The Role of SirT1 in Resveratrol Toxicity

By

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Abstract

SirT1 is a class III histone deacetylase that has beneficial roles in various diseases related to aging such as cancer, diabetes and neurodegenerative disease. Resveratrol is a natural compound that mimics most of the beneficial effects attributed to SirT1. Resveratrol has toxicity towards cancer cells and has been reported to be a direct activator of SirT1. Interestingly, SirT1 over-expression has also been reported to be toxic. We set out to determine if resveratrol toxicity is mediated through activation of SirT1. We have assessed resveratrol toxicity in embryonic stem cells and mouse embryonic fibroblast (MEFs) across different SirT1 genotypes. Our data indicates that SirT1 is not implicated in resveratrol toxicity in either normal or transformed MEFs. Thus, resveratrol toxicity does not appear to be mediated by SirT1.

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List of Abbreviation

AADPr Acetyl-ADP ribose

$A\beta_{42}$ Amyloid β peptide 42 amino acid length

ADP Adenosine diphosphate

ANOVA Analysis of variance

APC^{min} Adenomatous polyposis coli protein with the MIN mutation

APP Amyloid precursor protein

ATP Adenosine-5'-triphosphate

Bax Bcl-2–associated X protein

Bak Bcl-2 homologous antagonist/killer

Bcl-2 B-cell lymphoma 2 protein

BRCA-1 Breast cancer type 1 susceptibility protein

BSA Bovine serum albumin

CD95 Fas receptor

Cdk Cyclin-dependent kinases

CMV Cytomegalovirus promoter

COX-1 Cyclooxygenase-1

COX-2 Cyclooxygenase-1

CR Caloric restriction

CY3 Cyanide dyes 3(red)

DAPI 4',6-diamidino-2-phenylindole

DNA Deoxyribonucleic acid

DR4 Death receptor 4

DR5 Death receptor 5

E. Coli Escherichia coli

EDTA Ethylene di-amine tetra-acetic acid

ERC Extrachromosomal rDNA circle

ERK Extracellular-signal-regulated kinase

ES embryonic stem cells

FOXO Forkhead box protein O

GFP Green fluorescent protein

H1299 Human non-small cell lung carcinoma cell line

H3 Histone 3

H4 Histone 4

HDAC Histone deacetylase

HEK293T Human embryonic kidney 293

HML Hidden mating loci

HRP Horseradish peroxidase

HTRF Homogenous time resolve fluorescence

IPTG Isopropyl β-D-1-thiogalactopyranoside

JNK c-Jun N-terminal kinase

LB Luria-Bertani medium (lysogeny broth)

LD50 Lethal dose 50

LDS Lithium dodecyl sulfate

LTR Long terminal repeat

MEFs Mouse Embryonic Fibroblasts

MMTV Mouse mammary tumor virus promoter

MOPS 3-(N-morpholino)propanesulfonic acid buffer

NAD+ Nicotinamide adenine dinucleotide

NAM Nicotinamide

NFκB Nuclear factor kappa-light-chain-enhancer of activated B cells

NP-40 Octyl phenoxypolyethoxylethanol

OAADPr O-acetyl ADP-ribose

p38 MAPK P38 mitogen-activated protein kinase

p53 Tumor suppressor p53

PI3K Phosphatidylinositol 3-kinases

PBS Phosphate buffer saline

PGC1α Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PKC Protein kinase C

pkg Phosphoglycerate kinase promoter

PPARα Peroxisome proliferator-activated receptors α

PPARγ Peroxisome proliferator-activated receptors γ

PTEN Phosphatase and tensin homolog protein

PyMT Polyomavirus middle T antigen

RES Resveratrol

rDNA Ribosomal DNA

RPM Rotation per minute

SDS Sodium dodecyl sulfate

Sir2 Silent mating type information regulation 2

Sir3 Silent mating type information regulation 3

Sir4 Silent mating type information regulation 4

SirT1 Silent mating type information regulation 2 homolog 1

siRNA Small interfering RNA

Tag Large T antigen

TBST Tris-Buffered Saline with Tween 20 buffer

TNF-α Tumor necrosis factor-alpha

Tris Tris-(hydroxy-methyl) amino-methane

YFP Yellow fluorescent protein

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Chapter 1: Introduction

The history of Sirtuins

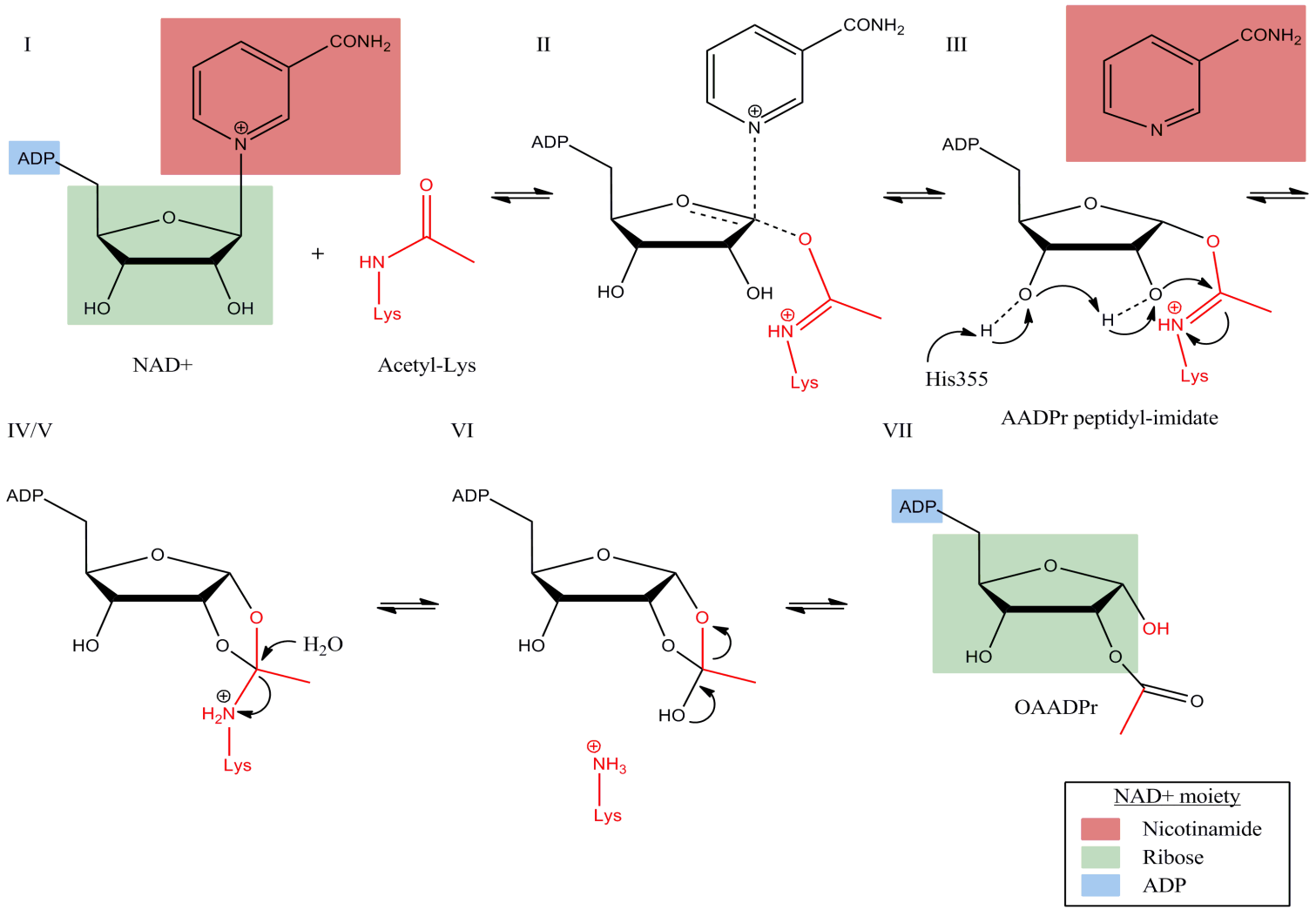
Sirtuins are a class of enzymes conserved from bacteria to mammals (Frye, 1999). They are characterized by an NAD+ dependant deacetylase and/or ADP mono-ribosyl transferase activity (Frye, 1999; Imai et al., 2000). The founding member of the family, Silent information regulator 2 (Sir2), was discovered in 1979 in the budding yeast *Saccharomyces cerevisiae* and was described as a transcriptional silencing factor at the Hidden Mating type Loci (HML), rDNA and telomeric regions (Aparicio et al., 1991; Klar et al., 1979; Smith and Boeke, 1997). Interestingly, it was shown that Sir2 silencing at the HML and telomeric regions required the formation of a multi-protein complex with Sir3 and Sir4 (Aparicio et al., 1991; Rine and Herskowitz, 1987) but not at the rDNA locus (Smith and Boeke, 1997). Transcriptional silencing by the Sir2 protein in yeast is mediated by deacetylation of the ε-amine of specific lysine residues on histone H3 and H4 tails which promote the spreading of a silent chromatin structure (Imai et al., 2000; Landry et al., 2000; Rusché et al., 2002). Since then, Sir2 has been implicated in many other important processes such as genomic stability (Bennett et al., 2001; Martin et al., 1999), segregation of protein aggregates (Liu et al., 2010) and metabolism (Starai et al., 2003).

The deacetylation mechanism of Sirtuins

Sirtuins were first described as ADP-ribosyl transferase enzymes (Tanny et al., 1999), but it has become clear that their major catalytic activity is deacetylation (Imai et al., 2000;

Landry et al., 2000). While sub-classified as a histone deacetylase (HDAC), it has recently been shown that these proteins can equally performed this reaction on non histone proteins (Haigis and Sinclair, 2010). Inherently, their deacetylation mechanism is quite different from Class I and Class II HDACs which hydrolyze the acetyl group from the ε-N lysine on histones. The deacetylation mechanism of Sirtuins or Class III HDACs is distinct, and has been extensively studied in yeast and bacteria. Except for certain details, the general mechanism of deacetylation has been resolved through crystallography and biochemical studies with different substrate analogues (Sauve, 2010). For a summary of this mechanism, see Figure 1. Briefly, NAD+ first binds to the catalytic core domain of Sirtuins. This allows the acetylated protein to bind (Sauve, 2010), and triggers a three dimensional conformational rearrangement. This in turn brings the acetyl group in close proximity to the NAD+ ribose moiety and pushes the nicotinamide group into a pocket that promotes NAD+ destabilization. At this point, the acetyl group attacks the NAD+ ribose moiety which is coupled with nicotinamide cleavage and release. The intermediate product formed is called acetyl-ADP-ribosyl peptidyl-imidate (AADPr-peptidy-imidate) which contains the ADP-ribose moiety from NAD+ and the acetylated protein (Sauve, 2010). Next, the intermediate undergoes intra-cyclisation which promotes deacetylation of the protein. This important step is driven by a conserved histidine residue in the catalytic core domain (Smith and Denu, 2006). While the catalytic requirement for the histidine has been demonstrated in both yeast and the bacteria, its involvement in the mammalian orthologue remains to be determined.

Figure 1. The mechanism of Sirtuin deacetylation.
The acetyl-lysine is represented in red while NAD+ is represented in black. NAD+ moieties are depicted by different color boxes: red for nicotinamide, green for ribose and blue for ADP. Roman numerals depict the steps involved the reaction. I) Binding of both substrates to sirtuins. II) 3D conformational rearrangement of the enzyme brings the substrates in close proximity. III) Nicotinamide cleavage coupled with the formation of the intermediate (AADPr peptidyl-imidate). IV/V) Free acetyl lysine cleavage and capture of a water molecule to form the tetrahedral intermediate in VI. VI/VII) Internal rearrangement and formation of the final OAADPr product. Modified from Sauvée A.A 2010.


I
II
III
ADP
CONH2
N
O
HO
OH
HN
Lys
NAD+
Acetyl-Lys
His355
AADPr peptidyl-imidate
IV/V
VI
VII
H2O
H2N
NH3
OAADPr
NAD+ moiety
Nicotinamide
Ribose
ADP

In the last step of the reaction, water molecules attack the remaining cyclic AADPR-peptidyl imidate compound to produce the final acyclic O-acetyl ADP ribose (OAADPr) product and randomly release the deacetylated protein and OAADPr.

