CoA and free acetoacetate is then utilized preferentially for fatty acid synthesis [165-167].

It is known that metformin activates AMPK by reducing the adenylate charge of the cell resulting in subsequent activation of the kinase via the AMPK γ subunit[147,148]. In contrast salicylate increases AMPK activity through an allosteric mechanism requiring the AMPK β1 subunit[47,49]. Given their distinct mechanism of AMPK activation we tested whether combining these two well-tolerated therapies could lead to the potentiation of the anti-tumor activity of salicylate in cancer cells. We found that the effects of metformin were either additive or synergistic with salicylate for inhibiting prostate and lung cancer cell survival. Using immunoblot analysis we found that at maximally achievable clinical concentrations of metformin (100 μM) there was no effect on the phosphorylation of AMPK, ACC or mTOR substrates[118]. However, when combined with 1mM salicylate, metformin enhanced the effects of salicylate to increase ACC phosphorylation and suppress mTOR in prostate and lung cancer cells. Consistent, with changes in ACC phosphorylation the rate of cellular lipogenesis and cell survival was significantly reduced with combination therapy. Importantly, the combined effects of salicylate and metformin on lipogenesis were dependent on the expression of the β1 subunit of AMPK and were eliminated when the media was supplemented with oleate and mevalonate. When metformin is combined with an allosteric activator of AMPK such as salicylate there is a potentiation of the allosteric response. These findings are consistent with our

recent observations in cell-free assays which demonstrate that AMP (generated in hepatocytes by metformin [168]) and salicylate together increase the activity of purified, non-phosphorylated AMPK α1β1γ1 heterotrimers by greater than 60-fold, but only have minor effects on allosteric activation when provided alone[169]. This is also consistent with the findings that the drug A769662, which binds to the same site as salicylate, dramatically potentiates allosteric activation of AMPK when applied in combination with AMP or the AMP mimetic AICAR[170,171]. The fact that metformin and salicylate appear to work collaboratively highlights the potential offered by combining low doses of both metformin and salicylate for inhibiting tumor cell growth.

5.1 Future Directions

This thesis has found that salicylate, the active compound in aspirin, is able to reduce the survival of prostate and lung cancer *ex vivo*. Also, this thesis highlighted that treatment with salicylate increases AMPK activity via the phosphorylation of Ser79 on ACC and conversely there is a reduction in mTOR signaling kinase p70S6K and its effector S6. Finally, salicylate inhibits cellular *de novo* lipognesis and when combined with metformin there is potentiation of the above effects. Further extension of this initial research should better characterize the importance of the β1 subunit of mediating the effect of salicylate. It would be important to have a stable knockdown of the β1 subunit in the 22RV-1 prostate and H1299 lung cancer cells. Although, this concept has been preliminarily explored in the MEF cells, they are not human and are non-cancerous cells as they do not share most of the cardinal features of transformation as describe by Hanahan and Weinberg, although it should be noted that

some MEF cell lines have chromosomal instability which enhances the cells ability for spontaneous immortalization.

Further strengthening the clinical applicability of the project, would be expansion to an *in vivo* murine model. Given that the prostate cancer cells appeared to be most responsive to the treatment with salicylate it would make sense to move forward with this cancer type. For this, the well-characterized transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model, which was developed on a C57BL/6 background, could be used. These mice will spontaneously develop intermediate and high-grade orthotopic prostatic lesions starting from about 10 weeks of age[172]. Importantly, these mice develop prostate cancer in a fashion that mirrors the development of human disease[172]. *In vitro* extensions of this mouse model has lead to the development of several immortalized cells lines including TRAMP-C1 and TRAMP-C2 both of which can be injected into C57BL/6 mice to induce ectopic tumors[173] and allow for primary screening on the efficacy of salicylate treatment as well as the combination of salicylate and metformin.

Mechanistically, it would be important to show the *in vivo* effect of salicylate on a β1 knockout murine model in the context of cancer progression. To this end, the β1 knockout mouse model that has been previously characterized by Dzamko et al[174] could be crossbred with the TRAMP mouse model. This would be a significant advancement, as it would produce a novel murine model that will faithfully develop prostate cancer in a similar fashion to humans but in the absence of the β1 subunit of AMPK. Important outcomes measures to follow up on are with regard to the proposed future directions are:

Is AMPK activity decreased in the tumor cells and is there a reduced phosphorylation of mTOR targets p70S6K and S6? Does salicylate reduce the tumor volume of treated versus control mice? Is there a statistically significant difference of the survival curve of mice injected with TRAMP-C1/2 cells treated with salicylate? Is there an elevated lipogenic rate of the tumor cells as measured by H^3 acetate uptake *in vivo* and is this affected by treatment with salicylate? Furthermore, can the *in vitro* effects of salicylate when combined with low dose metformin be replicated *in vivo?*

5.2 Limitations

The major limitation of this study is that all of the experiments have been conducted in an *in vitro* setting. To make the outcomes more clinically translatable it would be important as described above to move to an *in vivo* model of cancer progression. Mechanistically it would be important again, to use human cancer cells without the β1 subunit of AMPK as right now the stable knockdown is using murine cells. In a similar vein although the knockout is specific to the β1 subunit of AMPK there is clearly a strong inhibition of the α subunit. This may suggest that the knock out could be to the general level of AMPK activity and not the β1 subunit. To fully address this concern it would be important to use a specific knockout using a serine to alanine on amino acid 108. This would be important because there would still be a functional AMPK enzyme and would address issues related to the dependency of salicylate effects on serine 108 expression. Also, currently this study only addresses issues related to lipogenesis, this marker while important is only part of the picture for cancer metabolism. To help strengthen this component it would be important to look at fatty acid oxidation using

palmitate C^{14}. Theoretically, if lipogenesis is decreasing fatty acid oxidation should be increasing.

This study is the first to investigate the effects of therapeutic concentrations of salicylate on AMPK, ACC, mTORC1 and lipogenesis in prostate and lung cancer cells. Within the therapeutic window of salicylate-treatment the effects on downstream effectors ACC, S6 kinase and lipogenesis are dependent on the expression of the β1 subunit of AMPK; a subunit of AMPK which is highly expressed in most cancer cell types[175-177]. The inhibitory effects of salicylate on prostate and lung cancer survival are strongly linked to the synthesis of fatty acids and cholesterol suggesting that inhibition of this pathway may be vital for its therapeutic effects. Finally, we underscore the pharmacologic potential of combining low dose metformin with salicylate to reduce the survival of prostate and lung cancers. Future clinical studies evaluating whether well-tolerated salicylate based drugs such as salsalate (which does not induce bleeding like aspirin) also inhibits prostate and lung cancer development in pre-clinical models are warranted. Careful retrospective analysis of interactions between aspirin and metformin in clinical trials investigating prostate and lung cancer development may be informative to guide population-based chemopreventative strategies.

6.0 Table 1: Total protein expression relative to β-Actin in 22RV-1 and H1299 cancer cells in a salicylate dose response (data calculated from Figures 2 and 3).

	22RV-1						H1299					
	Salicylate Concentration[mM]						Salicylate Concentration[mM]					
	Control	0.25	0.50	1.00	3.00	5.00	Control	0.25	0.5	1.00	3.00	5.
AMPK	1.0	1.23±0.09	1.18±0.13	1.12±0.13	2.06±0.36*	1.90±0.38*	1.0	0.94±0.04	1.02±0.04	1.04±0.05	1.22±0.04	1.85±
ACC	1.0	1.83±0.49	1.74±0.49	1.19±0.23	2.37±0.54**	1.96±0.48*	1.0	0.84±0.15	0.94±0.11	0.86±0.15	0.74±0.15	0.69
p70S6K	1.0	1.12±0.02	1.15±0.08	1.07±0.09	1.67±0.54	1.99±0.55*	1.0	0.72±0.13	0.83±0.13	0.89±0.10	0.78±0.17	0.84
S6	1.0	1.13±0.08	1.09±0.12	1.04±0.09	1.46±0.25	1.95±0.63*	1.0	0.97±0.10	1.03±0.07	0.90±0.06	0.80±0.06	0.66

* =P<0.05, ** =P<0.01, *** =P<0.001

7.0 Table 2: Measured IC_{50} for colony survival of adenocarcinomas treated with salicylate, metformin or combination (data calculated from Figure 6).

Cell Line	IC_{50} Values [mM]			Combined Interaction
	Salicylate	Metformin	Combination	
PC3	0.67 ± 0.07	1.15 ±0.07	0.30 ± 0.04	*Synergistic*
22RV-1	0.30 ± 0.07	0.67 ± 0.07	0.15 ± 0.05	*Additive*
H1299	0.40 ± 0.07	0.98 ± 0.10	0.28 ± 0.07	*Additive*
A549	0.97 ± 0.14	0.42 ± 0.13	0.29 ± 0.10	*Additive*

8.0 Figures:

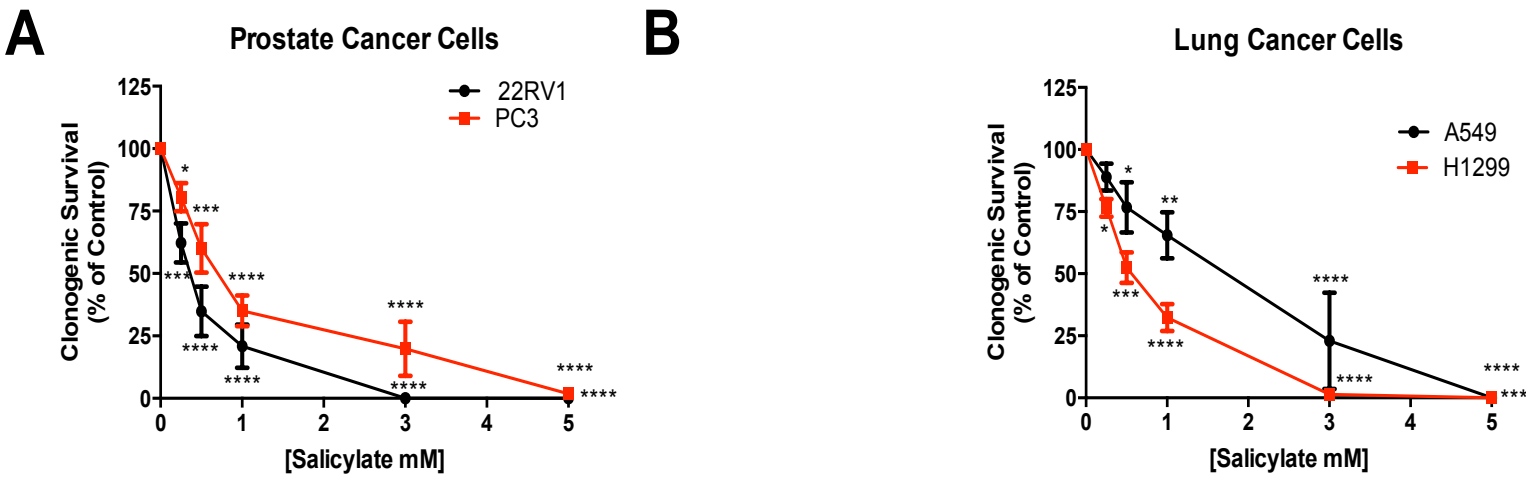

Figure 1: Salicylate reduces colony survival in prostate and lung and cancer cells. (A) Approximately 5x10^2 prostate (22RV-1 and PC3) or **(B)** lung (A549 and H1299) cancer cells were seeded into 12-well plates. The following day, these cells were treated with the indicated (mM) concentrations of salicylate and allowed to grow for 7 days. These cells were then subsequently fixed and stained with crystal violet and viable colonies >50 cells were counted under a microscope. The results are expressed as the mean and SE relative to untreated controls from at least 3 independent experiments. Mean±SE values of 3 independent experiments are shown. * =P<0.05, ** =P<0.01, *** =P<0.001, **** =P<0.0001 as calculated by one-way ANOVA.

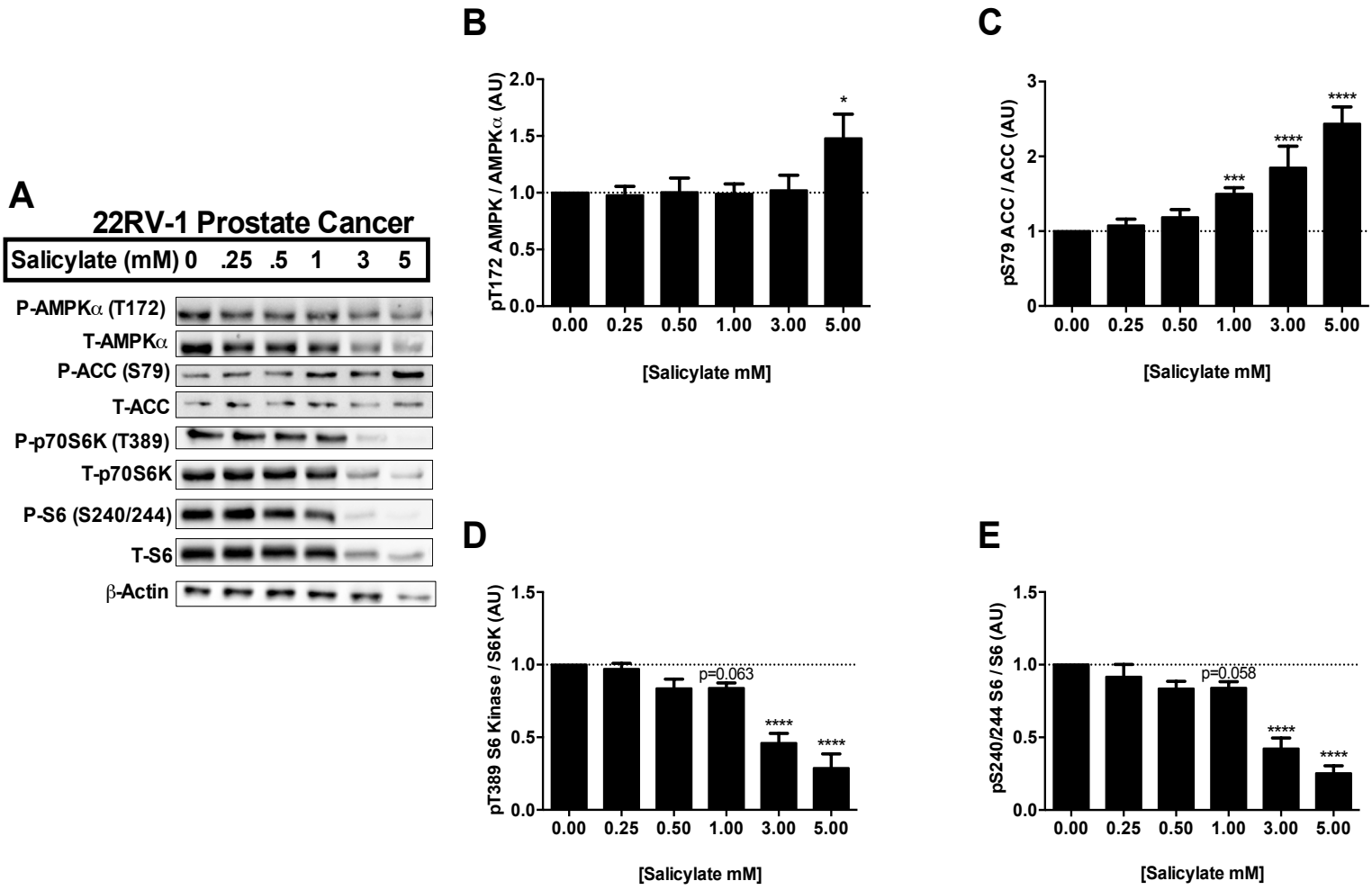

Figure 2: Salicylate activates AMPK and inhibits mTOR in 22RV-1 prostate cancer cells. Representative immunoblot using nitrocellulose membrane **(A)** and densitometry for indicated phosphorylation relative to total AMPK **(B)**, ACC **(C)** p70S6 kinase **(D)** and S6 **(E)**. Immunoblots were quantified using Image J software. Densitometry values are expressed as percent of control. Mean±SE values of 5-16 independent experiments are shown. * =P<0.05, *** =P<0.001, **** =P<0.0001 relative to control (0 mM salicylate) calculated using one-way ANOVA. Phosphorylated and total proteins were measured on different gels.

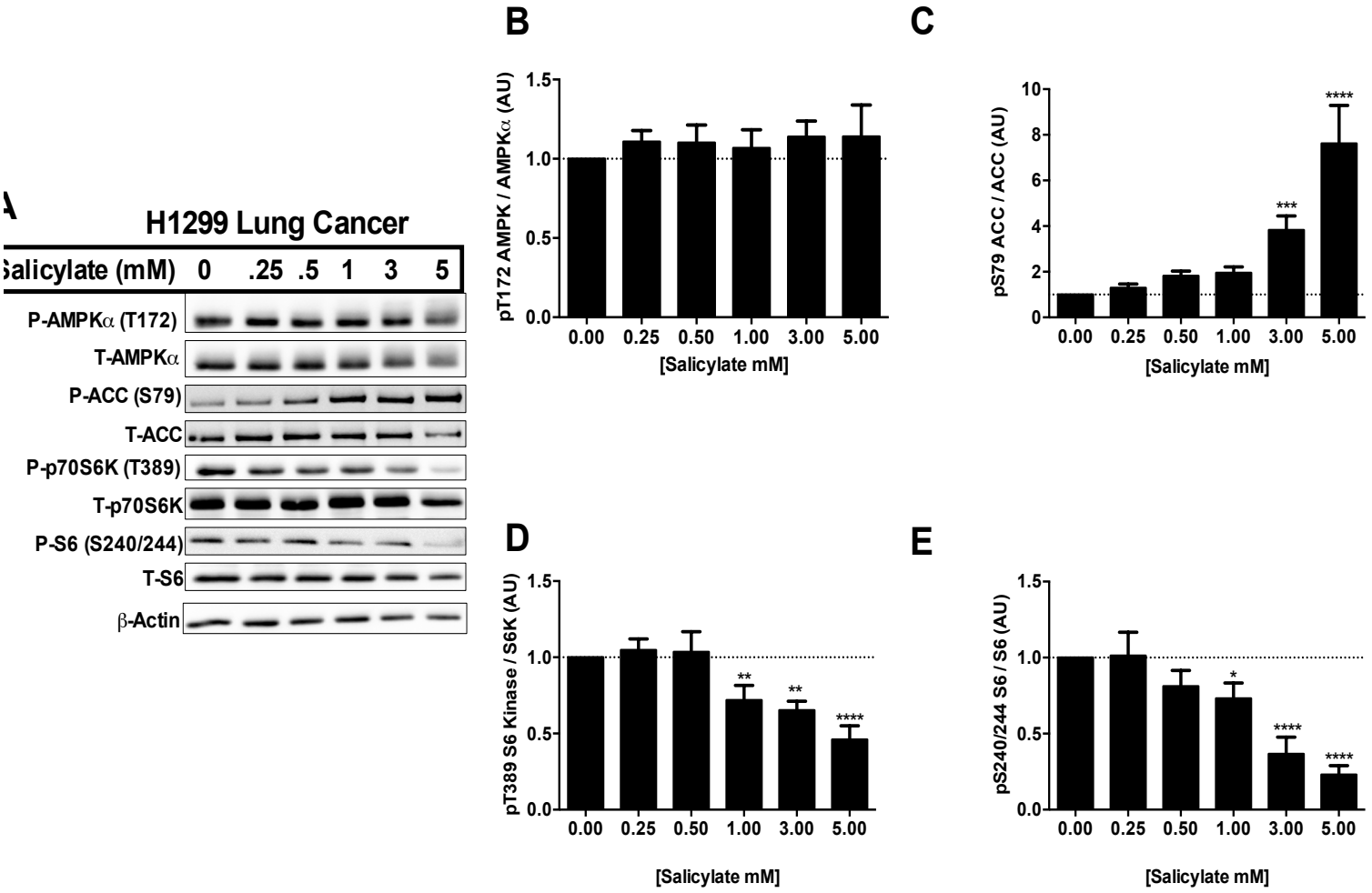

Figure 3: Salicylate activates AMPK and inhibits mTOR in H1299 lung cancer cells.

Representative immunoblot using nitrocellulose membrane **(A)** and densitometry for indicated phosphorylation and total AMPK **(B)**, ACC **(C)** p70S6 kinase **(D)** and S6 **(E)**. Immunoblots were quantified using Image J software. Densitometry values are expressed as percent of control. Mean±SE values of 5-12 independent experiments are shown. * =P<0.05, ** =P<0.01, ***=P<0.001, ****=P<0.0001 relative to control (0 mM salicylate) calculated using one-way ANOVA. Phosphorylated and total proteins were measured on different gels.