The activity of Sirtuins is regulated by NAD+ availability, a key metabolite whose level is believed to fluctuate depending on the energy and redox status of the cell (Houtkooper et al., 2010). Rodgers et al. were the first to demonstrate that a decrease in nutrient availability increases the level of NAD+ in mouse liver which subsequently increases expression and potentially the activity of the mammalian Sirtuin, SirT1 (Rodgers et al., 2005). Conversely, nicotinamide inhibits Sirtuins by non competitive inhibition (Bitterman et al., 2002; Gallo et al., 2004; Sauve and Schramm, 2003). This suggests that Sirtuins may function as stress sensors which modulate cellular activities depending on the redox status of the cell, a process well documented to be affected throughout aging (Gilca et al., 2007).

Mammalian SirT1

Interest in this family of protein really began when Kaeberlein M et al. in 1999 established their role in aging (Kaeberlein et al., 1999). Yeast mutants harboring an extra copy of the SIR2 gene had a prolonged replicative life span, a function attributed to the ability of Sir2 to inhibit the formation of extrachromosomal rDNA circle (ERC). This process is dependent on homologous recombination (Kaeberlein et al., 1999). Follow up studies in *C. elegans* and *D. melanogaster* (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001), further reinforced the biological function of Sir2 in aging. Since Sirtuins

are able to sense the energy status of cells, and are implicated in longevity, it was proposed that they might be able to regulate caloric restriction (CR) mediated lifespan extension (Lin et al., 2000; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). CR is a known dietary regimen that increases lifespan of many model organism including small mammals (Mair and Dillin, 2008). It consists in decreasing the number of calories consumed by 30-40% without malnutrition (Mair and Dillin, 2008). Interestingly, caloric restriction in mammals induces metabolic changes and conferred protection against age-related diseases such as cancer, diabetes mellitus, cardiovascular, and neurodegenerative diseases (Mair and Dillin, 2008). Interestingly, mounting evidence suggests that the mammalian Sirtuin orthologs are equally implicated in age-related diseases that are delayed by CR (Haigis and Sinclair, 2010). While its role in caloric restriction remains controversial, studies with transgenic mice showed that the mammalian Sirtuins ortholog, SirT1, is indeed important for physiological changes induces by CR (Boily et al., 2008; Bordone et al., 2007; Chen et al., 2005a) which indicates that SirT1 may in part be responsible for life span extension by caloric restriction. These studies provided evidence for a conserved biological function of Sirtuins in caloric restriction mediated lifespan extension and prompted researchers to study the mammalian homolog: SirT1.

In mammals, there are 7 genes (SIRT1-SIRT7) that harbor high sequence homology to the catalytic core domain of yeast SIR2 (Fig. 2) (Frye, 1999). They all have different sub-cellular localization and enzymatic activity (Haigis and Sinclair, 2010) (see Table 1). Based on their amino acid sequence homology, SirT1 is the true ortholog of the yeast Sir2 protein (Frye, 1999). Like Sir2, SirT1 also localized to the nucleus, but in contrast, it is excluded

Figure 2. Protein sequence comparison of the seven mouse sirtuins.
The conserved catalytic core domain is depicted in red, the NAD+ binding domain in green and the conserved histidine is depicted in blue. Non conserved region are represent in black and the scale represent the protein length in terms of amino acid number.

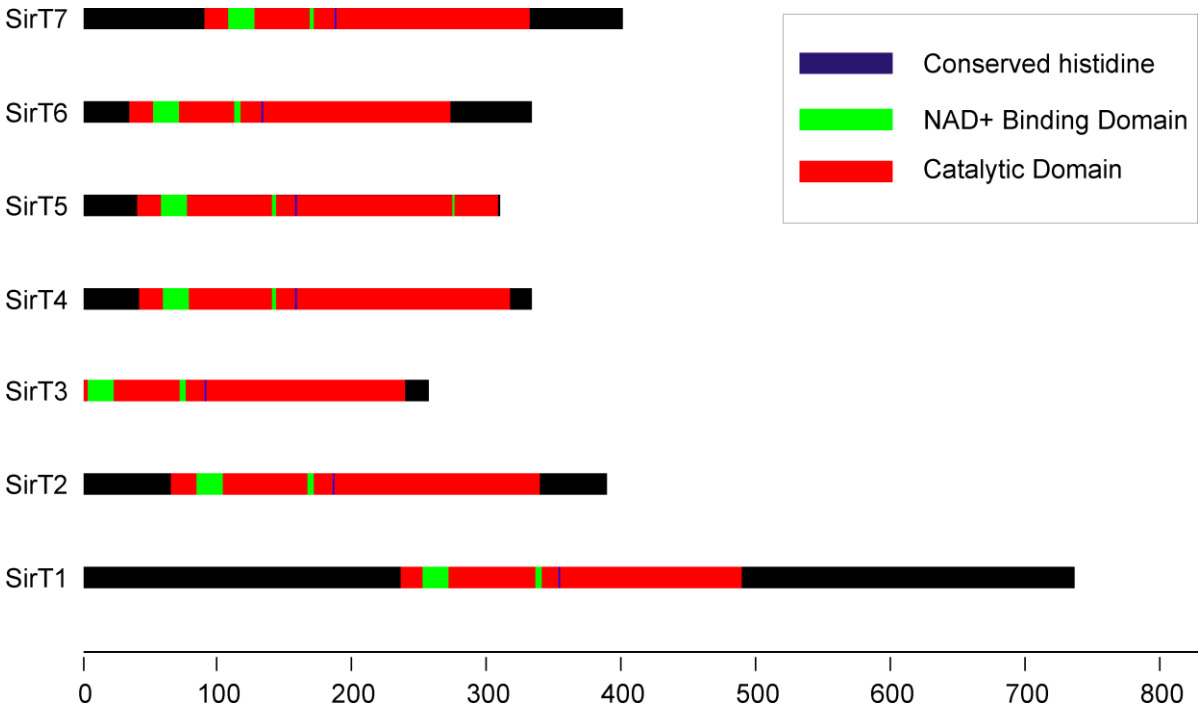

SirT7
SirT6
SirT5
SirT4
SirT3
SirT2
SirT1
Conserved histidine
NAD+ Binding Domain
Catalytic Domain
0
100
200
300
400
500
600
700
800

Table I

Cellular localization and enzymatic activity of mammalian Sirtuins

	Localization	**Activity**
SIRT1	Nucleus	Deacetylase
SIRT2	Cytoplasm	Deacetylase
SIRT3	Mitochondria	Deacetylase
SIRT4	Mitochondria	ADP-ribosyl transferase
SIRT5	Mitochondria	Deacetylase
SIRT6	Nucleus	ADP-ribosyl transferase and deacetylase
SIRT7	Nucleolus	Unknown

from heterochromatin region and does not take part of a large multi-protein complex (McBurney et al., 2003b). This suggests that SirT1 may have alternative functions. In fact, SirT1 has a plethora of targets including both histone and non-histone protein. Many of its substrates are transcription factors including PGC-1α (Nemoto et al., 2005; Rodgers et al., 2005), PPARγ (Picard et al., 2004), PPARα (Purushotham et al., 2009), FOXO (Brunet et al., 2004; Van Der Horst et al., 2004), p53 (Langley et al., 2002; Luo et al., 2001; Vaziri et al., 2001), NF-κB (Chen et al., 2005b; Yang et al., 2007; Yeung et al., 2004), Ku70 (Jeong et al., 2007), etc. Through its various substrates, SirT1 has been implicated in many cellular processes including; genomic stability (Oberdoerffer et al., 2008; Wang et al., 2008a), chromatin silencing (El Ramy et al., 2009; Vaquero et al., 2004), metabolism (Bordone et al., 2006; Picard et al., 2004; Rodgers et al., 2005), inflammation (Chen et al., 2005b; Pfluger et al., 2008; Yoshizaki et al., 2009), protein aggregates homeostasis (Wang et al., 2011; Westerheide et al., 2009), apoptosis (Brunet et al., 2004; Vaziri et al., 2001; Yeung et al., 2004) and autophagy (In et al., 2008; Kume et al., 2010).

SirT1 health beneficial effects

Age-related diseases are characterized by failure to maintain cellular and metabolic homeostasis due to environmental and/or genetics factors. Inflammatory processes underlie some age-related diseases and often contribute to the progression, aggravation and complication associated with the disease (Libby, 2002; Williams and Nadler, 2007; Wyss-Coray and Mucke, 2002). SirT1 mediates its beneficial effects on age-related diseases by

acting on specific targets important for the etiology and progression of different diseases. Importantly, SirT1 contributes to reducing inflammatory processes. By regulating both inflammatory processes, as well as disease specific markers, SirT1 may function as a master regulator of age related diseases.

SirT1 has been implicated in type II diabetes by its function across different metabolic tissues. Type II diabetes is characterized by impaired insulin sensitivity and secretion which leads to elevated blood glucose levels and subsequently to several health complications such as nephropathy, neuropathy and atherosclerosis (Williams and Nadler, 2007). SirT1 increases both insulin secretion and sensitivity by its direct action on the pancreas and peripheral metabolic tissue (Imai and Guarente, 2010; Liang et al., 2009). Furthermore, SirT1 also protects against β-cell destruction by controlling inflammatory processes in the pancreas and adipocytes (Lee et al., 2009; Yoshizaki et al., 2009).

Since CR protects against cardiovascular diseases, researchers investigated the impact of SirT1 in the context of these diseases. Cardiovascular diseases are characterized by formation of atherosclerotic plaques follow by atherothrombosis which is characterized by plaque rupture and formation of blood clots which restrict blood flow (Stein and Matter, 2011). This process leads to stroke and cardiac arrest (Stein and Matter, 2011). Endothelial cells within the arteries are subject to oxidative damage caused by high blood glucose or circulating oxidized low-density lipoprotein (oxLDL). Oxidative damage promotes inflammation and plaque formation by recruitment and accumulation of macrophages that have engulfed the oxLDL particles. SirT1 has been shown to protect endothelial cells from inflammation by reducing the level of inflammatory cytokines in both endothelial cells and macrophages through NF-κB inhibition (Stein and Matter, 2011). Some reports equally

indicate that SirT1 may help regulate specific mechanisms involved in the development of cardiovascular disease. These include the maintenance of endothelial vascular function, macrophages cholesterol uptake and plaque stabilization (Stein and Matter, 2011).

The role of SirT1 in neurodegenerative disease remains controversial and elusive. Most of the evidence comes from indirect activation of SirT1 through pharmacological compounds or through the NAD+ biosynthetic enzyme Nmnat1 (Conforti et al., 2007; Shindler et al., 2007; Yahata et al., 2009). Nonetheless, SirT1 seems important for regulating the formation of a small peptide ($A\beta_{42}$) responsible for the formation of amyloid plaques, a hallmark of Alzheimer disease. Amyloid plaques are formed by aberrant processing of the transmembrane protein; APP. SirT1 has been shown to promote the proper cleavage of APP which decreases the formation of the aberrant peptide ($A\beta_{42}$) found in plaques (Bonda et al., 2011). Furthermore, by controlling inflammation at the plaques site by inhibiting the inflammatory response by microglia through NF-κB inhibition SirT1 might equally be associated with a protective effect (Bonda et al., 2011). Clearly, more research is needed to uncover to role of SirT1 in neurodegenerative disease.

SirT1 and Cancer

The role of SirT1 in cancer is currently highly debated in the literature and it seems that SirT1 can either promote or inhibit cancer. The process of carcinogenesis occurs through a series of mutations in key genes which promote or inhibit growth, survival and invasion. There are two main class of key genes; tumor suppressors and oncogenes. Tumor suppressors are characterized by loss-of-function mutations which renders cells more

susceptible to cancer. On the other hand, oncogenes are often associated with mutations which promote their activation and/or up-regulation. As such these genes are typically involved in the control of cell survival, metabolism and cell cycle progression. *In vivo* experiments provided evidence that SirT1 might function as a tumor suppressor. Two different studies demonstrated that SirT1 protects against loss of heterozygosity at the p53 and APC^{min} locus (Wang et al., 2008a; Firestein et al., 2008). This effect may possibly be explained by its ability to promote DNA repair (Oberdoerffer et al., 2008; Wang et al., 2008a). In contrast, Boily et al. did not observed a difference in the number and size of polyps between $SirT1^{+/+}$ $APC^{min/+}$ and $SirT1^{-/-}$ $APC^{min/+}$ mice as well as in the 2 stage skin carcinogenesis model (Boily et al., 2009a). Consistent with SirT1 being a tumor suppressor, Wang RH et al. analyzed SirT1 expression levels across different cancer tissues and observed that SirT1 expression decreases in glioblastoma, bladder carcinomas, prostate carcinomas and ovarian cancers (Wang et al., 2008a). This result supports SirT1 functioning as a tumor suppressor. Furthermore, they showed that BRCA1-associated breast cancers have a lower level of SirT1 and restoration of SirT1 in these cells decreased their proliferation *in vitro* and tumor formation *in vivo* (Wang et al., 2008b).