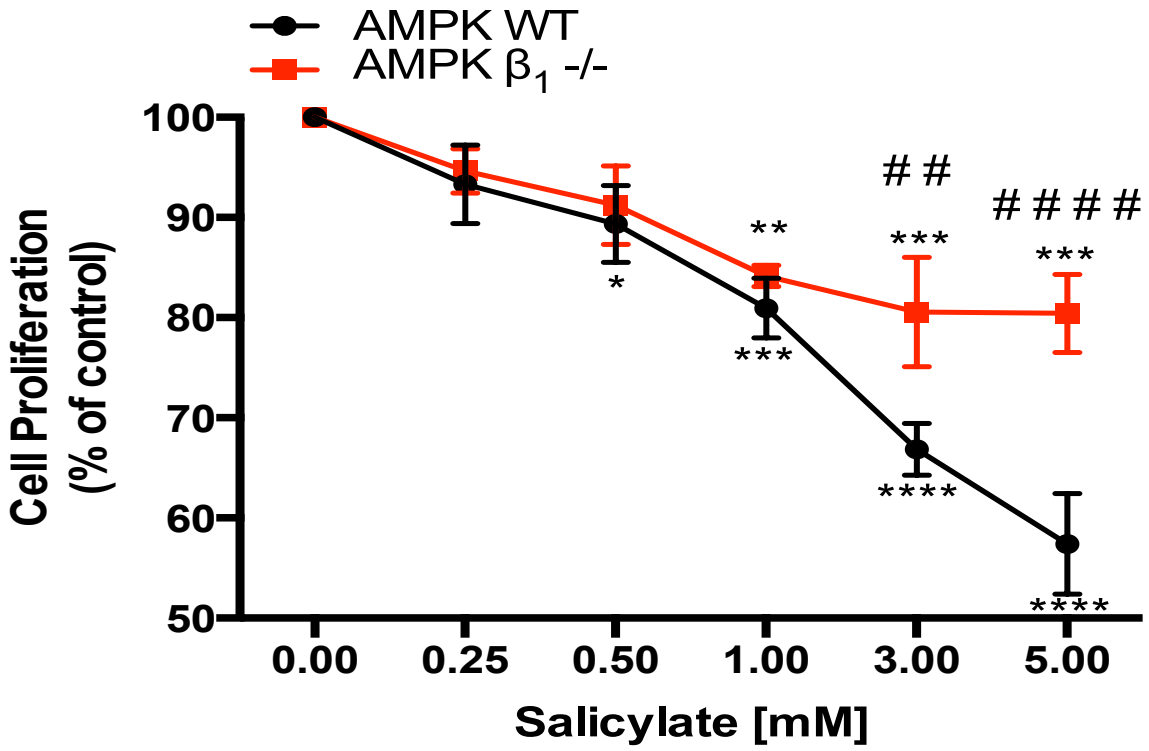

Figure 4: AMPK β1 enhances the anti proliferative action of salicylate. Approximately $1x10^3$ (MEF WT) or $5x10^2$ (MEF β1KO) cells were seeded into 96-well plate. The following day the cells were treated with the labelled amount of salicylate and left for 72 hours. After which the cells were fixed using 10% formalin and stained using crystal violet in 25% methanol. DNA content was quantified using an MBI plate reader at 570nm. The results expressed as a mean and S.E. relative to untreated control (0 mM salicylate) from at least 3 independent experiments. Mean±S.E. of 3 independent experiments are shown. * =P<0.05, ** =P<0.01, *** =P<0.001, **** =P<0.0001 relative to control within genotype; # # =P<0.01, # # # # =P<0.0001 between genotype as calculated by two-way ANOVA.

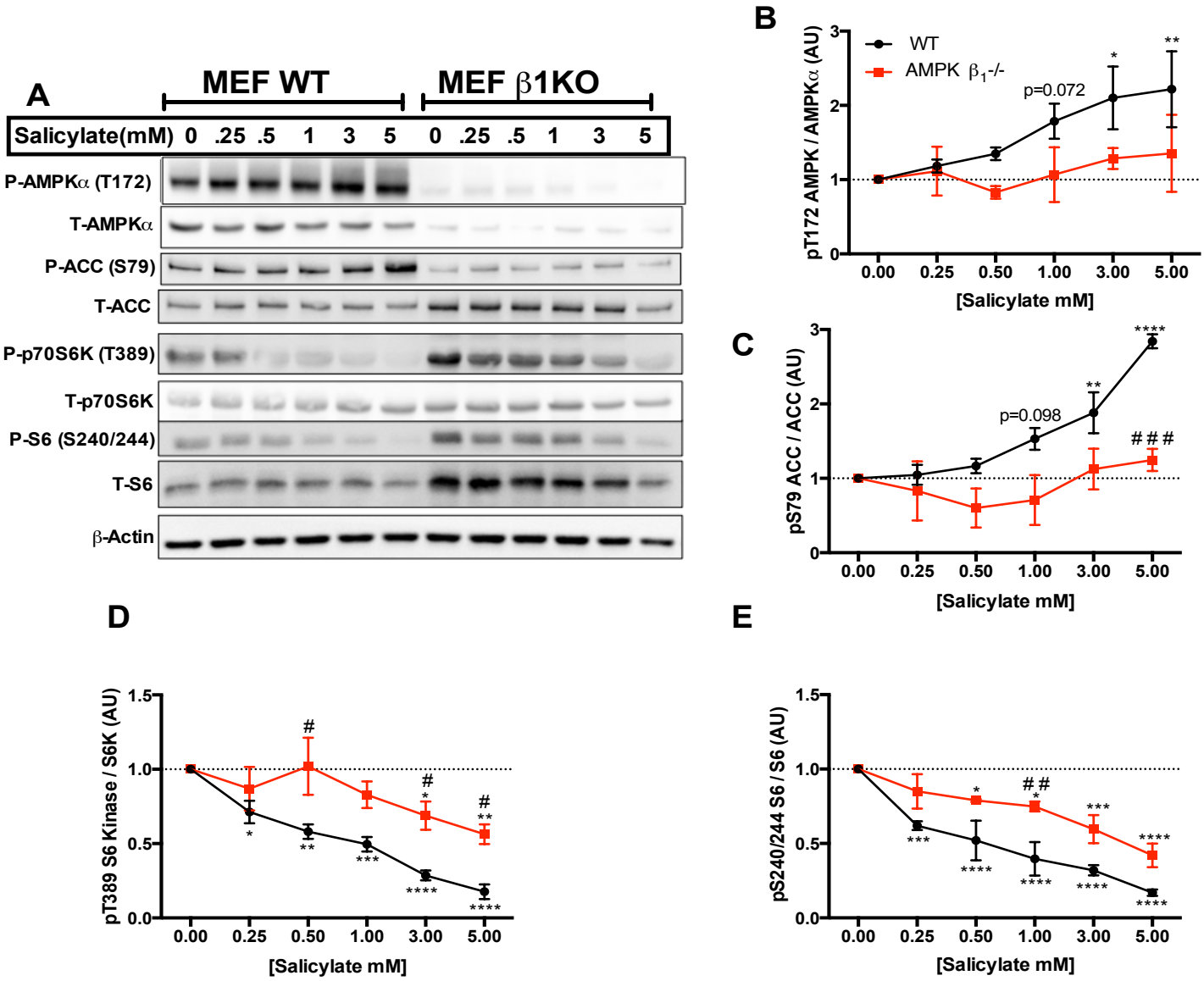

Figure 5: Salicylate activation of AMPK and inhibition of mTOR in MEFS requires AMPK β1 expression. Representative immunoblot using PVDF membrane **(A)** and densitometry for indicated phosphorylation and total AMPK **(B)**, ACC **(C)** p70S6 kinase **(D)** and S6 **(E)**. Immunoblots were quantified using Image J software. Densitometry values are expressed as percent of control. Mean±SE values of 4-6 independent experiments are shown. Indicated p-values are relative control (0 mM salicylate) within the same genotype; *=P<0.05, ** =P<0.01, *** =P<0.001, **** =P<0.0001 relative control (0 mM salicylate) within genotype; # =P<0.05, #

=P<0.01, # # # =P<0.001 relative to same concentration of salicylate in WT controls using two-way ANOVA Phosphorylated and total proteins were measured on different gels.