The first evidence that SirT1 may function as an oncogene was based on its effect in regulating the tumor suppressor: p53. It was shown that SirT1 can inhibit p53 mediated apoptosis through direct binding and deacetylation at lysine 382 (Luo et al., 2001; Vaziri et al., 2001). Several *in vitro* experiments performed on established cancer cell lines indicate that pharmacological inhibition or siRNA knockdown of SirT1 induced cell cycle arrest and apoptosis. In many of these studies, the phenomenon was dependent on p53 (Audrito et al., 2011; Kim et al., 2007; Ota et al., 2007; Zhao et al., 2011). However, other cell-based studies

indicate that specific SirT1 inhibition or siRNA knockdown did not affect p53 activation or cell viability (Ford et al., 2005; Solomon et al., 2006). Furthermore, experiments conducted in our laboratory, failed to identify a role for SirT1 in regulating p53-dependant biological functions in γ-irradiated splenocytes and thymocytes derived from knockout mice (Kamel et al., 2006). These data indicate that the effect of SirT1 on p53 might be dependent on the cell type and specific genetic alterations that occur throughout the process of carcinogenesis. Analysis of cancer tissues revealed that SirT1, like oncogenes, is over-expressed in many cancer including ovarian, skin, prostates, and leukemia (Bradbury et al., 2005; Hida et al., 2007; Huffman et al., 2007). These studies suggest that the function of SirT1 in cancer might be more complex than first anticipated. SirT1 might confer protection in early events of carcinogenesis by maintaining genomic stability (Firestein et al., 2008; Ford et al., 2005; Wang et al., 2008a) but promote survival at a later stage (Audrito et al., 2011; Ford et al., 2005; Ota et al., 2007). Clearly, further research is required in order to verify SirT1 involvement in the etiology and progression of the disease.

Resveratrol

The involvement of SirT1 in age-related diseases has urged scientists to find drugs that regulate SirT1 function. In a high-throughput drug screen, it was found that resveratrol increase SirT1 catalytic activity (Howitz et al., 2003a). Interestingly, many have shown that resveratrol has beneficial health effects overlapping those of SirT1 and equally mimics some aspects of caloric restriction (Baur, 2010). This polyphenolic natural compound is found in many plants such as peanuts, blueberries, pines and grapes (Shakibaei et al., 2009). Plants

synthesize this compound to protect themselves from fungal infections and UV irradiation (Shakibaei et al., 2009). In 1992, Renaud S. et Lorgeril M. discovered through an epidemiological study that French people had a lower risk of coronary heart disease despite a rich diet in saturated fat (Renaud and De Lorgeril, 1992). Importantly, their lower risk of coronary heart disease was correlated, to a certain extent, with their red wine consumption. A few years after, Ray PS et al. demonstrated that resveratrol was the natural compound responsible for this effect (Ray et al., 1999). Since then, many biological properties have been attributed to resveratrol including: anti-oxidant, anti-inflammatory, anti-carcinogenic, and antimicrobial activities (Pervaiz and Holme, 2009). The beneficial effects of this polyphenol have been mainly attributed to its anti-oxidant property but more recently through its action on various signaling molecules (Pervaiz and Holme, 2009). Resveratrol is considered a pleiotropic agent because it can act on a myriad of targets including: cell surface and intracellular receptors, signal transduction molecules, metabolic and DNA repair proteins as well as transcription factors (Pervaiz and Holme, 2009). It is believed that resveratrol modulates different targets depending on the dose, cell type and genetic variations (Mukherjee et al., 2010). At a low concentration, resveratrol protects against diabetes mellitus, cardiovascular and neurodegenerative diseases (Mukherjee et al., 2010). However at high a concentration, resveratrol has chemotherapeutic effects by inducing cell cycle arrest and apoptosis in cancer cells (Mukherjee et al., 2010).

Resveratrol health beneficial effects

The beneficial effects of resveratrol in diabetes are very similar to that of SirT1. Resveratrol modulates insulin secretion, preserves β-cells from cytokines destruction and oxidative stress and improves insulin sensitivity in peripheral tissue (Szkudelski and Szkudelska, 2011). Importantly, the effects of resveratrol on insulin secretion and sensitivity have been shown to be mediated via SirT1 (Lee et al., 2009; Sun et al., 2007).

The protective action of resveratrol on cardiovascular disease was well known before its action on SirT1. Resveratrol has been shown to prevent the initial oxidative injury, to control the inflammatory process and to inhibit plaque aggregation which eventually leads to thrombosis (Ramprasath and Jones, 2010). In the literature, many studies suggest the involvement of SirT1 in cardiovascular disease. However, most of these studies rely on pharmacological modulation of SirT1 through various small molecules such as resveratrol, a limitation that is widespread within the Sirtuins literature. Nevertheless, two groups have demonstrated that SirT1 is directly implicated in resveratrol-mediated cardio-protection (Csiszar et al., 2008; Ungvari et al., 2009).

The effect of resveratrol in neurodegenerative diseases has been attributed to its ability to protect neurons against various stresses (Alvira et al., 2007; Kim et al., 2006; Sharma and Gupta, 2002). In general, resveratrol protects neurons from apoptosis mediated by oxidative stress and inflammation (Candelario-Jalil et al., 2007; Sharma and Gupta, 2002). In Alzheimer disease, resveratrol increases the clearance of Aβ peptides by promoting proteasomal degradation but not APP processing as observed with SirT1 (Albani et al., 2010). By impairing Aβ peptide aggregation resveratrol decreased plaque formation (Albani et al., 2010).

Resveratrol chemopreventive properties

In 1997 Jang M et al. showed for the first time that resveratrol act as a chemopreventive agent in all three stages of carcinogenesis: initiation, promotion and progression (Jang et al., 1997). Later, Boily et al. found that these effects were in part mediated by SirT1 (Boily et al., 2009b). Cancer initiation is characterized by insults which lead to DNA alterations. Many carcinogens need to be metabolically activated by phase I enzymes in order to form DNA adducts (Delmas et al., 2006). Resveratrol not only inhibits phase I but also activate phase II enzymes which increase carcinogen detoxification (Delmas et al., 2006). In addition, resveratrol protects DNA from oxidative damage through is free radical scavenging properties (Alarcón De La Lastra and Villegas, 2007).

Resveratrol inhibits tumor promotion and progression by controlling different inflammatory processes which promote cell growth and migration (Kundu and Surh, 2008). It is well recognized that pro-inflammatory prostaglandins are implicated in tumor promotion (Wang and DuBois, 2004). Fortunately, resveratrol decreases the biosynthesis of pro-inflammatory prostaglandins through inhibition of COX-1 and COX-2 enzymes through different mechanisms (Delmas et al., 2006). Pro-inflammatory cytokine levels are equally decreased by resveratrol action on NF-κB (Bishayee et al., 2010). Overall, it would appear that decreased inflammation by resveratrol is a hallmark that protects against cancer promotion.

Resveratrol is equally able to control cell proliferation by modulating key regulators of the cell cycle machinery including cyclins, cyclins dependent kinases (Cdk), cyclin inhibitors and check point kinases (Delmas et al., 2006). Also, resveratrol can inhibit tumor

growth by blocking DNA synthesis directly and by inhibiting the translation machinery (Kundu and Surh, 2008). This chemopreventive/chemotherapeutic effect was observed in several cancer cell lines and even in some *in vivo* model (Baarine et al., 2011; Bishayee et al., 2010; Boily et al., 2009a; Clément et al., 1998; Dörrie et al., 2001; Jang et al., 1997; Lu et al., 2001). Overall, it would appear that resveratrol can block tumor development and progression through a plethora of different mechanisms.

Resveratrol chemotherapeutic properties

Chemotherapeutic drugs exert their antitumor effects mainly by inducing apoptosis in cancer cells. Interestingly, resveratrol has been reported to induce cell death in various cancer cells but remains fairly non toxic for normal cells (Baarine et al., 2011; Clément et al., 1998; Dörrie et al., 2001; Lu et al., 2001). The function of resveratrol as a chemotherapeutic agent is typically attributed to its anti-proliferative, pro-apoptotic, anti-angiogenic and anti-metastatic properties (Pervaiz and Holme, 2009).

Apoptosis is a programmed cell death mechanism which consists in activation of a cellular signaling cascade that leads to activation of specific proteases named caspases. Caspases cleave key proteins which lead to the degradation of structural proteins, inactivation of enzymes in the DNA repair pathway and eventually cell death. In normal tissue, apoptosis is tightly controlled via a multitude of pro-apoptotic and pro-survival proteins. In many diseases apoptosis is deregulated where an imbalance towards pro-survival or pro-apoptotic proteins will dictate the fate of the cell. Aberrant processes which activate apoptosis lead to tissues damage in many diseases including cardiovascular, ischemic and

neurodegenerative diseases. However certain diseases, such as cancer, tip the balance towards a pro-survival state to avoid apoptosis. This in turn ultimately leads to hyper-proliferation and loss of tissue function. Apoptosis can be initiated by two main pathways 1) the intrinsic/mitochondrial pathway and/or 2) the extrinsic/ligand pathway. Both pathways require events that initialize, mediate and execute apoptosis (Fulda and Debatin, 2006).

Depending on the cell line studied resveratrol is able to induce apoptosis by both the intrinsic and the extrinsic pathway through its action on different apoptotic signaling molecules that initialize and mediate apoptosis (Fulda and Debatin, 2006). The induction of intrinsic cell death by resveratrol is believed to be mediated by its direct action on pro-apoptotic (Bax and Bak) and anti-apoptotic (Bcl2 and survivin) proteins (Delmas et al., 2006). Moreover, resveratrol can disrupt mitochondrial function and induce apoptosis by inhibiting the enzyme responsible for ATP synthesis, ATP synthase (Gledhill et al., 2007). Indirectly, resveratrol can mediate the intrinsic apoptotic pathway by modulating signaling molecule such as AKT/PI3K, PKC, ERK, JNK, P38 MAPK kinases and transcription factors like p53, FOXO, AP-1 and NF-κB (Delmas et al., 2006). Resveratrol is equally known to stimulate the extrinsic pathway by promoting localization of the death receptor (CD95, DR4 and DR5) to lipid rafts (Delmas et al., 2006) and by increasing ceramide signaling (Scarlatti et al., 2003). Interestingly, resveratrol sensitizes cells to TNF-α induced apoptosis through SirT1 action on NF-κB (Yeung et al., 2004). SirT1 shares many of the known resveratrol targets including p53, NF-κB, AP-1, FOXO and survivin (Luo et al., 2001; Wang et al., 2008b; Yeung et al., 2004; Zhang et al., 2010) and might in turn dictate cellular fate through a complex balancing of the different resveratrol cell survival and cell death signals.

SirT1 and Resveratrol: Duality in cytotoxic effects:

Recently, Alcendor et al. have created heart specific transgenic mice over-expressing SirT1 and have observed that a low to moderate increase in SirT1 expression (2.5-7.5 fold) provided a beneficial effect against oxidative stress. However, at high levels of SirT1 (12.5 fold) expression induced oxidative stress and myocardiopathy (Alcendor et al., 2007). Similarly, neuron specific SirT1 over-expression leads to memory deficiencies in mice, indicating that high level of SirT1 might be detrimental for neurons (Kakefuda et al., 2009). These reports suggest that SirT1 expression or activity level is crucial and must be tightly regulated in order to get the beneficial effects without inducing toxicity. Very similar to SirT1, resveratrol has hormetic properties. While low concentration confer protection against apoptosis, oxidative stress and inflammation, high concentration is toxic and induce cell death. Although, the similarities between SirT1 and resveratrol are nonetheless impressive, recent studies have demonstrated that SirT1 is not directly activated by resveratrol like previously reported by Howitz et al. (Howitz et al., 2003a; Beher et al., 2009; Borra et al., 2005; Kaeberlein et al., 2005; Pacholec et al., 2010). We in turn believe that resveratrol activation of SirT1 may be indirect. While the indirect association has been tested to a certain degree in diabetes and cardiovascular diseases, the role of SirT1 in resveratrol toxicity and tumor specificity remain to be characterized.

Hypothesis

We hypothesize that resveratrol toxicity is due to SirT1 over-activation in normal and/or cancer cells. This is based on the observation that high levels of SirT1 expression are toxic, that resveratrol phenocopies activation of SirT1 and that many of resveratrol's beneficial effects require SirT1 activity (Boily et al., 2009a; Csiszar et al., 2008; Ungvari et al., 2009; Lee et al., 2009; Sun et al., 2007).

Specific aims

We set out to evaluate resveratrol toxicity in embryonic stem (ES) cells. We have knocked out both SirT1 allele in R1 ES cells by homologous recombination and we have obtained R1($SirT1^{-/-}$) cells referred as B6 ES cells (McBurney et al., 2003a). We have generate knockout mice from these ES cells and derived mouse embryonic fibroblast (MEFs) from $SirT1^{+/+}$, $SirT1^{+/-}$, and $SirT1^{-/-}$ embryos. We have also generated by a similar methodology $SirT1^{Y/Y}$ mice which have their endogenous SirT1 alleles replace by an alleged catalytic dead mutation (histidine 355 to tyrosine). As a preamble, I will present the catalytic activity of the mutant $SirT1^{H355Y}$, since the loss-of-function point mutation has never been characterized, but rather inferred from studies of sir2 proteins from lower organisms (Hoff et al., 2006; Min et al., 2001; Smith and Denu, 2006). Characterization of SirT1 point mutant activity was crucial for the laboratory, since we have generated transgenic mice having both their SirT1 allele replace with the point mutant. This part of my work contributed to a publishable work (Seifert et al., 2011).

In order to test our hypothesis we planned to:

1) Test the catalytic activity of the SirT1 point mutant SirT1^{H355Y} expressed in mammalian cells.
2) Determine the catalytic activity of the purified His tagged SirT1 and SirT1^{H355Y} synthesized in bacteria
3) Assess resveratrol toxicity in both ES and MEFs cells by performing dose response curves.
4) Assess the importance of SirT1 catalytic activity in resveratrol toxicity by using nicotinamide a known inhibitor of SirT1 and the catalytic point mutant SirT1^{H355Y}.

Chapter 2: Materials and Methods

Cell culture. HEK293T and MEFs cell lines were cultured with Dulbecco′s Modified Eagle′s Medium (Thermo Scientific, Cat # SH30243.01, Logan, USA) supplemented with 10% fetal bovine serum (Thermo Scientific, Cat #SH30396.03, Logan, USA). Cells were incubated at 37°C in 5% CO_2. Embryonic stem cells from R1 ($SirT1^{+/+}$) (Nagy et al., 1993) and B6 ($SirT1^{-/-}$) (McBurney et al., 2003b) were culture in Dulbecco′s Modified Eagle′s Medium (Thermo Scientific, Cat # SH30243.01, Logan, USA) and supplemented with 15% fetal bovine serum (Thermo Scientific, Cat #SH30396.03, Logan, USA), LIF, non-essential amino-acids (Invitrogen, Cat # 11140-050,Burlington, Canada), β-mercaptoethanol (EMD Chemicals, Cat # 6010, Gibbstown, USA). Embryonic stem cells were incubated at 37°C in 10% CO_2.

MEFs retro-viral infection. $SirT1^{-/-}$ mouse embryonic fibroblasts were isolated from day 13 embryos and cultured until spontaneous immortalization occurred. MEFs infection was performed in two steps. First, HEK293T were transfected with a plasmid containing the retroviral backbone genes gag and pol (pHIT60), ecotropic env (pHIT123) in combination with the plasmid containing the gene of interest pKJ321 (SirT1), pKJ322 ($SirT1^{H355Y}$) or pLPCx(YFP) using the calcium phosphate transfection procedure (Chen and Okayama, 1987). Transfected HEK293T cells were incubated for 48 hours and left to produce viral particles containing the gene of interest. Subsequently, MEFs cells were co-cultured with HEK293T cells for an extra 48 hours. Mouse embryonic fibroblasts (MEFs) were selected

over the human embryonic kidney (HEK293T) cells by addition of 30μM of ouabain (Sigma Aldrich, Cat # O-3125, Oakville, Canada). Finally, the cells carrying the gene of interest were selected with puromycin (Sigma, Cat # P8833, Oakville, Canada) treatment (2.0 μg/ml).

MEFs transformation. Prior to retro-viral infection a fraction of MEFs SirT1$^{-/-}$ cells derived from embryo day 13 were transfected with a plasmid containing the large T antigen from Simian vacuolating virus (graciously donated by Jiahu Wang). Retro-viral infection of this cell line required 15μM of ouabain and 1μg/ml of puromycin for selection.

Western blot analysis. Cells were lysed in Ripa buffer (20mM Tris, 150mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1mM EDTA, 0.1% SDS and protease inhibitor cocktail (Roche,Cat # 11836153001, Mannhein, Germany)) or lysis EA buffer (see section deacetylation assay) and clarified by centrifugation at 14,000 rpm, 4 °C for 10 min and stored at − 80 °C. Protein quantification was performed with Bio-rad protein assay (Bio-rad laboratories inc., Cat # 500-0006, Mississauga, ON, Canada) and protein were prepared in 4X Nupage LDS buffer (Invitrogen, Cat # NP0007, Burlington, Canada) with 1mM Dithiothreitol and boiled at 95°C for 5 minutes. Sample and SeeBlue plus 2 ladder (Invitrogen, Cat # LC5925, Burlington,Canada) were loaded into NuPage precast 4–12% gels (Invitrogen, Cat # NP0335BOX, Burlington, Canada) and run for 90 min at 120 V in MOPS buffer (Invitrogen, Cat # NP0001, Burlington, Canada). Proteins were electrotransferred onto nitrocellulose membranes (Amerhsam Biosciences, Cat # RPN 303 E,

Québec, Canada) for 120min at 30V. Membranes were blocked with 5% powdered milk in TBST (20mM Tris pH 7.5, 200mM NaCl, 1% Tween-20) for 1 h at room temperature followed by immunoblotting overnight at 4°C in 2% powdered milk in the presence of either SirT1 (1/1000)(Cell signaling, Cat # 2028S, New England Biolabs, Pickering, Canada), Large T antigen Pab101 (1/500)(Santa Cruz, Cat # sc-147, Santa Cruz, USA) or β-actin (1/5000) (Sigma-Aldrich, Cat # A-5316, Oakville, Canada) primary antibodies. After 3 washes with TBST, membranes were incubated for 1 hour at room temperature with HRP Goat anti-rabbit secondary antibody (1/5000) (Sigma Aldrich, Cat # A0545, Oakville, Canada) or HRP Goat anti-mouse secondary antibody (1/5000) (Bio-rad laboratories inc., Cat #172-1011,Mississauga, ON, Canada). Signal was developed with chemoluminescence reagent as recommended by the manufacturer (Luminata Forte, Millipore, Cat # WBLUF0100, Billerica, USA). Detection was performed on X-ray film (Kodak X-OMAT-LS, Carestream Health, Cat # 864-6770, Rochester, USA).

Immunofluorescence. MEFs cells were grown overnight on a cover slip. The next day, cells were fixed with 4% paraformaldehyde (Fisher Scientific, Cat # T353-500, Ottawa, Canada) and permeabilized with 0.3% Triton-X (EMD Chemicals, Cat # 8603, Gibbstown, USA). Slides were blocked with 1% BSA (Fisher Scientific, Cat # BP1605-100, Ottawa, Canada) in PBS for 20 minutes at room temperature and stained with the SirT1 antibody (1/400) (Cell signaling, New England Biolabs, Cat # 2028S, Pickering, Canada) overnight at 4°C. Cells were washed in PBS and CY3 coupled goat anti-rabbit secondary antibody (1/400) (Jackson immunoResearch laboratories, GE health care Biosciences, Cat # 111-165-003, West Grove, USA) overnight at 4°C. Cover slips were mounted onto microscope slides with DAPI

containing mounting media (Vectashield, Vector Laboratories inc., Cat # H-1200, Burlington, Canada) prior to microscopy (Zeiss Axioskop2). The percentage of positive cells was determined by densitometry analysis performed with ImageJ 1.42J (developed by Wayne Rasband, National institute of Health, public domain software)

Dose response curve. MEFs or ES cells were plated in 96 well plates and incubated for 4 hours at 37°C under 5% CO_2 prior to resveratrol (Sigma Aldrich, Cat # R5010-100, Oakville, Canada) treatment. Resveratrol concentration ranged from 0.1953-800uM depending on the cell line. Cell viability was assessed after 48 hours incubation at 37°C under 5% CO_2 in presence of resveratrol with 10% alamarBlue (Invitrogen, Cat #DAL1100, Burlington, Canada). Detection was performed by fluorescence (Fluoroskan Ascent FL, Thermo Scientific, Logan, USA) at 595nm after 3-24 hours of incubation depending on the cell line. Prior to alamarBlue use, a standard viability curve was performed in order to test the linearity domain of alamarBlue signal (Fig. S1). AlamarBlue reagent contains the dye Resazurin which is reduced by different cytoplasmic diaphorases such as NQO1, Flavin reductase and NADH dehydrogenase (O'Brien et al., 2000).

Nonlinear regression. Non linear regression was performed with GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego California USA). The curves were fit to this equation: Y= bottom + (Top-Bottom)/(1+10^((logLD50-X)*Slope Coefficient)). Top and bottom value were fixed to 100 and 0%, respectively.

Deacetylation assay. HEK293T cells were transfected with plasmids containing wild type SirT1(pKJ321), SirT1-GFP(pKJ344), a catalytic point mutant $SirT1^{H355Y}$(pKJ322), $SirT1^{H355Y}$-GFP(pKJ345) or empty vector (PLPCx or PPOP) by the calcium phosphate procedure (Chen and Okayama, 1987). Cells were lysed in lysis EA buffer (50mM HEPES pH 8.00, 300mM NaCl) containing 1mM β-mercaptoethanol, 0.5% NP-40 and protease inhibitor cocktail Free-EDTA (Roche,Cat # 11836170001, Mannhein, Germany). Lysates were clarified by centrifugation at 14,000 rpm, 4 °C for 10 min. Total protein quantification was performed with Bio-rad protein assay (Bio-rad laboratories inc., Cat # 500-0006, Mississauga, Canada). For each cell lysate, a 3mg/ml total protein stock solution was prepared. Sirtuin activity was assessed directly by performing serial dilution of the 3mg/ml cell lysate. Deacetylation activity of both non his-tagged and his-tagged protein was monitored using the SirT1 HTRF assay kit (Cisbio, Cat # 64SI1PEB, Bedford, USA) in absence and presence of 5mM nicotinamide (BDH Chemicals, Cat # 44068, Poole, England). The homogeneous time resolve fluorescence (HTRF) signal was measured with Synergy2 reader (BioTek, Winooski, USA) coupled with the Gen5 data analysis software (BioTek, Winooski, USA). Purified human recombinant His-SirT1 (Enzo Life Sciences, Cat # BML-SE239-0100, Plymouth, UK) was used to perform a standard curve in order to determine the enzymatic activity of each construct (Fig. S2).

SirT1 purification. *E. coli* were transformed with the plasmid pET28a-c(+) containing $His_{(6)}$-$SirT1^{wt}$(pKJ347) or $His_{(6)}$-$SirT1^{H355Y}$(pKJ348) were grown overnight in 1 ml LB broth[10g/L NaCl, 10g/L tryptone, 5g/L yeast extract pH 7.00] containing 0.05 mg/ml kanamycin. A 250µl fraction of the overnight culture was grown for an extra 2 hours in 5ml LB. SirT1

expression was induced for 2 hours by addition of 1mM IPTG. Cell were collected and lysed in lysis EA buffer (50mM HEPES pH 8.00, 300mM NaCl) containing 1mM β-mercaptoethanol, 0.5% NP-40 and protease inhibitor cocktail Free-EDTA (Roche,Cat # 11836170001, Mannhein, Germany) supplemented with 10mM imidazole. Cell lysates were sonicated and clarified by centrifugation at 14,000 rpm, 10min, 4°C. Total protein quantification was performed with the Bio-rad protein assay (Bio-rad laboratories inc., Cat # 500-0006, Mississauga, Canada). For each cell lysate, a 1mg/ml total protein stock solution was prepared. Histidine tagged SirT1 recombinant proteins were purified with cobalt beads as described by the manufacturer (Clontech laboratories inc. Cat# 635501, Mountain view, USA). The wash/equilibration buffer had the same composition as the lysis buffer and the elution buffer contain 150mM imidazole. Deacetylation assay was performed as described above.