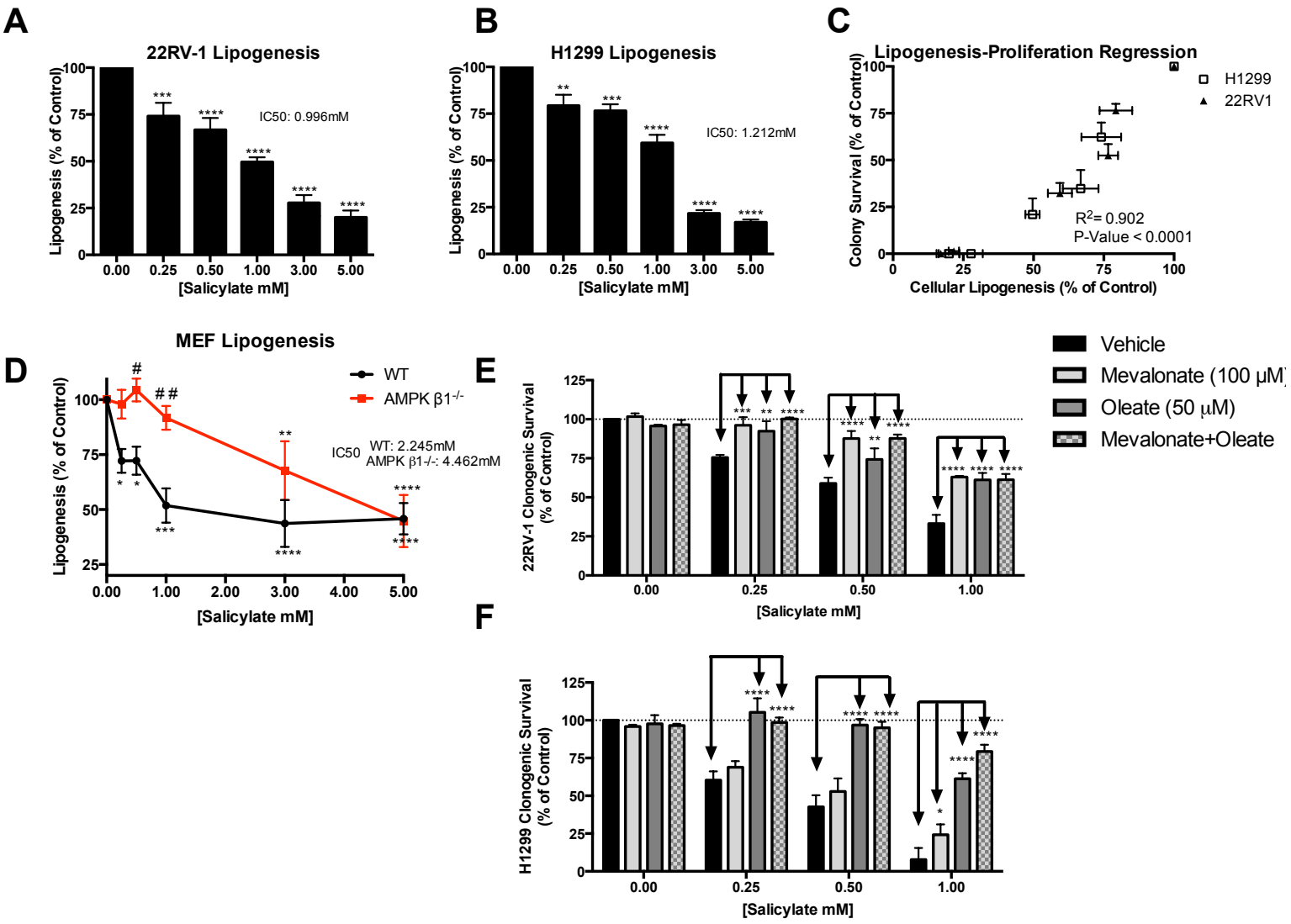

Figure 6: Salicylate induced inhibition of cell survival in prostate and lung cancer involves the inhibition of *de novo* fatty acid and cholesterol synthesis. (A) 22RV-1 or **(B)** H1299 cells were treated with the indicated concentrations of salicylate for 48h. ^{3}H-acetate incorporation into cellular lipids was then evaluated. The results are expressed as nmol of acetate per mg of protein per hour. ** = P<0.01 *** = P<0.001 **** = P<0.0001 relative to control as determined by one-way ANOVA analysis. **(C)** A linear regression analysis was conducted in 22RV-1 and H1299 cells comparing suppression of lipogenesis with clonogenic survival. **(D)** MEF WT or AMPK

β1-/-) were treated with the indicated concentrations of salicylate for 48h. ^{3}H-acetate incorporation into lipids was measured. The results are expressed as nmol of acetate per mg of protein per hour normalized to the control, * = P<0.05 ** = P<0.01 *** = P<0.001 ****=P<0.0001 within genotype; # = P<0.05, # # =P<0.001 within treatment group as calculated by two-way ANOVA. 22RV-1 **(E)** or H1299 **(F)** cancer cells were seeded into 12-well plates. The following day, these cells were treated with the indicated (mM) concentrations of salicylate along with mevalonate/oleate or both and allowed to grow for 7 days. These cells were then subsequently fixed and stained with crystal violet and viable colonies >50 cells were counted under a microscope. The results are expressed as the mean and SE relative to treated vehicle from at least 3 independent experiments. * =P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001 relative to salicylate treatment group as calculated by one-way ANOVA.

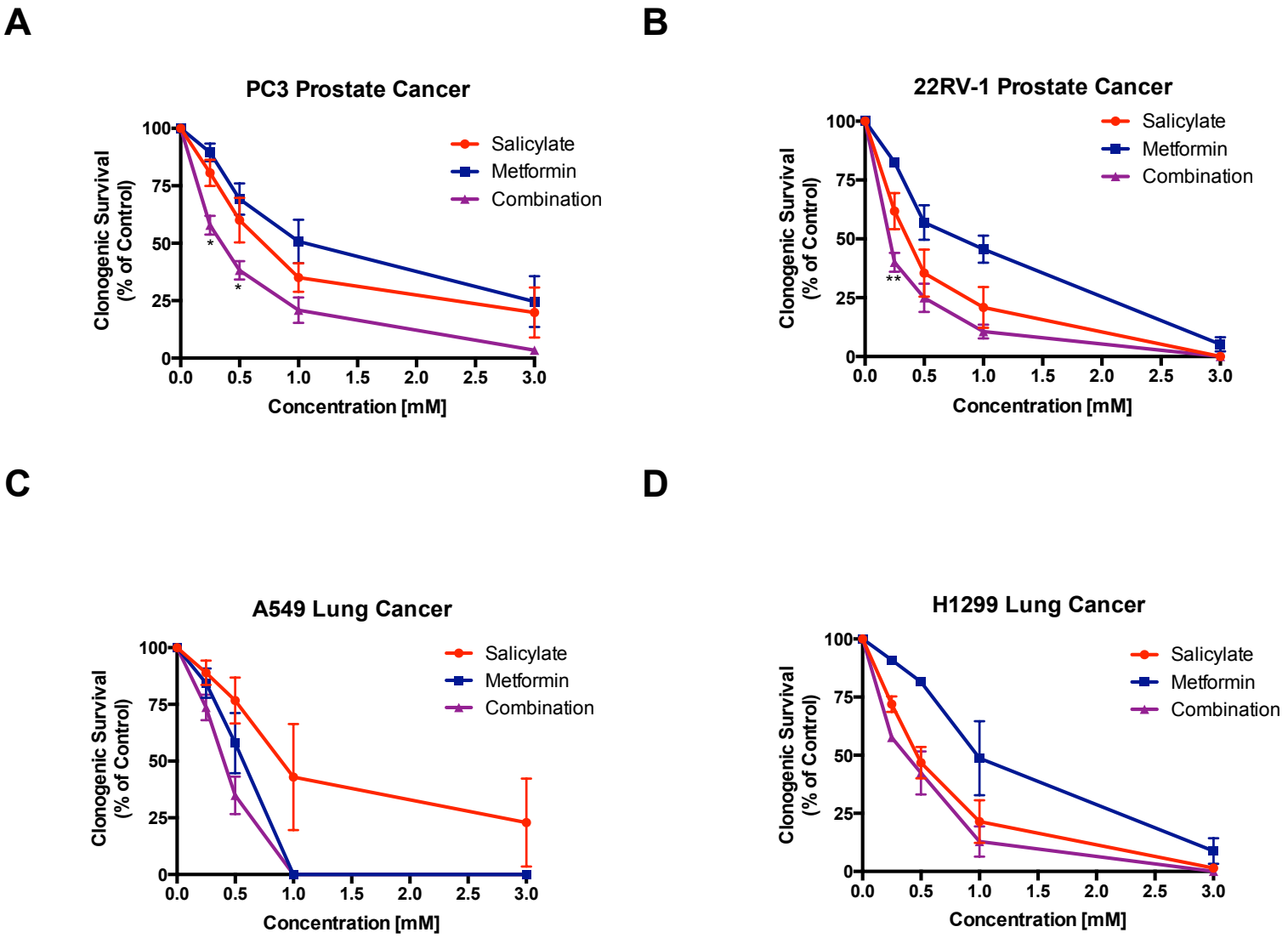

Figure 7: Salicylate and metformin synergistically or additively reduce clonogenic survival of prostate and lung cancer cells. PC3 **(A)**, 22RV-1 **(B)**, A549 **(C)** and H1299 **(D)** cancer cells were seeded into 12-well plates. The following day, these cells were treated with the indicated equivalent concentrations of salicylate and/or metformin and allowed to grow for 7 days. These cells were then subsequently fixed and stained with crystal violet and viable colonies >50 cells were counted under a microscope. The results are expressed as the mean and SE relative to untreated controls from at least 3 independent experiments. * = $P<0.05$, ** = $P<0.01$ salicylate vs. combination calculated using two-way ANOVA.

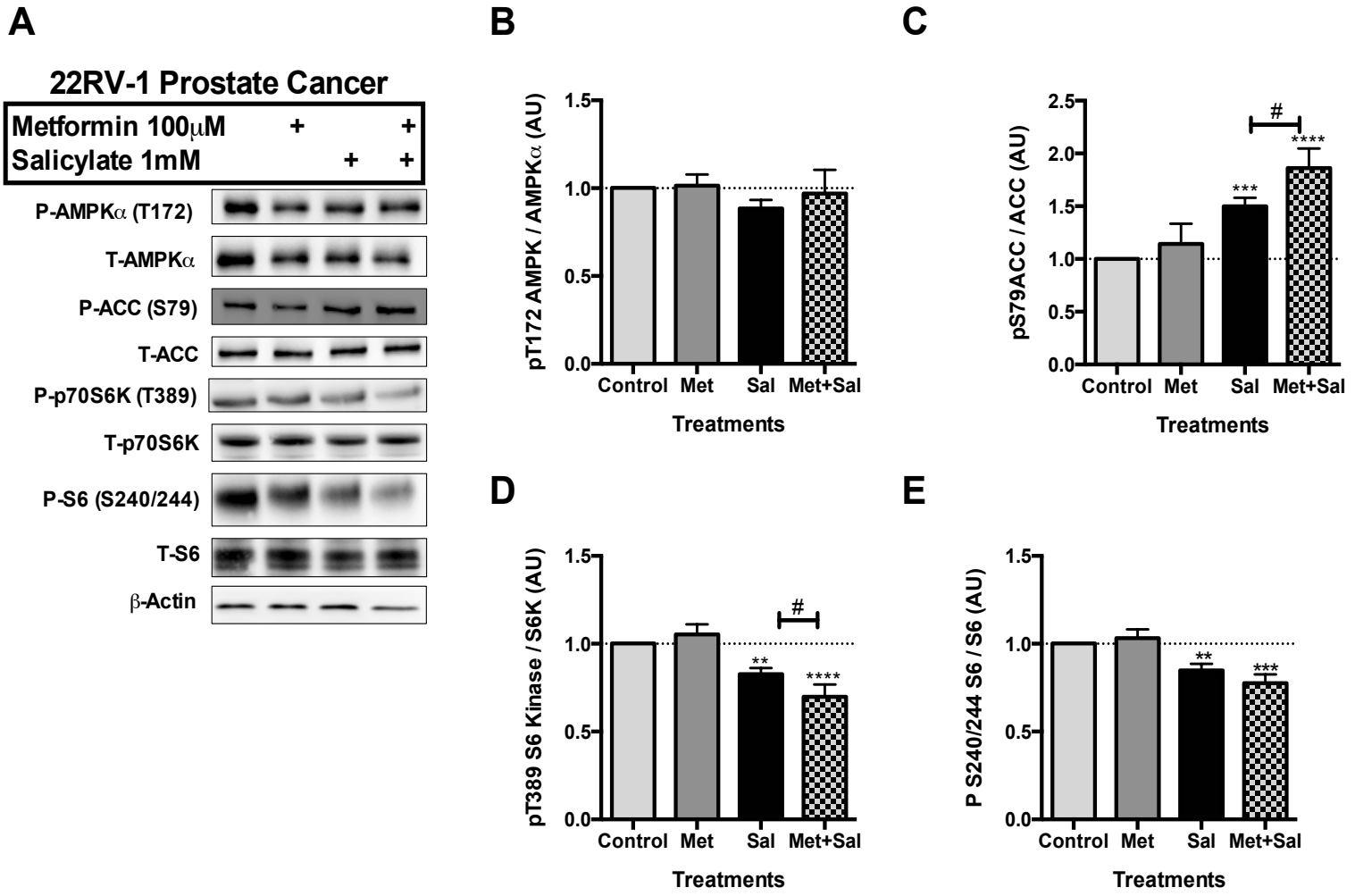

Figure 8: Combined metformin and salicylate treatment potentiates AMPK activity and mTOR inhibition in 22RV-1 prostate cancer cells. Representative immunoblot using nitrocellulose membrane **(A)** and densitometry for indicated phosphorylation and total AMPK **(B)**, ACC **(C)** p70S6 kinase **(D)** and S6 **(E)**. Immunoblots were quantified using Image J software. Densitometry values are expressed as percent of control. Mean±SE values of at least 10-16 independent experiments are shown. ** = P<0.01 *** =P<0.001, ****=P<0.0001 relative to control using one-way ANOVA; # P<0.05 between treatment group calculated using one-way ANOVA. Phosphorylated and total proteins were measured on different gels.

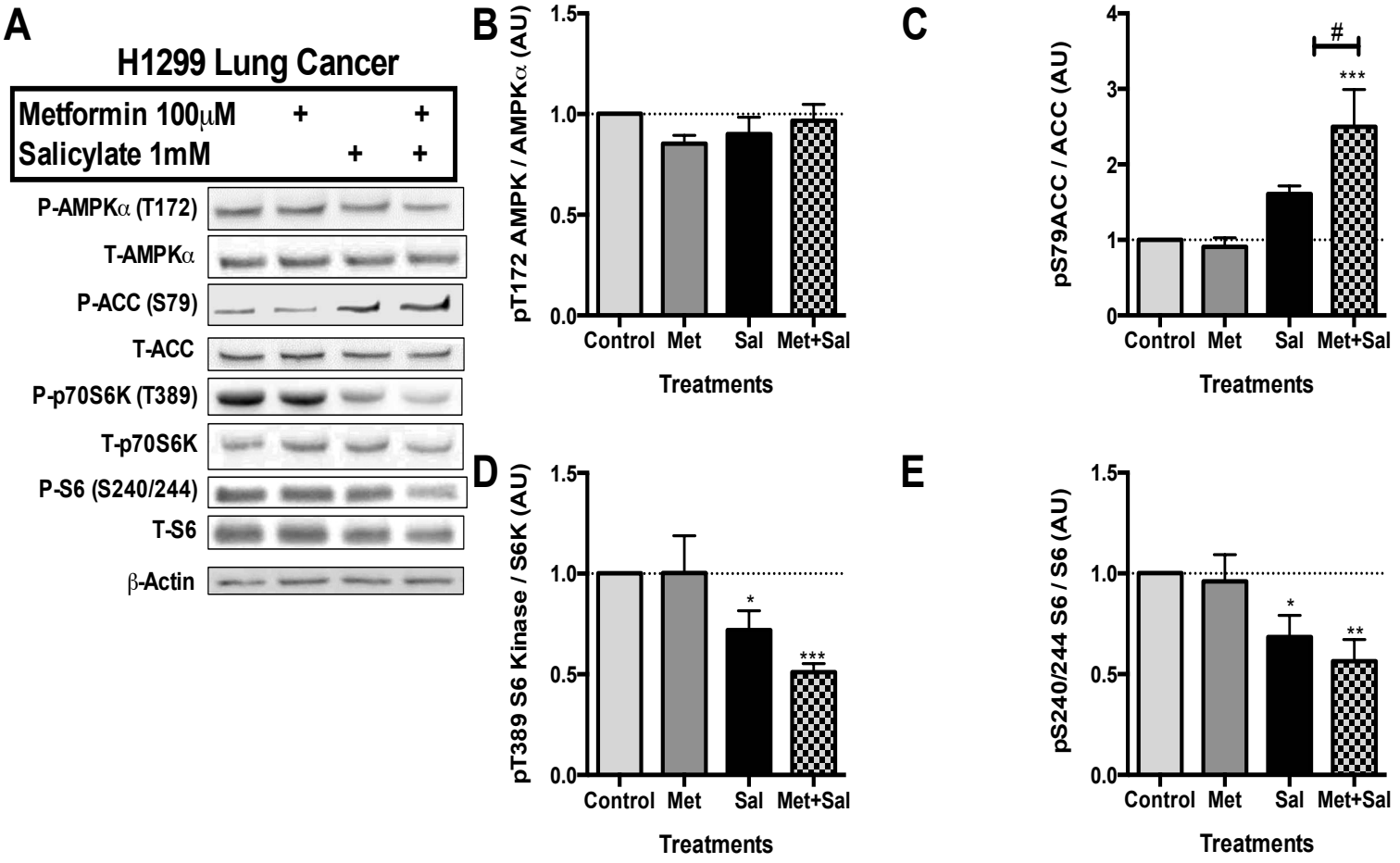

Figure 9: Combined metformin and salicylate treatment potentiates AMPK activity and mTOR inhibition in H1299 lung cancer cells. Representative immunoblot using PVDF membrane **(A)** and densitometry for indicated phosphorylation and total AMPK **(B)**, ACC **(C)** p70S6 kinase **(D)** and S6 **(E)**. Immunoblots were quantified using Image J software. Densitometry values are expressed as percent of control. Mean±SE values of at least 7-12 independent experiments are shown. * =P<0.05, **=P<0.01, *** =P<0.001 relative to control using one-way ANOVA; # =P<0.05 between treatment group calculated using one-way ANOVA. Phosphorylated and total proteins were measured on different gels.

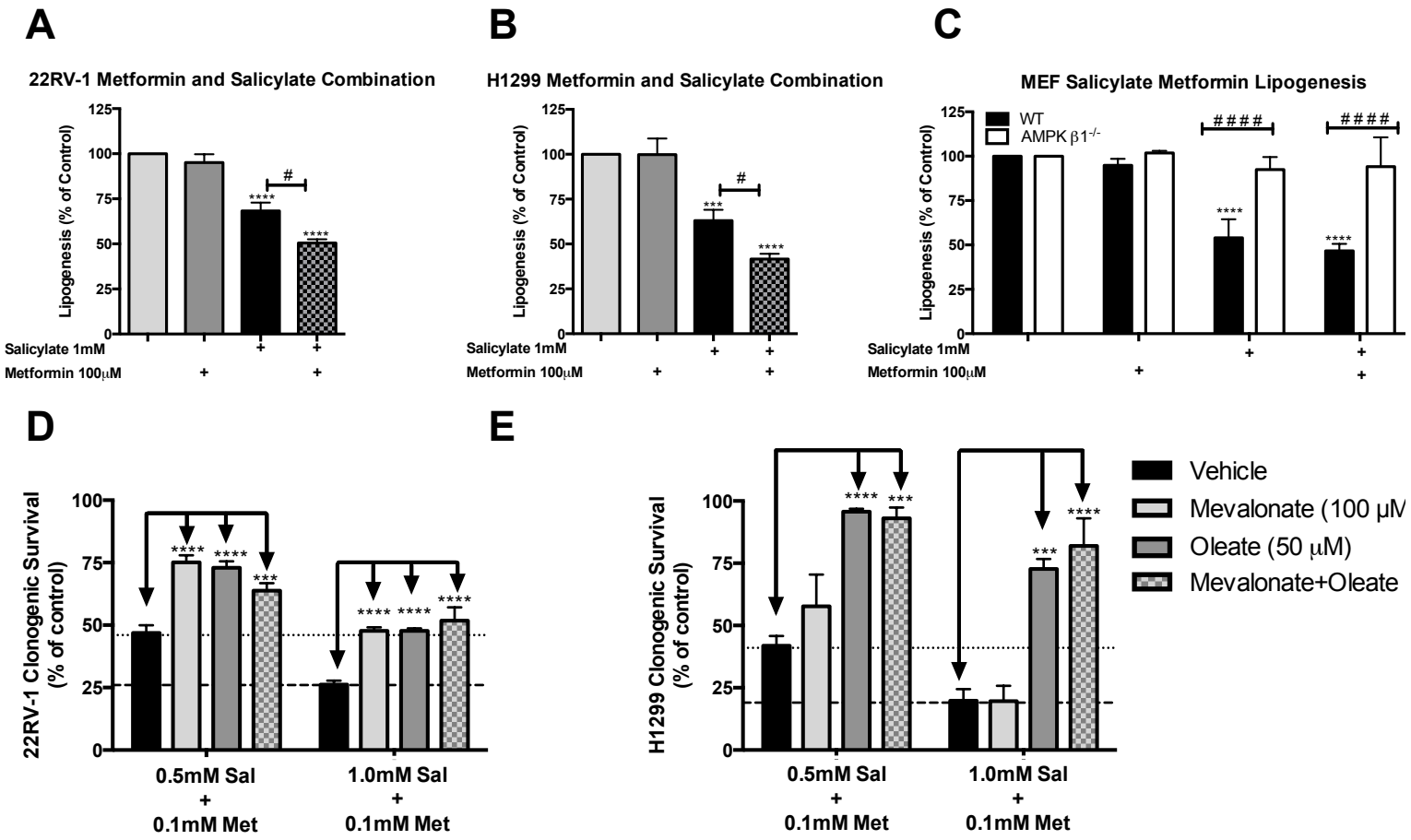

Figure 10: Metformin augments salicylate-induced inhibition of lipogenesis. 22RV-1 prostate **(A)** or H1299 lung **(B)** cancer cells were treated with vehicle, salicylate (1mM), metformin (0.1mM) or both salicylate and metformin for 48h. ^{3}H-acetate incorporation into lipids was then evaluated. The results are expressed as nmol of acetate per mg of protein per hour. *** = P<0.001 **** = P<0.0001 relative to control using one-way ANOVA; # =P<0.05 within treatment group as determined by two-way ANOVA analysis. **(C)** MEFS (WT or AMPK β1-/-) were treated with the vehicle, salicylate (1mM), metformin (0.1mM) or both salicylate and metformin for 48h. ^{3}H-acetate incorporation into lipids was measured. The results are expressed

as nmol of acetate per mg of protein per hour normalized to the control, **** = P<0.0001 within genotype; # # # # = P<0.0001 within treatment group as calculated by two-way ANOVA. Approximately $5x10^2$ 22RV-1 **(D)** or H1299 **(E)** cancer cells were seeded into 12-well plates. The following day, these cells were treated with the indicated (mM) concentrations of salicylate, 0.1 mM metformin or both along with mevalonate, oleate or mevalonate and oleate, the cells were allowed to grow for 7 days. These cells were then subsequently fixed and stained with crystal violet and viable colonies >50 cells were counted under a microscope. The results are expressed as the mean and SE relative to untreated controls from at least 3 independent experiments. *** = P<0.001, **** = P<0.0001 all values were calculated using one-way ANOVA.