***Statistical analysis*.** Linear regression, ANOVA and t-tests were performed with GraphPad Prism version 3.02 for Windows, GraphPad Software. Implementation of the linear mixed effect model was performed with R software version 2.11.1.

Chapter 3: Results

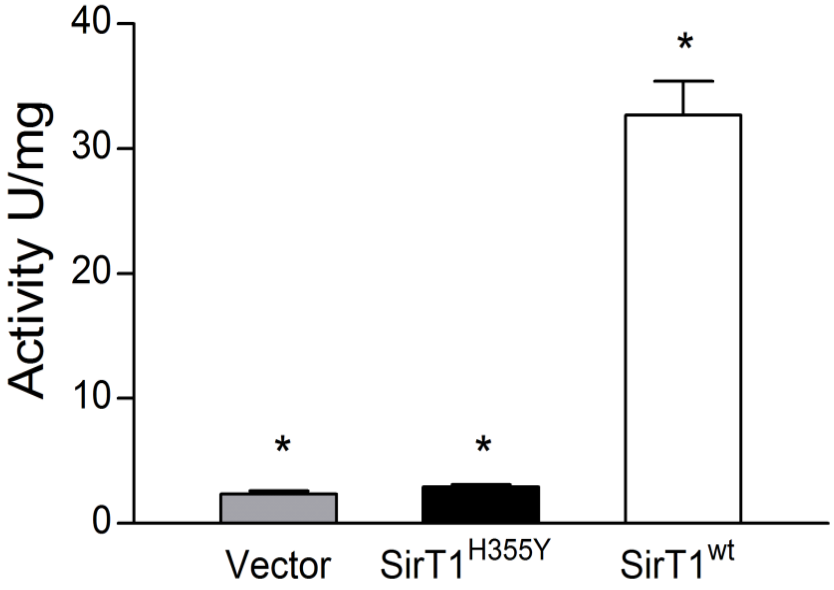
Catalytic activity of the SirT1^{H355Y} mutant in mammalian cells

To determine the catalytic activity of our SirT1^{H355Y} mutant protein, we transfected HEK293T cells with expression vectors encoding SirT1^{wt}, SirT1^{H355Y} or the control vector. We prepared stock solutions of known protein concentrations for each cell extract. Following proper dilution of the stock solution, we assessed the activity in these cell extracts in presence and absence of 5mM nicotinamide using homogenous time resolved fluorescence (HTRF) SirT1 deacetylase kit available from Cisbio Bioassays. Nicotinamide is used to inhibit sirtuin deacetylase activity and to distinguish this activity from that of endogenous HDAC type I and II activities against the acetylated p53 peptide used in the assay. Using this experimental design, we observed a 10 fold decrease in deacetylase activity in cell extracts transfected with our SirT1^{H355Y} construct relative to extracts from cells that got SirT1^{wt} ($P<0.001$) (Fig. 3A). Despite clear over-expression of SirT1^{H355Y} mutant (Fig. 3B), its signal remained undistinguishable from our control vector (Fig. 3A). We also verified the activity of our SirT1-GFP construct using the same methodology. The wild type SirT1-GFP had more than 30 fold greater activity relative to the SirT1^{H355Y}GFP construct (Fig. 4A and 4B). Based on the sensitivity obtained with our over-expressing constructs in the mammalian whole cell extract, we concluded that SirT1^{H355Y} mutant has less than 3% of the activity of the wildtype enzyme.

Figure 3. Enzymatic activity of the SirT1^{H355Y} point mutant expressed in mammalian cells.

A) Deacetylase activity of HEK293T whole cell lysates transfected with plasmid encoding wild type SirT1(white bar), SirT1 point mutant H355Y (black bar) or control vector (grey bar) (N=4). Signal was measured with SirT1 HTRF assay kit from Cisbio Bioassays in presence and absence of 5mM nicotinamide. *ANOVA Tuckey post hoc test revealed a statistical difference between SirT1 and both SirT1^{H355Y} and the control vector ($P<0.001$). B) Western blot analysis of SirT1 expression following construct transfection in HEK293T cells along with the actin loading control. 25μg of total protein was loaded for HEK293T transfected with control vector while only 1μg was loaded for SirT1 expressing vector. Longer exposure is required in order to visualize endogenous SirT1 in HEK293T cells form control vector and to visualized β-actin in HEK293T cells transfected with SirT1 constructs.

A


40
30
20
10
0
Activity U/mg
*
*
*
Vector
SirT1H355Y
SirT1wt

B


Vector
SirT1H355Y
SirT1wt
SirT1
actin

Catalytic activity of purified SirT1^{H355Y} from bacteria

To reduce the background signal from the endogenous sirtuins that we were observing in the HEK293T cell extract, we have generated (Karen Jardine) a SirT1 bacterial expression vector that contains a hexa-histidine tag ($His_{(6)}$) at the N-terminus of SirT1^{wt} and SirT1^{H355Y}. We were able to induce SirT1 expression in *E. coli* and purified both His-SirT1 and His-SirT1^{H355Y} with cobalt beads (Fig. 5A, B and C). A 400 fold difference ($P < 0.001$) in total activity between His-SirT1 and His-SirT1^{H355Y} was observed for the same amount of purified protein (Fig. 6A and B). The enzymatic activity of the mutant was undistinguishable from the non-induced control. With this analysis we were able to conclude that SirT1^{H355Y} mutant has less than 0.25% remaining activity compare with wild type.

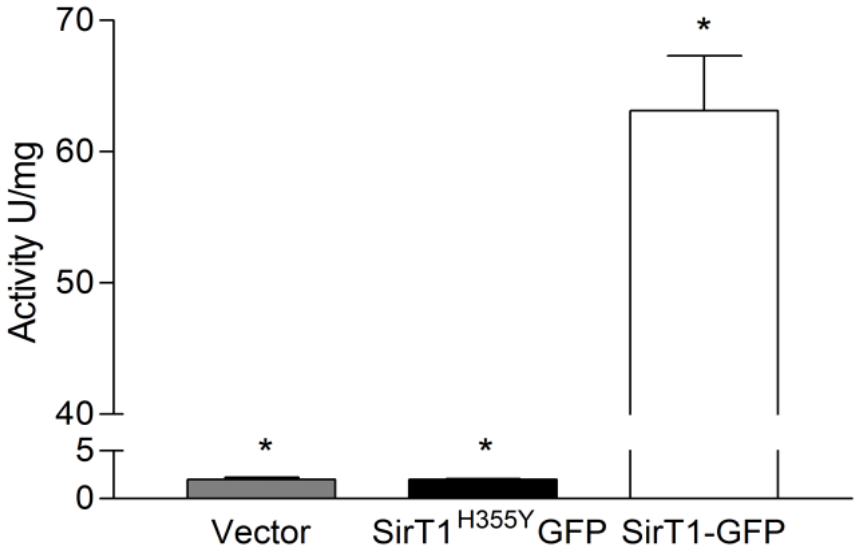
Deacetylase activity of SirT1$^{+/+}$ and SirT1$^{-/-}$ embryonic stem cells

We were next interested in evaluating endogenous levels of Sirtuins activity. We first assessed the deacetylation activity in mouse embryonic stem cells from the same origin that include the R1 line (SirT1$^{+/+}$) (Nagy et al., 1993) and the B6 line (SirT1$^{-/-}$). The B6 line was generated by knocking out both SirT1 alleles in R1 line by homologous recombination (McBurney et al., 2003b). ES cells were analyzed as they highly express SirT1 (Fig. S3A). Using these cell lines we were able to detect a Sirtuin-dependent (NAM inhibitable) signal in both R1 and B6 ES cells (Fig. 7). Interestingly, SirT1 positive cells have a significant 2 fold higher deacetylation activity ($P<0.01$) compare with SirT1 negative cells. This data suggests that SirT1 accounts for about half to the total Sirtuins activity in ES cells. We also tested different tissues to see if we were able to assess SirT1 activity.

Figure 4. Enzymatic activity of the SirT1^{H355Y}-GFP point mutant expressed in mammalian cells.

A) Deacetylase activity of HEK293T whole cell lysates transfected with plasmid encoding SirT1-GFP (white bar), SirT1-GFP point mutant H355Y (black bar) or control vector (grey bar) (N=4). Signal was measured with SirT1 HTRF assay kit from Cisbio Bioassays in presence and absence of 5mM nicotinamide. ANOVA Tuckey post hoc test revealed a statistical difference between SirT1, SirT1^{H355Y} and control vector ($P<0.001$). B) Western blot analysis of SirT1 expression following construct transfection in HEK293T cells along with the actin loading control. 25µg of total protein was loaded for HEK293T cells transfected with control vector while only 2µg was loaded for HEK293T cells transfected with SirT1 containing vector.

A


70
60
50
40
5
0
Activity U/mg
*
*
*
Vector
SirT1 H355Y GFP
SirT1-GFP

B

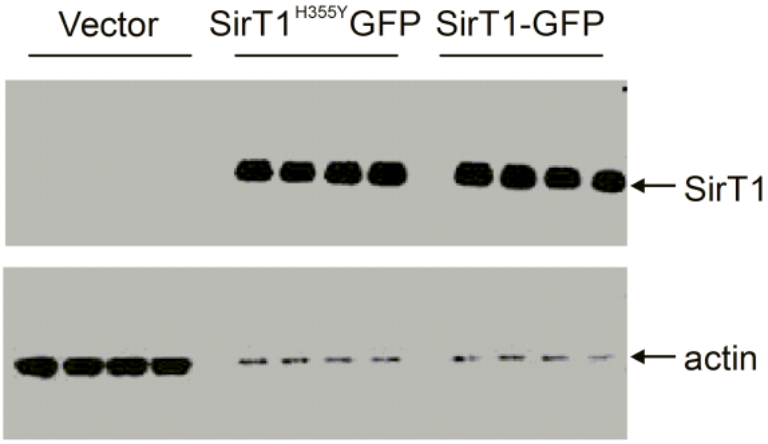

Vector
SirT1 H355Y GFP
SirT1-GFP
SirT1
actin

Figure 5. SirT1 expression and purification from bacteria.
A) Western blot analysis of SirT1 expression following IPTG induction of transformed *E. Coli* containing either $His_{(6)}$-$SirT1^{wt}$ or His(6)-$SirT1^{H355Y}$ plasmids (N=2). B) His tag purification of induced (IPTG(+)) and non induced(IPTG(-)) $SirT1^{wt}$ and C) $SirT1^{H355Y}$. NP = non purified, P = purified, S = Supernatant, W_1 = wash 1, W_2 = wash 2. Blot was probed for SirT1.

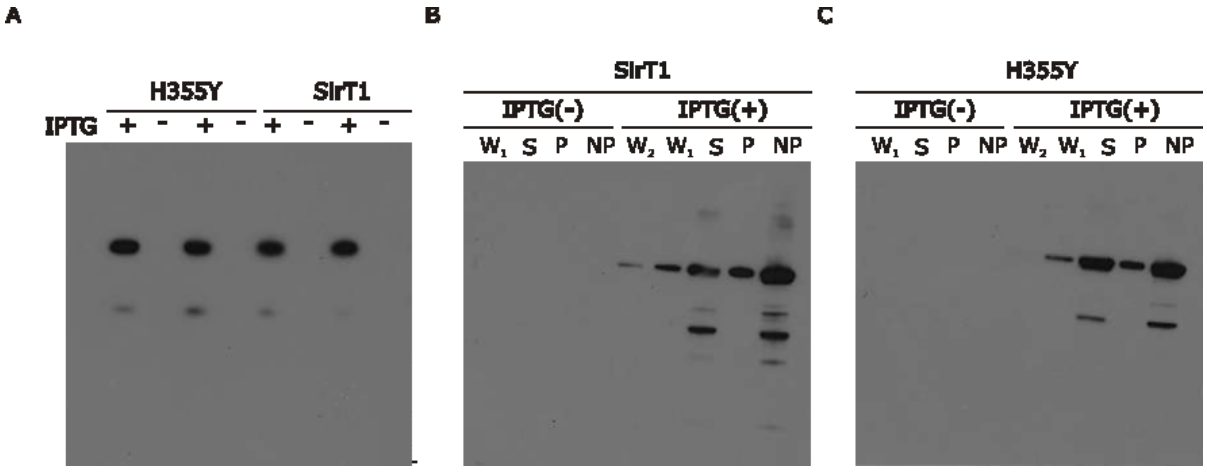
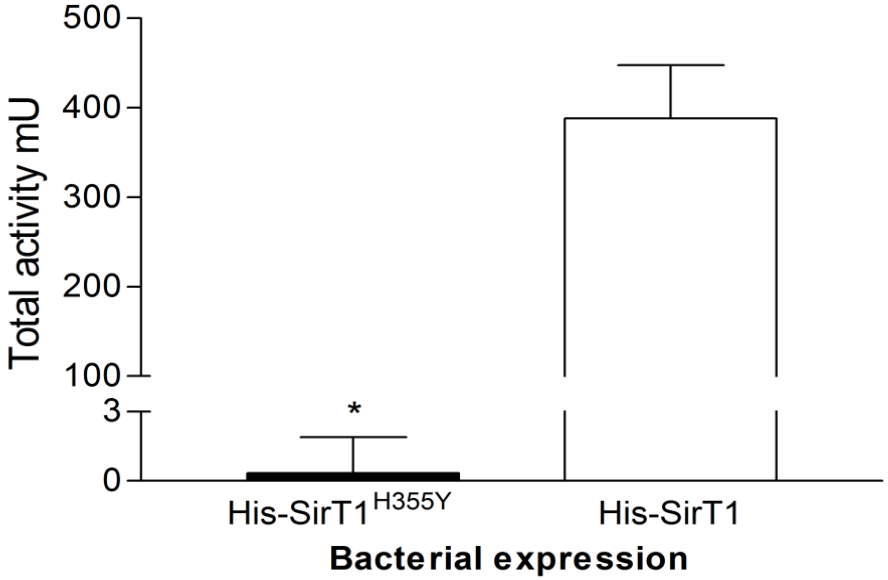