9.0 References:

1. Siegel, R., Ward, E., Brawley, O. & Jemal, A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA: a cancer journal for clinicians* **61**, 212-236 (2011).
2. Stavridis, S., Saidi, S., Lekovski, L., Dohcev, S. & Spasovski, G. Screening for prostate cancer: a controversy or fact. *Hippokratia* **14**, 170-175 (2010).
3. Jemal, A.*, et al.* Global cancer statistics. *CA: a cancer journal for clinicians* **61**, 69-90 (2011).
4. Center, M.M.*, et al.* International variation in prostate cancer incidence and mortality rates. *European urology* **61**, 1079-1092 (2012).
5. Ezzati, M. & Lopez, A.D. Estimates of global mortality attributable to smoking in 2000. *Lancet* **362**, 847-852 (2003).
6. Ezzati, M., Henley, S.J., Lopez, A.D. & Thun, M.J. Role of smoking in global and regional cancer epidemiology: current patterns and data needs. *International journal of cancer. Journal international du cancer* **116**, 963-971 (2005).
7. Shi, Y. & Hu, F.B. The global implications of diabetes and cancer. *The Lancet* **383**, 1947-1948 (2014).
8. Yang, W.*, et al.* Projected supply of and demand for oncologists and radiation oncologists through 2025: an aging, better-insured population will result in shortage. *Journal of Oncology Practice* **10**, 39-45 (2014).
9. Haslam, D.W. & James, W.P. Obesity. *Lancet* **366**, 1197-1209 (2005).

10. Mokdad, A.H., *et al.* Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *JAMA* **289**, 76-79 (2003).

11. Kabat, G.C., Miller, A.B. & Rohan, T.E. Body mass index and lung cancer risk in women. *Epidemiology* **18**, 607-612 (2007).

12. Khandekar, M.J., Cohen, P. & Spiegelman, B.M. Molecular mechanisms of cancer development in obesity. *Nature Reviews Cancer* **11**, 886-895 (2011).

13. Samuel, V.T., Petersen, K.F. & Shulman, G.I. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet* **375**, 2267-2277 (2010).

14. Michels, K.B., *et al.* Type 2 diabetes and subsequent incidence of breast cancer in the Nurses' Health Study. *Diabetes care* **26**, 1752-1758 (2003).

15. Ma, J., *et al.* A prospective study of plasma C-peptide and colorectal cancer risk in men. *Journal of the National Cancer Institute* **96**, 546-553 (2004).

16. Ma, J., *et al.* Prediagnostic body-mass index, plasma C-peptide concentration, and prostate cancer-specific mortality in men with prostate cancer: a long-term survival analysis. *The Lancet. Oncology* **9**, 1039-1047 (2008).

17. Pollak, M. Insulin and insulin-like growth factor signalling in neoplasia. *Nature reviews. Cancer* **8**, 915-928 (2008).

18. Chan, J.M., *et al.* Plasma insulin-like growth factor-I and prostate cancer risk: a prospective study. *Science* **279**, 563-566 (1998).

19. Moorehead, R.A., Sanchez, O.H., Baldwin, R.M. & Khokha, R. Transgenic overexpression of IGF-II induces spontaneous lung tumors: a model for human lung adenocarcinoma. *Oncogene* **22**, 853-857 (2003).

20. Hay, N. The Akt-mTOR tango and its relevance to cancer. *Cancer cell* **8**, 179-183 (2005).

21. Engelman, J.A. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nature reviews. Cancer* **9**, 550-562 (2009).

22. Calle, E.E. & Kaaks, R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nature Reviews Cancer* **4**, 579-591 (2004).

23. Menendez, J.A. & Lupu, R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. *Nature reviews. Cancer* **7**, 763-777 (2007).

24. Nguyen, P.L.*, et al.* Fatty acid synthase polymorphisms, tumor expression, body mass index, prostate cancer risk, and survival. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 3958-3964 (2010).

25. Rossi, S.*, et al.* Fatty acid synthase expression defines distinct molecular signatures in prostate cancer. *Molecular cancer research : MCR* **1**, 707-715 (2003).

26. Visca, P.*, et al.* Fatty acid synthase (FAS) is a marker of increased risk of recurrence in lung carcinoma. *Anticancer research* **24**, 4169-4173 (2004).

27. Kuhajda, F.P.*, et al.* Fatty acid synthesis: a potential selective target for antineoplastic therapy. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 6379-6383 (1994).

28. Bandyopadhyay, S.*, et al.* FAS expression inversely correlates with PTEN level in prostate cancer and a PI 3-kinase inhibitor synergizes with FAS siRNA to induce apoptosis. *Oncogene* **24**, 5389-5395 (2005).

29. Van de Sande, T., De Schrijver, E., Heyns, W., Verhoeven, G. & Swinnen, J.V. Role of the phosphatidylinositol 3'-kinase/PTEN/Akt kinase pathway in the overexpression of fatty acid synthase in LNCaP prostate cancer cells. *Cancer research* **62**, 642-646 (2002).

30. Ning, J. & Clemmons, D.R. AMP-activated protein kinase inhibits IGF-I signaling and protein synthesis in vascular smooth muscle cells via stimulation of insulin receptor substrate 1 S794 and tuberous sclerosis 2 S1345 phosphorylation. *Molecular Endocrinology* **24**, 1218-1229 (2010).

31. Salt, I.P., Johnson, G., Ashcroft, S.J. & Hardie, D.G. AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. *The Biochemical journal* **335 (Pt 3)**, 533-539 (1998).

32. Hardie, D.G. AMPK: a key regulator of energy balance in the single cell and the whole organism. *International journal of obesity* **32 Suppl 4**, S7-12 (2008).

33. Gauthier, M.S.*, et al.* Decreased AMP-activated protein kinase activity is associated with increased inflammation in visceral adipose tissue and with whole-body insulin resistance in morbidly obese humans. *Biochem Biophys Res Commun* **404**, 382-387 (2011).

34. Hardie, D.G., Ross, F.A. & Hawley, S.A. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nature reviews. Molecular cell biology* **13**, 251-262 (2012).

35. Yeh, L.A., Lee, K.H. & Kim, K.H. Regulation of rat liver acetyl-CoA carboxylase. Regulation of phosphorylation and inactivation of acetyl-CoA carboxylase by the adenylate energy charge. *The Journal of biological chemistry* **255**, 2308-2314 (1980).

36. Kahn, B.B., Alquier, T., Carling, D. & Hardie, D.G. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell metabolism* **1**, 15-25 (2005).

37. Steinberg, G.R. & Kemp, B.E. AMPK in Health and Disease. *Physiological reviews* **89**, 1025-1078 (2009).

38. Hawley, S.A.*, et al.* Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *Journal of biology* **2**, 28 (2003).

39. Shaw, R.J.*, et al.* The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3329-3335 (2004).

40. Hemminki, A. The molecular basis and clinical aspects of Peutz-Jeghers syndrome. *Cellular and molecular life sciences : CMLS* **55**, 735-750 (1999).

41. Hemminki, A.*, et al.* A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature* **391**, 184-187 (1998).

42. Stahmann, N., Woods, A., Carling, D. & Heller, R. Thrombin activates AMP-activated protein kinase in endothelial cells via a pathway involving Ca2+/calmodulin-dependent protein kinase kinase beta. *Molecular and cellular biology* **26**, 5933-5945 (2006).

43. Tamas, P.*, et al.* Regulation of the energy sensor AMP-activated protein kinase by antigen receptor and Ca2+ in T lymphocytes. *The Journal of experimental medicine* **203**, 1665-1670 (2006).

44. Inokuchi-Shimizu, S.*, et al.* TAK1-mediated autophagy and fatty acid oxidation prevent hepatosteatosis and tumorigenesis. *The Journal of clinical investigation* **124**, 3566 (2014).

45. Horman, S.*, et al.* Insulin antagonizes ischemia-induced Thr172 phosphorylation of AMP-activated protein kinase alpha-subunits in heart via hierarchical phosphorylation of Ser485/491. *The Journal of biological chemistry* **281**, 5335-5340 (2006).

46. Hawley, S.A.*, et al.* Phosphorylation by Akt within the ST loop of AMPK-alpha1 down-regulates its activation in tumour cells. *The Biochemical journal* **459**, 275-287 (2014).

47. Hawley, S.A.*, et al.* The ancient drug salicylate directly activates AMP-activated protein kinase. *Science* **336**, 918-922 (2012).

48. Corton, J.M., Gillespie, J.G., Hawley, S.A. & Hardie, D.G. 5-Aminoimidazole-4-Carboxamide Ribonucleoside. *European Journal of Biochemistry* **229**, 558-565 (1995).

49. Gowans, G.J., Hawley, S.A., Ross, F.A. & Hardie, D.G. AMP is a true physiological regulator of AMP-activated protein kinase by both allosteric activation and enhancing net phosphorylation. *Cell metabolism* **18**, 556-566 (2013).

50. Xiao, B.*, et al.* Structure of mammalian AMPK and its regulation by ADP. *Nature* **472**, 230-233 (2011).

51. Huang, X.*, et al.* Important role of the LKB1-AMPK pathway in suppressing tumorigenesis in PTEN-deficient mice. *Biochem. J* **412**, 211-221 (2008).

52. Zadra, G.*, et al.* A novel direct activator of AMPK inhibits prostate cancer growth by blocking lipogenesis. *EMBO molecular medicine* (2014).

53. Xiang, X., Saha, A.K., Wen, R., Ruderman, N.B. & Luo, Z. AMP-activated protein kinase activators can inhibit the growth of prostate cancer cells by multiple mechanisms. *Biochemical and biophysical research communications* **321**, 161-167 (2004).

54. Zhou, J.*, et al.* Inactivation of AMPK alters gene expression and promotes growth of prostate cancer cells. *Oncogene* **28**, 1993-2002 (2009).

55. Zadra, G., Priolo, C., Patnaik, A. & Loda, M. New strategies in prostate cancer: targeting lipogenic pathways and the energy sensor AMPK. *Clinical Cancer Research* **16**, 3322-3328 (2010).

56. Storozhuk, Y.*, et al.* Metformin inhibits growth and enhances radiation response of non-small cell lung cancer (NSCLC) through ATM and AMPK. *British journal of cancer* **108**, 2021-2032 (2013).

57. Sanli, T.*, et al.* Ionizing radiation activates AMP-activated kinase (AMPK): a target for radiosensitization of human cancer cells. *International Journal of Radiation Oncology* Biology* Physics* **78**, 221-229 (2010).

58. Shackelford, D.B.*, et al.* LKB1 inactivation dictates therapeutic response of non-small cell lung cancer to the metabolism drug phenformin. *Cancer cell* **23**, 143-158 (2013).