A
B
C
H355Y
SlrT1
IPTG + - + - + - + -
SlrT1
IPTG(-)
IPTG(+)
W1 S P NP W2 W1 S P NP
H355Y
IPTG(-)
IPTG(+)
W1 S P NP W2 W1 S P NP

Figure 6. Enzymatic activity of purified SirT1^{H355Y} point mutant expressed in bacteria. A) Deacetylase activity was measured on purified point mutant His$_{(6)}$-SirT1^{H355Y} (black bar) and His$_{(6)}$-SirT1 (white bar) (N=3) expressed in bacteria. Signal was measured with SirT1 HTRF assay kit from Cisbio Bioassays in presence and absence of 5mM nicotinamide. *Unpaired t-test with Welch's correction revealed a statistical difference between SirT1^{H355Y} and SirT1 ($P<0.01$). B) Western blot analysis of SirT1 protein purified from bacteria expressing either His$_{(6)}$-SirT1^{H355Y} or His$_{(6)}$-SirT1.

A


500
400
300
200
100
3
0
Total activity mU
*
His-SirT1 H355Y
His-SirT1
Bacterial expression

B

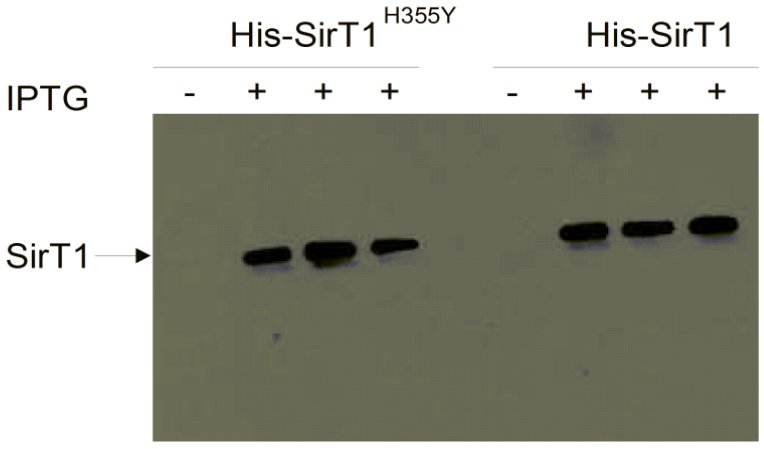
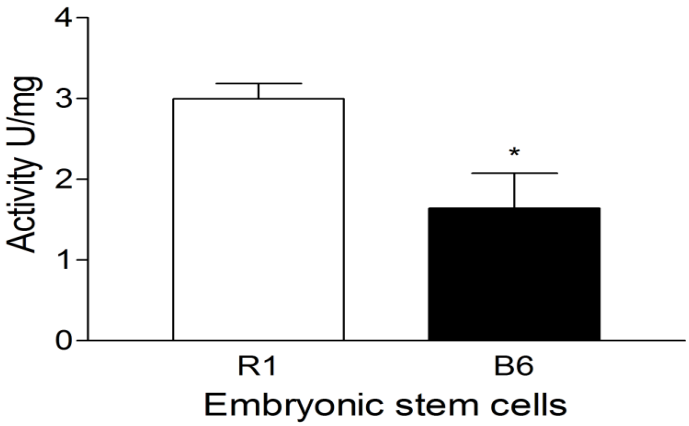

His-SirT1 H355Y
His-SirT1
IPTG
- + + + - + + +
SirT1

Figure 7. Enzymatic activity of endogenous Sirtuins.
A) Deacetylase activity of $SirT1^{+/+}$(R1, white bar) and $SirT1^{-/-}$(B6, black bar) from ES whole cells extract (N=4). Signal was measured with SirT1 HTRF assay kit from Cisbio Bioassays in presence and absence of 5mM nicotinamide. *Student t-test revealed a statistical difference between $SirT1^{+/+}$ and $SirT1^{-/-}$ ($P<0.01$).

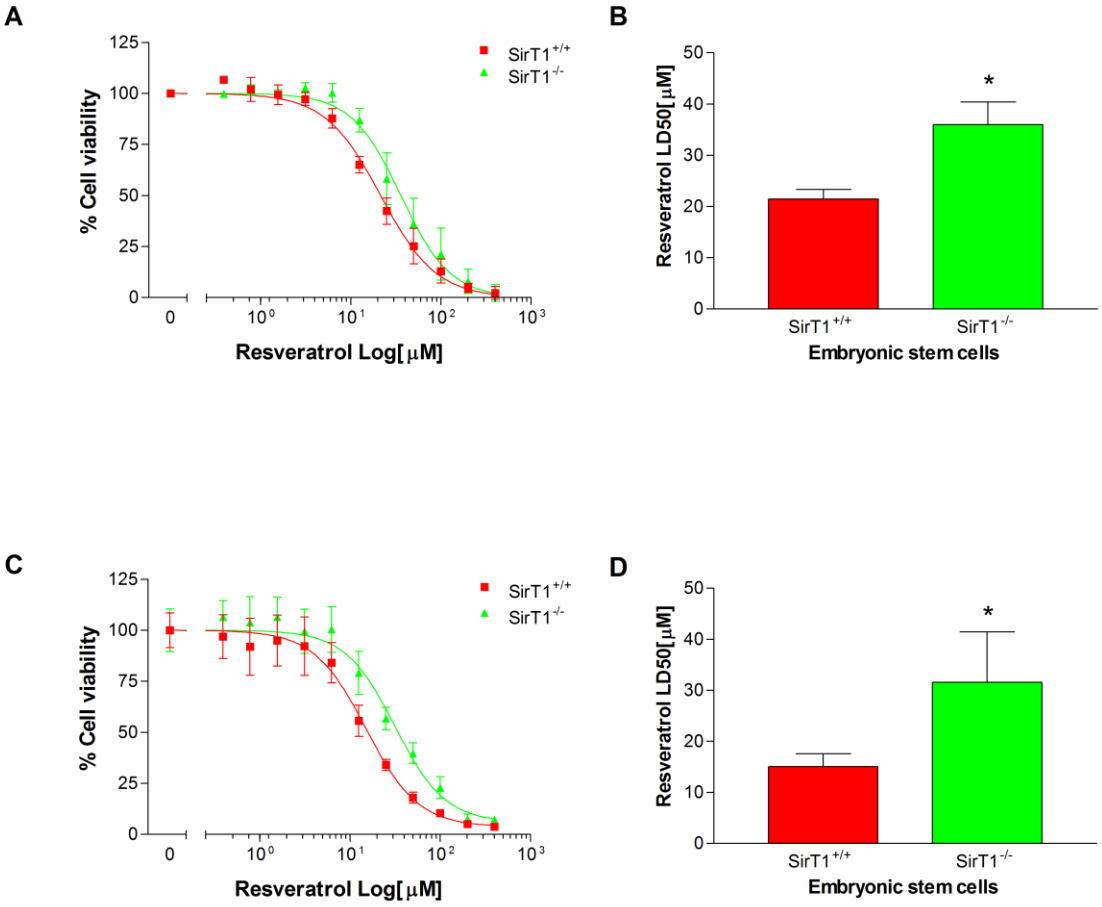

4
3
2
1
0
Activity U/mg
*
R1
B6
Embryonic stem cells

Unfortunately, we were not able to detect a differential signal between $SirT1^{+/+}$ and $SirT1^{Y/Y}$ (animal harboring $SirT1^{H355Y}$ mutation in both allele) in testis, epididymides and fetal liver (Fig. S4 A, B and C). Since, we were unable to detect a signal that could be attributed to SirT1 in these tissues; we concluded that the kit cannot be used to assess enzymatic activity in tissue.

Resveratrol toxicity in embryonic stem cells

Embryonic stem cells were used as a surrogate cancer cell model since they possess a transcriptional signature very similar to cancerous cells (Wong et al., 2008), and are well known to induce teratomas in immuno-compromised mice (Blum and Benvenisty, 2008). To evaluate the sensitivity of our cell lines towards resveratrol, we measured cell viability after 48 hours exposure to different drug concentration. The dose response curves obtained were used to calculate the dose at which 50% of the cells are killed (LD50). We have obtained resveratrol dose response curves for both R1 ES line ($SirT1^{+/+}$) (Nagy et al., 1993) and their B6 counterpart ($SirT1^{-/-}$) (McBurney et al., 2003b). Here, we observed that $SirT1^{+/+}$ cells are slightly more sensitive to this agent compare to $SirT1^{-/-}$ cells (t-test $P<0.05$) (Fig. 8A and B). In order to verify that SirT1 deacetylase activity was responsible for this difference, we performed resveratrol dose response curves in the presence of nicotinamide, a known inhibitor of SirT1 activity (Bitterman et al., 2002; Sauve and Schramm, 2003).

Figure 8. Resveratrol toxicity in mouse embryonic stem cells.
A) Resveratrol dose response curves and B) Lethal Dose 50 for $SirT1^{+/+}$ (red) and $SirT1^{-/-}$ (green) ES cells (N=3). C) Resveratrol dose response curves and D) Lethal Dose 50 in $SirT1^{+/+}$ (red) and $SirT1^{-/-}$ (green) ES cells in presence of 5mM nicotinamide (N=5). *Linear mixed effect model revealed a statistical difference between $SirT1^{+/+}$ and $SirT1^{-/-}$ in both absence and presence of nicotinamide ($P<0.05$).


A
% Cell viability
125
100
75
50
25
0
0
10^0
10^1
10^2
10^3
Resveratrol Log[μM]
SirT1+/+
SirT1-/-
B
Resveratrol LD50[μM]
50
40
30
20
10
0
*
SirT1+/+
SirT1-/-
Embryonic stem cells
C
% Cell viability
125
100
75
50
25
0
0
10^0
10^1
10^2
10^3
Resveratrol Log[μM]
SirT1+/+
SirT1-/-
D
Resveratrol LD50[μM]
50
40
30
20
10
0
*
SirT1+/+
SirT1-/-
Embryonic stem cells

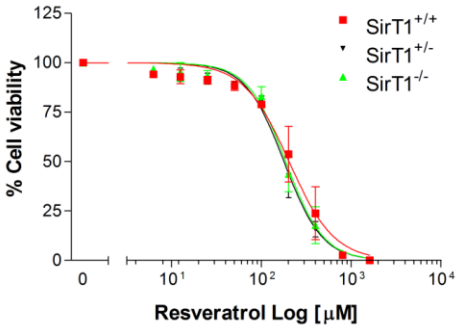
Nicotinamide dosage was determined by performing dose response curves in both R1 and B6 line (Fig. S5). Interestingly, nicotinamide did not decrease resveratrol sensitivity in $SirT1^{+/+}$ cells (Fig. 8C and D). This result, suggests that SirT1 protein is implicated in resveratrol toxicity but not its catalytic activity, or that the small differences in resveratrol sensitivity are not due to the mutations in the SirT1 genes but to some other differences in the 2 cells being compared.