59. Laplante, M. & Sabatini, D.M. mTOR signaling in growth control and disease. *Cell* **149**, 274-293 (2012).

60. Gwinn, D.M.*, et al.* AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Molecular cell* **30**, 214-226 (2008).

61. Nojima, H.*, et al.* The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. *The Journal of biological chemistry* **278**, 15461-15464 (2003).

62. Schalm, S.S., Fingar, D.C., Sabatini, D.M. & Blenis, J. TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. *Current biology : CB* **13**, 797-806 (2003).

63. Inoki, K., Zhu, T. & Guan, K.L. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115**, 577-590 (2003).

64. Manning, B.D. & Cantley, L.C. United at last: the tuberous sclerosis complex gene products connect the phosphoinositide 3-kinase/Akt pathway to mammalian target of rapamycin (mTOR) signalling. *Biochemical Society transactions* **31**, 573-578 (2003).

65. Li, Y., Corradetti, M.N., Inoki, K. & Guan, K.-L. TSC2: filling the GAP in the mTOR signaling pathway. *Trends in biochemical sciences* **29**, 32-38 (2004).

66. Law, B.K.*, et al.* Salicylate-induced growth arrest is associated with inhibition of p70s6k and down-regulation of c-myc, cyclin D1, cyclin A, and proliferating cell nuclear antigen. *The Journal of biological chemistry* **275**, 38261-38267 (2000).

67. Lissa, D.*, et al.* Resveratrol and aspirin eliminate tetraploid cells for anticancer chemoprevention. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 3020-3025 (2014).

68. Din, F.V.*, et al.* Aspirin inhibits mTOR signaling, activates AMP-activated protein kinase, and induces autophagy in colorectal cancer cells. *Gastroenterology* **142**, 1504-1515 e1503 (2012).

69. Vincent, E.E.*, et al.* Differential effects of AMPK agonists on cell growth and metabolism. *Oncogene* (2014).

70. Majumder, P.K.*, et al.* Prostate intraepithelial neoplasia induced by prostate restricted Akt activation: the MPAKT model. *Proceedings of the National Academy of Sciences* **100**, 7841-7846 (2003).

71. Carracedo, A.*, et al.* Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *The Journal of clinical investigation* **118**, 3065 (2008).

72. Seki, N.*, et al.* Prognostic significance of expression of eukaryotic initiation factor 4E and 4E binding protein 1 in patients with pathological stage I invasive lung adenocarcinoma. *Lung cancer* **70**, 329-334 (2010).

73. Yoshizawa, A.*, et al.* Overexpression of phospho-eIF4E is associated with survival through AKT pathway in non-small cell lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**, 240-248 (2010).

74. Gingras, A.C., Raught, B. & Sonenberg, N. Regulation of translation initiation by FRAP/mTOR. *Genes & development* **15**, 807-826 (2001).

75. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).

76. Fullerton, M.D.*, et al.* Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. *Nature medicine* **19**, 1649-1654 (2013).

77. Smith, M.J. The effects of salicylate on the metabolism of acetate in the rat. *The Journal of biological chemistry* **234**, 144-147 (1959).

78. Fullerton, M.D.*, et al.* Salicylate improves macrophage cholesterol homeostasis via activation of Ampk. *Journal of lipid research* (2015).

79. Hatzivassiliou, G.*, et al.* ATP citrate lyase inhibition can suppress tumor cell growth. *Cancer cell* **8**, 311-321 (2005).

80. Migita, T.*, et al.* ATP Citrate Lyase: Activation and Therapeutic Implications in Non–Small Cell Lung Cancer. *Cancer research* **68**, 8547-8554 (2008).

81. Mashima, T., Seimiya, H. & Tsuruo, T. De novo fatty-acid synthesis and related pathways as molecular targets for cancer therapy. *British journal of cancer* **100**, 1369-1372 (2009).

82. Rothwell, P.M.*, et al.* Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *The Lancet* **377**, 31-41 (2011).

83. Liu, Y.*, et al.* Effect of aspirin and other non-steroidal anti-inflammatory drugs on prostate cancer incidence and mortality: a systematic review and meta-analysis. *BMC medicine* **12**, 55 (2014).

84. Shebl, F.M.*, et al.* Aspirin but not ibuprofen use is associated with reduced risk of prostate cancer: a PLCO study. *British journal of cancer* **107**, 207-214 (2012).

85. Thun, M.J., Namboodiri, M.M., Calle, E.E., Flanders, W.D. & Heath, C.W. Aspirin use and risk of fatal cancer. *Cancer research* **53**, 1322-1327 (1993).

86. Din, F.V.*, et al.* Effect of aspirin and NSAIDs on risk and survival from colorectal cancer. *Gut* **59**, 1670-1679 (2010).

87. Higgs, G.A., Salmon, J.A., Henderson, B. & Vane, J.R. Pharmacokinetics of aspirin and salicylate in relation to inhibition of arachidonate cyclooxygenase and

antiinflammatory activity. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 1417-1420 (1987).

88. Vlot, A.C., Dempsey, D.M.A. & Klessig, D.F. Salicylic acid, a multifaceted hormone to combat disease. *Annual review of phytopathology* **47**, 177-206 (2009).

89. Vlot, A.C., Dempsey, D.A. & Klessig, D.F. Salicylic Acid, a multifaceted hormone to combat disease. *Annual review of phytopathology* **47**, 177-206 (2009).

90. Vane, J.R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature: New biology* **231**, 232-235 (1971).

91. Preston, S.J., Arnold, M., Beller, E., Brooks, P. & Buchanan, W. Comparative analgesic and anti‐inflammatory properties of sodium salicylate and acetylsalicylic acid (aspirin) in rheumatoid arthritis. *British journal of clinical pharmacology* **27**, 607-611 (1989).

92. Yin, M.-J., Yamamoto, Y. & Gaynor, R.B. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature* **396**, 77-80 (1998).

93. Frantz, B., O'Neill, E.A., Ghosh, S. & Kopp, E. The effect of sodium salicylate and aspirin on NF-kappaB. (American Association for the Advancement of Science, 1995).

94. Veitonmäki, T., Tammela, T.L., Auvinen, A. & Murtola, T.J. Use of aspirin, but not other non-steroidal anti-inflammatory drugs is associated with decreased prostate cancer risk at the population level. *European journal of cancer* **49**, 938-945 (2013).

95. Vidal, A.C.*, et al.* Aspirin, NSAID and risk of prostate cancer: Results from the REDUCE study. *Clinical cancer research : an official journal of the American Association for Cancer Research* (2014).

96. Jacobs, E.J.*, et al.* Daily aspirin use and prostate cancer-specific mortality in a large cohort of men with nonmetastatic prostate cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **32**, 3716-3722 (2014).

97. Dhillon, P.K., Kenfield, S.A., Stampfer, M.J. & Giovannucci, E.L. Long‐term aspirin use and the risk of total, high‐grade, regionally advanced and lethal prostate cancer in a prospective cohort of health professionals, 1988–2006. *International Journal of Cancer* **128**, 2444-2452 (2011).

98. Elwood, P.C., Gallagher, A.M., Duthie, G.G., Mur, L.A. & Morgan, G. Aspirin, salicylates, and cancer. *The Lancet* **373**, 1301-1309 (2009).

99. Lloyd, F.P., Slivova, V., Valachovicova, T. & Sliva, D. Aspirin inhibits highly invasive prostate cancer cells. *International journal of oncology* **23**, 1277-1283 (2003).

100. Kohnen, P.W. & Drach, G.W. Patterns of inflammation in prostatic hyperplasia: a histologic and bacteriologic study. *The Journal of urology* **121**, 755-760 (1979).

101. Suh, J.*, et al.* Mechanisms of constitutive NF‐κB activation in human prostate cancer cells. *The Prostate* **52**, 183-200 (2002).

102. Moysich, K.B.*, et al.* Regular aspirin use and lung cancer risk. *BMC cancer* **2**, 31 (2002).

103. Van Dyke, A.L.*, et al.* Regular adult aspirin use decreases the risk of non-small cell lung cancer among women. *Cancer Epidemiology Biomarkers & Prevention* **17**, 148-157 (2008).

104. Akhmedkhanov, A., Toniolo, P., Zeleniuch-Jacquotte, A., Koenig, K.L. & Shore, R.E. Aspirin and lung cancer in women. *British journal of cancer* **87**, 49-53 (2002).

105. Wolff, H.*, et al.* Expression of cyclooxygenase-2 in human lung carcinoma. *Cancer research* **58**, 4997-5001 (1998).

106. Hida, T.*, et al.* Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer research* **58**, 3761-3764 (1998).

107. Lim, W.Y.*, et al.* Aspirin and non-aspirin non-steroidal anti-inflammatory drug use and risk of lung cancer. *Lung cancer* **77**, 246-251 (2012).

108. Steinberg, G.R., Dandapani, M. & Hardie, D.G. AMPK: mediating the metabolic effects of salicylate-based drugs? *Trends in endocrinology and metabolism: TEM* (2013).

109. Pollak, M. Overcoming Drug Development Bottlenecks With Repurposing: Repurposing biguanides to target energy metabolism for cancer treatment. *Nature medicine* **20**, 591-593 (2014).

110. Blandino, G.*, et al.* Metformin elicits anticancer effects through the sequential modulation of DICER and c-MYC. *Nature communications* **3**, 865 (2012).

111. Barba, M. & Muti, P. Is it time to test metformin in breast cancer prevention trials? A reply to the authors. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **18**, 2565; author reply 2565 (2009).

112. Muti, P.*, et al.* Metformin, diet and breast cancer: an avenue for chemoprevention. *Cell cycle* **8**, 2661 (2009).

113. Barba, M.*, et al.* The effects of metformin on endogenous androgens and SHBG in women: a systematic review and meta-analysis. *Clinical endocrinology* **70**, 661-670 (2009).

114. Anisimov, V.N.*, et al.* Effect of metformin on life span and on the development of spontaneous mammary tumors in HER-2/neu transgenic mice. *Experimental gerontology* **40**, 685-693 (2005).

115. Dowling, R.J., Goodwin, P.J. & Stambolic, V. Understanding the benefit of metformin use in cancer treatment. *BMC medicine* **9**, 33 (2011).

116. Evans, J.M., Donnelly, L.A., Emslie-Smith, A.M., Alessi, D.R. & Morris, A.D. Metformin and reduced risk of cancer in diabetic patients. *Bmj* **330**, 1304-1305 (2005).

117. Pollak, M. Metformin and other biguanides in oncology: advancing the research agenda. *Cancer Prev Res (Phila)* **3**, 1060-1065 (2010).

118. Pollak, M. Potential applications for biguanides in oncology. *The Journal of clinical investigation* **123**, 3693-3700 (2013).

119. Shaw, R.J.*, et al.* The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* **310**, 1642-1646 (2005).

120. Foretz, M.*, et al.* Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *The Journal of clinical investigation* **120**, 2355-2369 (2010).

121. Pollak, M. The insulin and insulin-like growth factor receptor family in neoplasia: an update. *Nature Reviews Cancer* **12**, 159-169 (2012).

122. Algire, C.*, et al.* Diet and tumor LKB1 expression interact to determine sensitivity to anti-neoplastic effects of metformin in vivo. *Oncogene* **30**, 1174-1182 (2011).

123. Preston, M.A.*, et al.* Metformin use and prostate cancer risk. *European urology* **66**, 1012-1020 (2014).

124. Margel, D.*, et al.* Metformin use and all-cause and prostate cancer–specific mortality among men with diabetes. *Journal of Clinical Oncology* **31**, 3069-3075 (2013).

125. Wright, J.L. & Stanford, J.L. Metformin use and prostate cancer in Caucasian men: results from a population-based case–control study. *Cancer causes & control* **20**, 1617-1622 (2009).

126. Tseng, C.H. Metformin significantly reduces incident prostate cancer risk in Taiwanese men with type 2 diabetes mellitus. *European journal of cancer* **50**, 2831-2837 (2014).

127. Akinyeke, T.*, et al.* Metformin targets c-MYC oncogene to prevent prostate cancer. *Carcinogenesis* **34**, 2823-2832 (2013).

128. Xu, H.*, et al.* Will metformin postpone high-fat diet promotion of TRAMP mouse prostate cancer development and progression? *International urology and nephrology* **46**, 2327-2334 (2014).

129. Sahra, I.B.*, et al.* The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene* **27**, 3576-3586 (2008).

130. Sahra, I.B.*, et al.* Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells. *Cancer research* **70**, 2465-2475 (2010).

131. Rothermundt, C.*, et al.* Metformin in chemotherapy-naive castration-resistant prostate cancer: a multicenter phase 2 trial (SAKK 08/09). *European urology* **66**, 468-474 (2014).

132. Babcook, M.A.*, et al.* Synergistic simvastatin and metformin combination chemotherapy for osseous metastatic castration-resistant prostate cancer. *Molecular cancer therapeutics* **13**, 2288-2302 (2014).

133. Sakoda, L.C.*, et al.* Metformin use and lung cancer risk in patients with diabetes. *Cancer prevention research* **8**, 174-179 (2015).

134. Wu, Y., Liu, H.B., Shi, X.F. & Song, Y. Conventional hypoglycaemic agents and the risk of lung cancer in patients with diabetes: a meta-analysis. *PloS one* **9**, e99577 (2014).

135. Zhang, Z.J.*, et al.* Reduced risk of lung cancer with metformin therapy in diabetic patients: a systematic review and meta-analysis. *American journal of epidemiology* **180**, 11-14 (2014).

136. Lin, J.J.*, et al.* Survival of Stage IV Lung Cancer Patients with Diabetes Treated with Metformin. *American journal of respiratory and critical care medicine* (2014).

137. Algire, C.*, et al.* Diet and tumor LKB1 expression interact to determine sensitivity to anti-neoplastic effects of metformin in vivo. *Oncogene* **30**, 1174-1182 (2011).

138. Memmott, R.M.*, et al.* Metformin prevents tobacco carcinogen--induced lung tumorigenesis. *Cancer Prev Res (Phila)* **3**, 1066-1076 (2010).

139. Salani, B.*, et al.* Caveolin-1 is essential for metformin inhibitory effect on IGF1 action in non-small-cell lung cancer cells. *FASEB J* **26**, 788-798 (2012).

140. Sanli, T.*, et al.* Ionizing radiation regulates the expression of AMP-activated protein kinase (AMPK) in epithelial cancer cells: modulation of cellular signals regulating cell cycle and survival. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* **102**, 459-465 (2012).

141. Morgillo, F.*, et al.* Synergistic effects of metformin treatment in combination with gefitinib, a selective EGFR tyrosine kinase inhibitor, in LKB1 wild-type NSCLC cell lines. *Clin Cancer Res* **19**, 3508-3519 (2013).

142. Kitazono, S.*, et al.* Effect of metformin on residual cells after chemotherapy in a human lung adenocarcinoma cell line. *Int J Oncol* **43**, 1846-1854 (2013).

143. Rocha, G.Z*., et al.* Metformin amplifies chemotherapy-induced AMPK activation and antitumoral growth. *Clin Cancer Res* **17**, 3993-4005 (2011).

144. Iliopoulos, D., Hirsch, H.A. & Struhl, K. Metformin decreases the dose of chemotherapy for prolonging tumor remission in mouse xenografts involving multiple cancer cell types. *Cancer Res* **71**, 3196-3201 (2011).

145. Birsoy, K*., et al.* Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. *Nature* (2014).

146. Wheaton, W.W*., et al.* Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *eLife* **3**, e02242 (2014).

147. Hawley, S.A., Gadalla, A.E., Olsen, G.S. & Hardie, D.G. The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* **51**, 2420-2425 (2002).

148. Zhou, G*., et al.* Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of clinical investigation* **108**, 1167-1174 (2001).

149. DeBerardinis, R.J., Lum, J.J., Hatzivassiliou, G. & Thompson, C.B. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell metabolism* **7**, 11-20 (2008).

150. Vander Heiden, M.G., Cantley, L.C. & Thompson, C.B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* **324**, 1029-1033 (2009).

151. Sanli, T.*, et al.* Ionizing radiation activates AMP-activated kinase (AMPK): a target for radiosensitization of human cancer cells. *International journal of radiation oncology, biology, physics* **78**, 221-229 (2010).

152. Chou, T.C., Motzer, R.J., Tong, Y. & Bosl, G.J. Computerized quantitation of synergism and antagonism of taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a rational approach to clinical protocol design. *J Natl Cancer Inst* **86**, 1517-1524 (1994).

153. Blacklock, C.*, et al.* Salicylic acid in the serum of subjects not taking aspirin. Comparison of salicylic acid concentrations in the serum of vegetarians, non-vegetarians, and patients taking low dose aspirin. *Journal of clinical pathology* **54**, 553-555 (2001).

154. Ruffin, M.T.t.*, et al.* Suppression of human colorectal mucosal prostaglandins: determining the lowest effective aspirin dose. *Journal of the National Cancer Institute* **89**, 1152-1160 (1997).

155. Pearlman, B.L. & Gambhir, R. Salicylate intoxication: a clinical review. *Postgraduate medicine* **121**, 162-168 (2009).

156. Beynen, A.C. & Geelen, M.J. Short-term inhibition of fatty acid biosynthesis in isolated hepatocytes by mono-aromatic compounds. *Toxicology* **24**, 183-197 (1982).

157. Hundal, R.S.*, et al.* Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes. *The Journal of clinical investigation* **109**, 1321-1326 (2002).

158. Watt, M.J., *et al.* Rapid upregulation of pyruvate dehydrogenase kinase activity in human skeletal muscle during prolonged exercise. *Journal of applied physiology* **97**, 1261-1267 (2004).

159. Algra, A.M. & Rothwell, P.M. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *The Lancet. Oncology* **13**, 518-527 (2012).

160. Zhuang, Y., Chan, D.K., Haugrud, A.B. & Miskimins, W.K. Mechanisms by which low glucose enhances the cytotoxicity of metformin to cancer cells both in vitro and in vivo. *PloS one* **9**, e108444 (2014).

161. Yin, J., *et al.* Effects of berberine on glucose metabolism in vitro. *Metabolism* **51**, 1439-1443 (2002).

162. Pizer, E.S., *et al.* Increased fatty acid synthase as a therapeutic target in androgen-independent prostate cancer progression. *The Prostate* **47**, 102-110 (2001).

163. Swinnen, J.V., Esquenet, M., Goossens, K., Heyns, W. & Verhoeven, G. Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP. *Cancer research* **57**, 1086-1090 (1997).

164. Piyathilake, C.J., *et al.* The expression of fatty acid synthase (FASE) is an early event in the development and progression of squamous cell carcinoma of the lung. *Human pathology* **31**, 1068-1073 (2000).

165. Edmond, J. & Popjak, G. Transfer of carbon atoms from mevalonate to n-fatty acids. *Journal of Biological Chemistry* **249**, 66-71 (1974).

166. Fogelman, A., Edmond, J. & Popjåk, G. Metabolism of mevalonate in rats and man not leading to sterols. *Journal of Biological Chemistry* **250**, 1771-1775 (1975).

167. Schwabauer, R.A., Li, C., Adams, G. & Gamble, W. Utilization of mevalonate by aorta for the synthesis of medium-chain< i> n</i>-fatty acids (C< sub> 8</sub>, C< sub> 10</sub>) and acylglycerols. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* **960**, 139-147 (1988).

168. El-Mir, M.Y.*, et al.* Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *The Journal of biological chemistry* **275**, 223-228 (2000).

169. Ford, R.J.*, et al.* Metformin and salicylate synergistically activate liver AMPK, inhibit lipogenesis and improve insulin sensitivity. *The Biochemical journal* (2015).

170. Scott, J.W.*, et al.* Small Molecule Drug A-769662 and AMP Synergistically Activate Naive AMPK Independent of Upstream Kinase Signaling. *Chem Biol* (2014).

171. Ducommun, S.*, et al.* Enhanced activation of cellular AMPK by dual-small molecule treatment: AICAR and A769662. *American journal of physiology. Endocrinology and metabolism* **306**, E688-696 (2014).

172. Greenberg, N.M.*, et al.* Prostate cancer in a transgenic mouse. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 3439-3443 (1995).

173. Hurwitz, A.A., Foster, B.A., Allison, J.P., Greenberg, N.M. & Kwon, E.D. The TRAMP mouse as a model for prostate cancer. *Current protocols in immunology*, 20.25. 21-20.25. 23 (2001).

174. Dzamko, N.*, et al.* AMPK beta1 deletion reduces appetite, preventing obesity and hepatic insulin resistance. *The Journal of biological chemistry* **285**, 115-122 (2010).

175. Feng, Z.*, et al.* The regulation of AMPK β1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. *Cancer research* **67**, 3043-3053 (2007).

176. Sanli, T., Linher-Melville, K., Tsakiridis, T. & Singh, G. Sestrin2 modulates AMPK subunit expression and its response to ionizing radiation in breast cancer cells. *PloS one* **7**, e32035 (2012).

177. Li, J., Jiang, P., Robinson, M., Lawrence, T.S. & Sun, Y. AMPK-beta1 subunit is a p53-independent stress responsive protein that inhibits tumor cell growth upon forced expression. *Carcinogenesis* **24**, 827-834 (2003).