Resveratrol toxicity in primary MEFs cells

Since cancer cells are more sensitive to resveratrol treatment compared to their normal counterpart, we next decided to evaluate its toxicity in normal primary MEFs cells derived from day 13 embryos. We obtained $SirT1^{+/+}$, $SirT1^{+/-}$ and $SirT1^{-/-}$ MEFs cells. We performed dose response curves in these cells at three different passages (P4 to P6). We did not detect any significant differences between genotypes at any passage (Fig. 9). We have also analyzed $SirT1^{+/-}$ and $SirT1^{-/-}$ MEFs that were immortalized but still at an early passages (P33 to P36) (Fig. 10). Confirmation of these cells was done by genotyping (Karen Jardine) and western blot analysis (Fig. S3C). Linear mixed effect model did not reveal a statistical difference in LD50 between $SirT1^{+/-}$ and $SirT1^{-/-}$ in either the presence or absence of nicotinamide (Fig. 10A, B, C and D). This data suggests that SirT1 is not implicated in resveratrol toxicity in normal primary MEFs.

Figure 9. Resveratrol toxicity in primary MEFs.
A) Resveratrol dose response curves and B) Lethal Dose 50 in primary MEFs SirT1$^{+/+}$ (red), MEFs SirT1$^{+/-}$ (black) and MEFs SirT1$^{-/-}$ (green) cells (N=3). Linear mixed effect model did not capture a statistical difference between genotypes.

A


125
100
75
50
25
0
% Cell viability
0
10^1
10^2
10^3
10^4
Resveratrol Log [μM]
SirT1$^{+/+}$
SirT1$^{+/-}$
SirT1$^{-/-}$

B

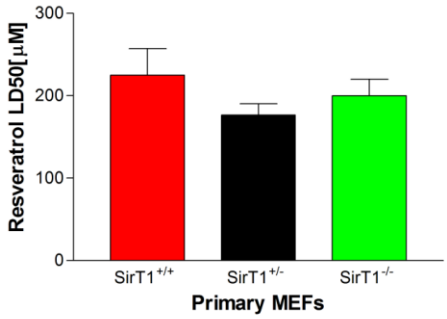
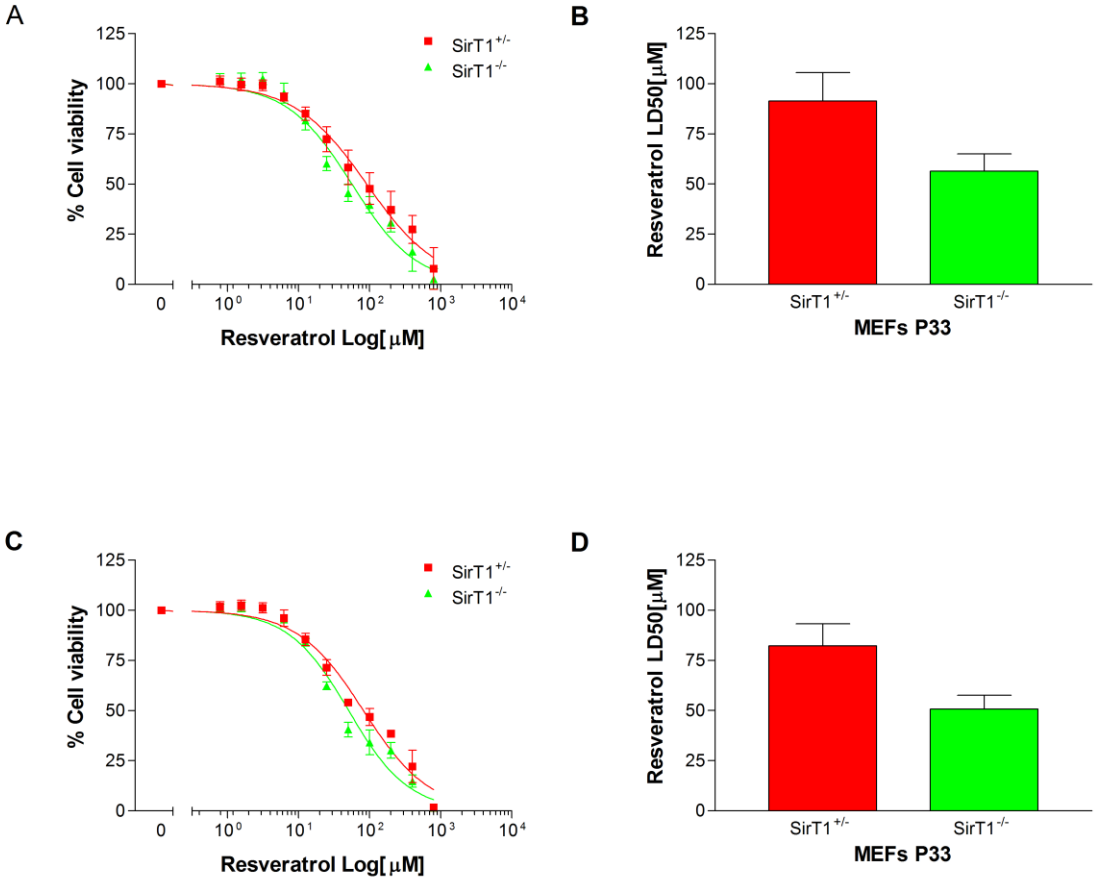

300
200
100
0
Resveratrol LD50[μM]
SirT1$^{+/+}$
SirT1$^{+/-}$
SirT1$^{-/-}$
Primary MEFs

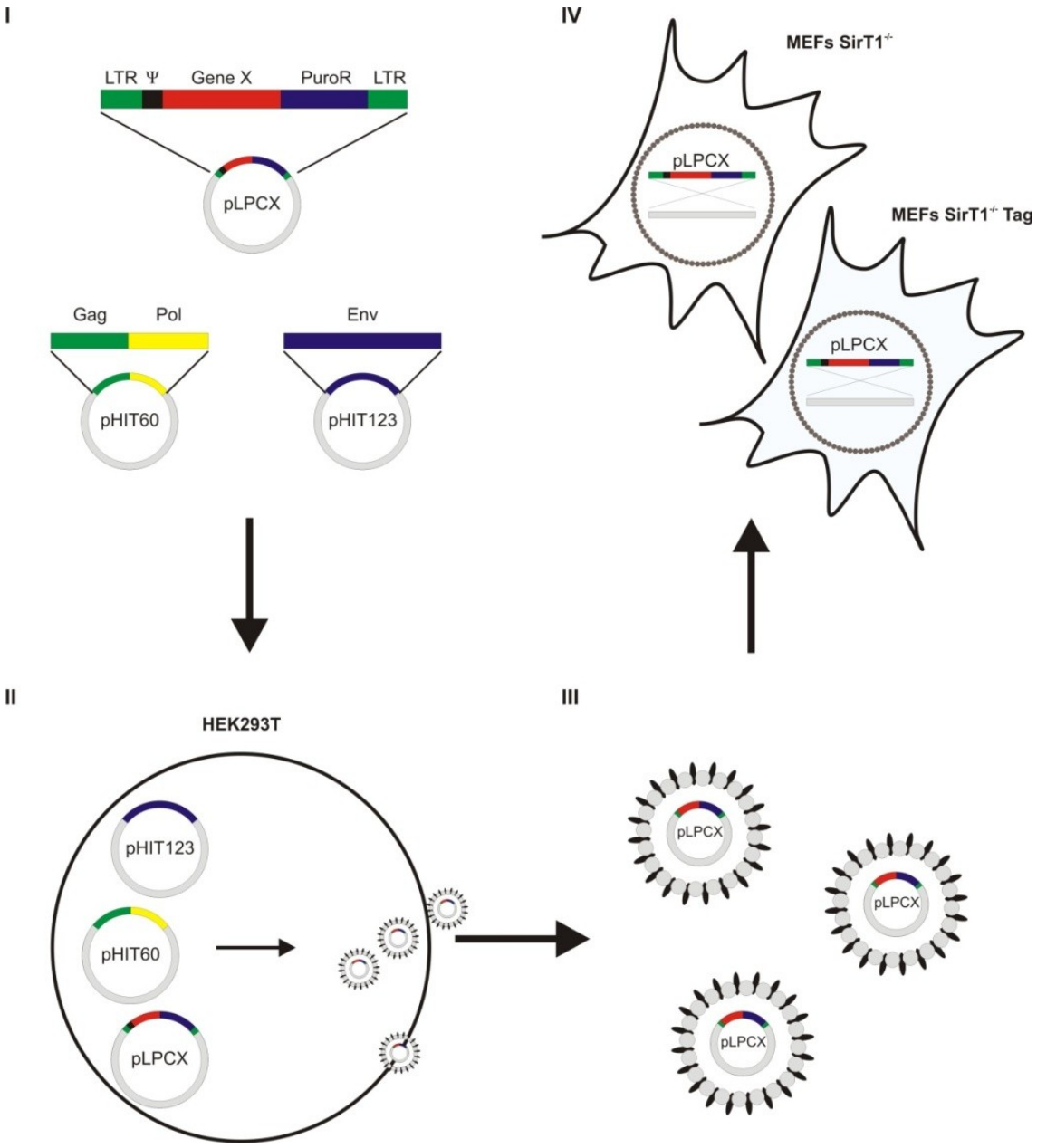
Figure 10. Resveratrol toxicity in low passage MEFs.
A) Resveratrol dose response curves B) Lethal Dose 50 in passage 33 MEFs SirT1$^{+/-}$ (red) and SirT1$^{-/-}$ (green) cells (N=3) in absence of nicotinamide. C) Resveratrol Dose response curves and D) Lethal Dose 50 in MEFs SirT1$^{+/-}$ (red) and SirT1$^{-/-}$ (green) cells (N=3) in presence of 5mM nicotinamide. Linear mixed effect model did not capture statistical difference between MEFs SirT1$^{+/-}$ and MEFs SirT1$^{-/-}$.


A
B
C
D
% Cell viability
Resveratrol Log[μM]
SirT1+/-
SirT1-/-
Resveratrol LD50[μM]
MEFs P33

Mouse embryonic fibroblast retro-viral mediated gene transformation

Given that resveratrol toxicity has been reported to be specific towards cancerous cells (Baarine et al., 2011; Clément et al., 1998; Dörrie et al., 2001; Lu et al., 2001), we next investigated the role of SirT1 in resveratrol toxicity in neoplastic transformed cells derived from the same origin. This was tested by restoring SirT1 protein into Mouse Embryonic Fibroblasts (MEFs) derived from SirT1 knockout embryos. To do so, we isolated $MEFs^{-/-}$ cells from a single knockout embryo and subcultured them until spontaneous immortalization occurred. Subsets of these cells were transfected with large T antigen (Tag), a viral protein from Simian vacuolating virus known to induce neoplastic transformation in MEFs cells (Ahuja et al., 2005). We confirmed the absence of SirT1 in $MEFs^{-/-}$ as well as the presence of Tag by western blot analysis (Fig. S3B). We were able to restore SirT1 and YFP in both "normal" and "transformed" MEFs referred from now on as MEFs Normal and MEFs Tag, respectively. MEFs cells are known to be difficult to transfect. To accomplish this challenging task, we have developed a retro-viral infection methodology. First, we transfect Human Embryonic Kidney cells (HEK293T) cells with plasmids harboring the structural genes (gag, pol, env) from murine leukemia virus (Fig. 11). This enables HEK293T cells to serve as a retroviral particle factory (Fig. 11). By co-transfecting these constructs along with plasmids harboring the encapsulate recognition site (Ψ), as well as the SirT1 or YFP gene between the viral 5' and 3' LTR, we enable the formation of a retroviral particle containing the gene of interest (Fig. 11). By co-culturing transfected HEK293T cells with Tag or Normal MEFs, we enable our fibroblasts to become infected by the viral particle, allowing for stable integration of the constructs into recipient cells (Fig. 11).

Figure 11. Retro-viral infection methodology in MEFs.
Roman numerals depict the steps involved the infection process. I) Plasmid used for the transfection. pLPCX contains the packaging signal Ψ, the puromycin resistant gene (PuroR) and the gene of interest X (SirT1 , $SirT1^{H355Y}$ or YFP) between the viral 5' and 3' long terminal repeat (LTR) regions. pHIT60 contains the murine leukemia viral gene gag, pol while pHIT123 encodes the viral env protein. II) The three plasmids are introduced in the packaging line (HEK293T cells) by calcium phosphate transfection. HEK293T cells are left to produce viral particles containing the gene of interest for 48hrs. III) MEFs $SirT1^{-/-}$ cells or MEFs $SirT1^{-/-}$ Tag cells are co-cultured with HEK293T for an extra 48 hrs. MEFs are selected over HEK293T cells by addition of ouabain (2-3days). IV) MEFs harbouring the provirus genome are selected by puromycin treatment.

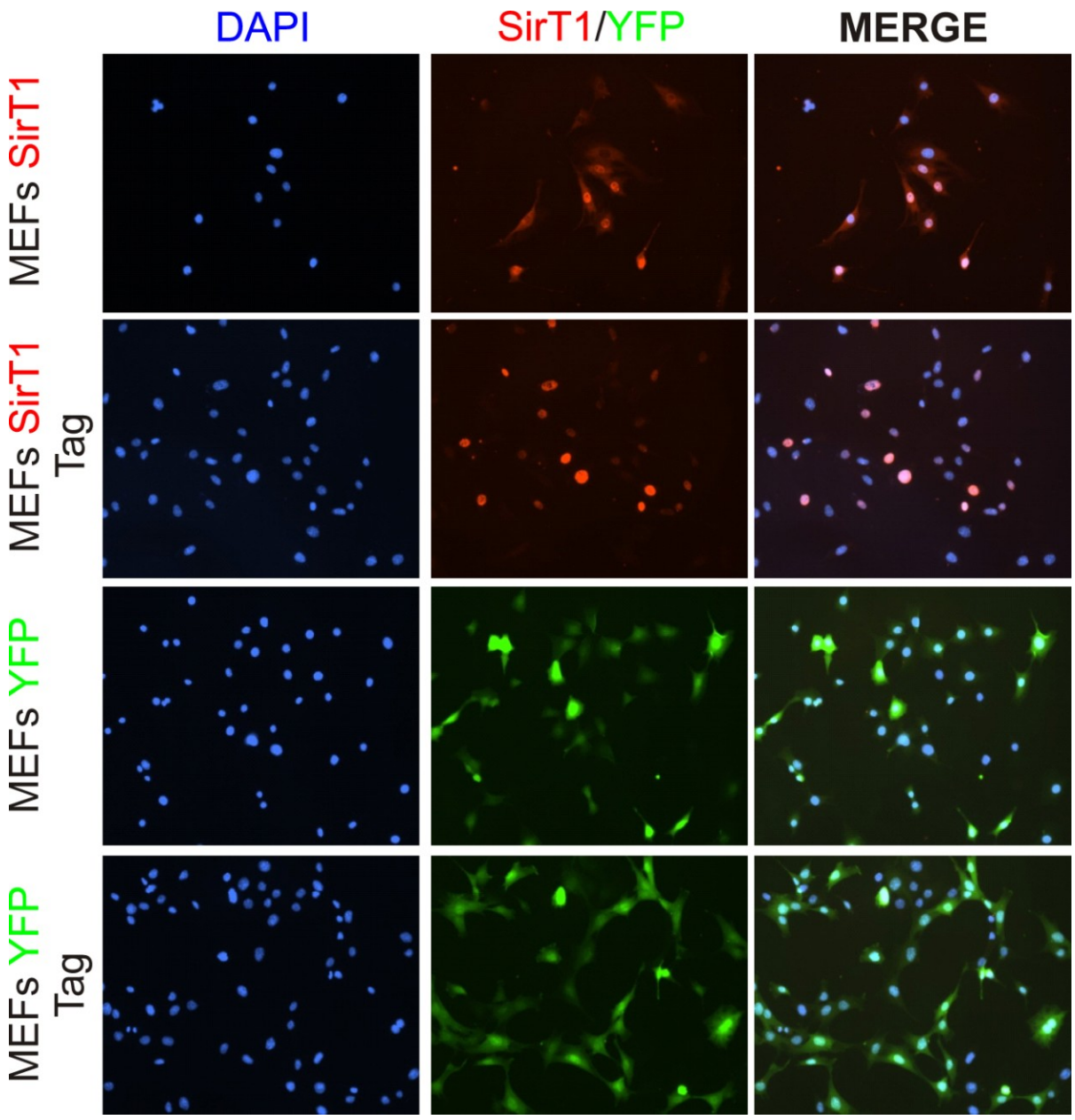

I
LTR Ψ
Gene X
PuroR
LTR
pLPCX
Gag
Pol
pHIT60
Env
pHIT123
II
HEK293T
pHIT123
pHIT60
pLPCX
III
pLPCX
pLPCX
pLPCX
IV
MEFs SirT1-/-
pLPCX
MEFs SirT1-/- Tag
pLPCX

The result in turn allows for the generation of 4 MEFs strains derived from an isogenic population. MEFs SirT1 and MEFs SirT1 Tag have SirT1 expression restored, while MEFs YFP and MEFs YFP Tag rather express the YFP. To eliminate residual HEK293T cells from the co-culture, we utilize ouabain selection since human cells are more sensitive to this agent relative to their mouse counterparts. Further puromycin selection is required to select for cells carrying and expressing the retroviral provirus. Subsequently, immunofluorescence staining was performed to confirm that SirT1 and YFP expression was restored in at least 70% of the population (Fig. 12). Importantly, SirT1 localization remained in the nucleus and excluded from the nucleolus (Fig. 12) as also seen in $MEFs^{wt}$ (Fig. S6) and as previously reported (McBurney et al., 2003b). As expected, YFP expression is found throughout the cells (Fig. 12). We have verified the specificity of our SirT1 antibody by staining $MEFs^{wt}$ and $MEFs^{-/-}$ and observed strong Sirt1-specific nuclear staining in WT cells but weak cytoplasmic staining in both $MEFs^{wt}$ and $MEFs^{-/-}$ associated with a non specific signal (Fig. S6).

Resveratrol toxicity in infected mouse embryonic fibroblast

To confirm the results obtained in ES and MEFs cells, we performed resveratrol dose response curves in both normal and transformed MEFs cells generated by retro-viral infection. No difference was observed between cells infected with SirT1 or YFP in the "Normal" MEFs background (Fig. 13A and B).

Figure 12. SirT1 expression in MEFs following retroviral infection.
(Top) MEFs SirT1 and SirT1 Tag are confirmed to express the SIRT1 protein through staining with SirT1 antibody (red). (Bottom) MEFs YFP and YFP Tag expression is confirmed through YFP fluorescence (green). DNA is counter stained with DAPI (blue). In the above example, SirT1 expression is observed in ~50% of the cells while YFP is observed in over 80% of the cells.


DAPI
SirT1/YFP
MERGE
MEFs SirT1
MEFs SirT1 Tag
MEFs YFP
MEFs YFP Tag

However, we did observed a significant difference ($P<0.005$) between SirT1 and YFP in the MEFs Tag background (Fig. 13C and D). To confirm that MEFs SirT1 Tag cells are more sensitive to resveratrol than MEFs YFP Tag, we performed resveratrol dose response curve in presence of nicotinamide. Nicotinamide concentration was determined by performing dose response curves in $MEFs^{wt}$, $MEFs^{-/-}$ and $MEFs^{-/-}$ Tag (Fig. S7).We selected a dose at which 5-10% of the cells were killed (Fig. S7). If SirT1 catalytic activity was implicated in resveratrol toxicity in MEFs Tag cells, it was expected that SirT1 inhibition by nicotinamide would increase their LD50. Again, nicotinamide treatment did not modulate the sensitivity of SirT1 positive cells (Fig. 13E and F). In corroboration with our previous findings in ES cells, this results, suggests that SirT1 protein, but not its catalytic activity, is implicated in resveratrol toxicity in cancer cells models.

To confirm that these effects are associated with SirT1 and not secondary mutations, we repeated the retro-viral infection. This experiment equally included the catalytic dead mutant $SirT1^{H355Y}$. Again, we have successfully restored protein expression and localization and generated 6 different cells lines which include: MEFs SirT1, MEFs $SirT1^{H355Y}$, MEFs YFP and their MEFs Tag counterpart (Fig. 14).

Figure 13. Resveratrol toxicity in the first round of infected MEFs.
A, C and E) Resveratrol dose response curves and B, D and F) Lethal Dose 50 across cell lines. Measurements were performed across (A/B) MEFs SirT1(red) and YFP (green) cells (N=3), (C/ D) MEFs SirT1 Tag(red) and MEFs YFP Tag(green) cells (N=3), and (E/ F) MEFs SirT1 Tag(red) and MEFs YFP Tag(green) cells in absence(solid line) and presence(dashed line) of 5mM nicotinamide (N=5). *Linear mixed effect model identified a statistical difference between MEFs SirT1 Tag and MEFs YFP Tag in both the presence and absence of nicotinamide ($P<0.005$).

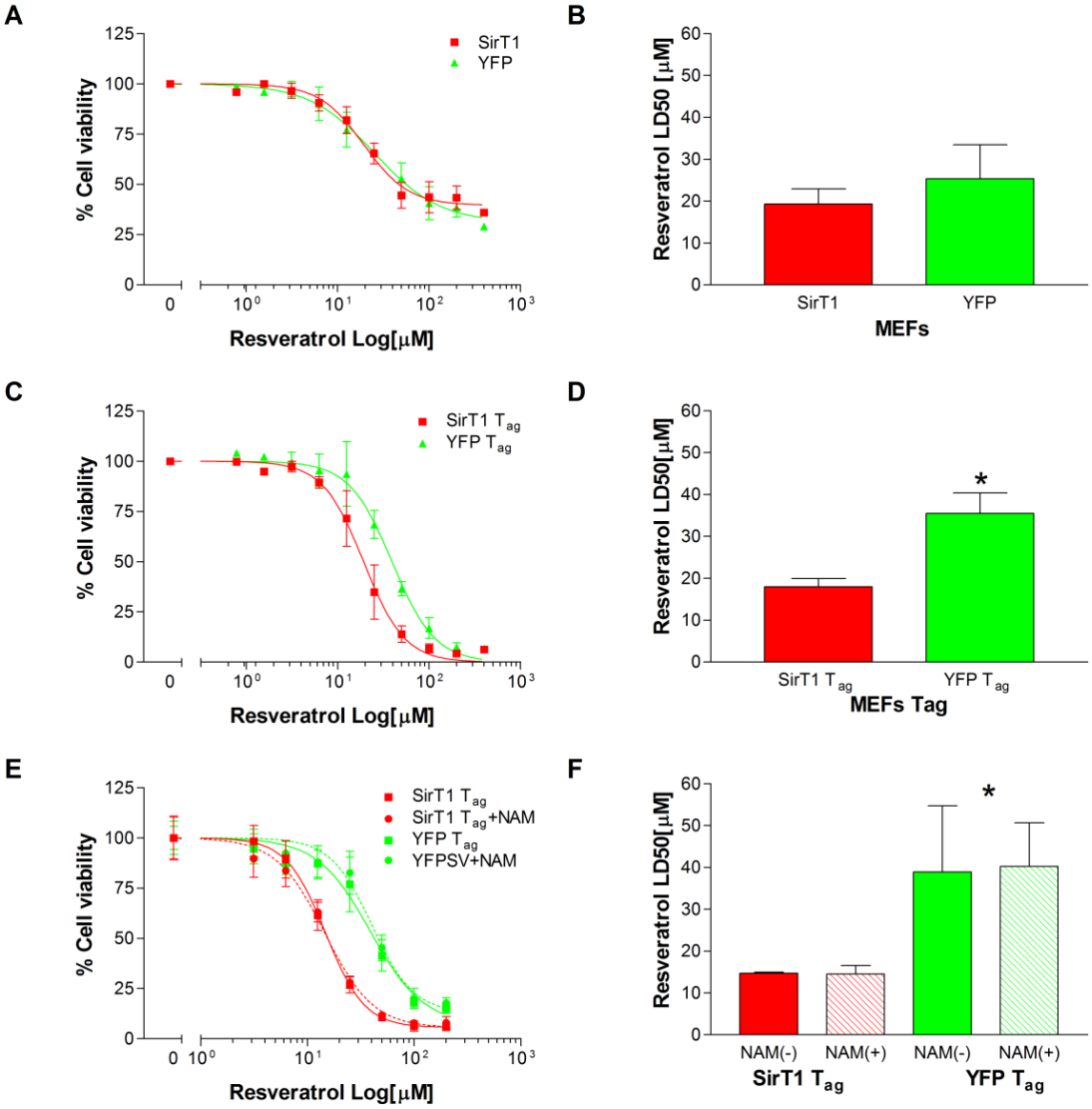
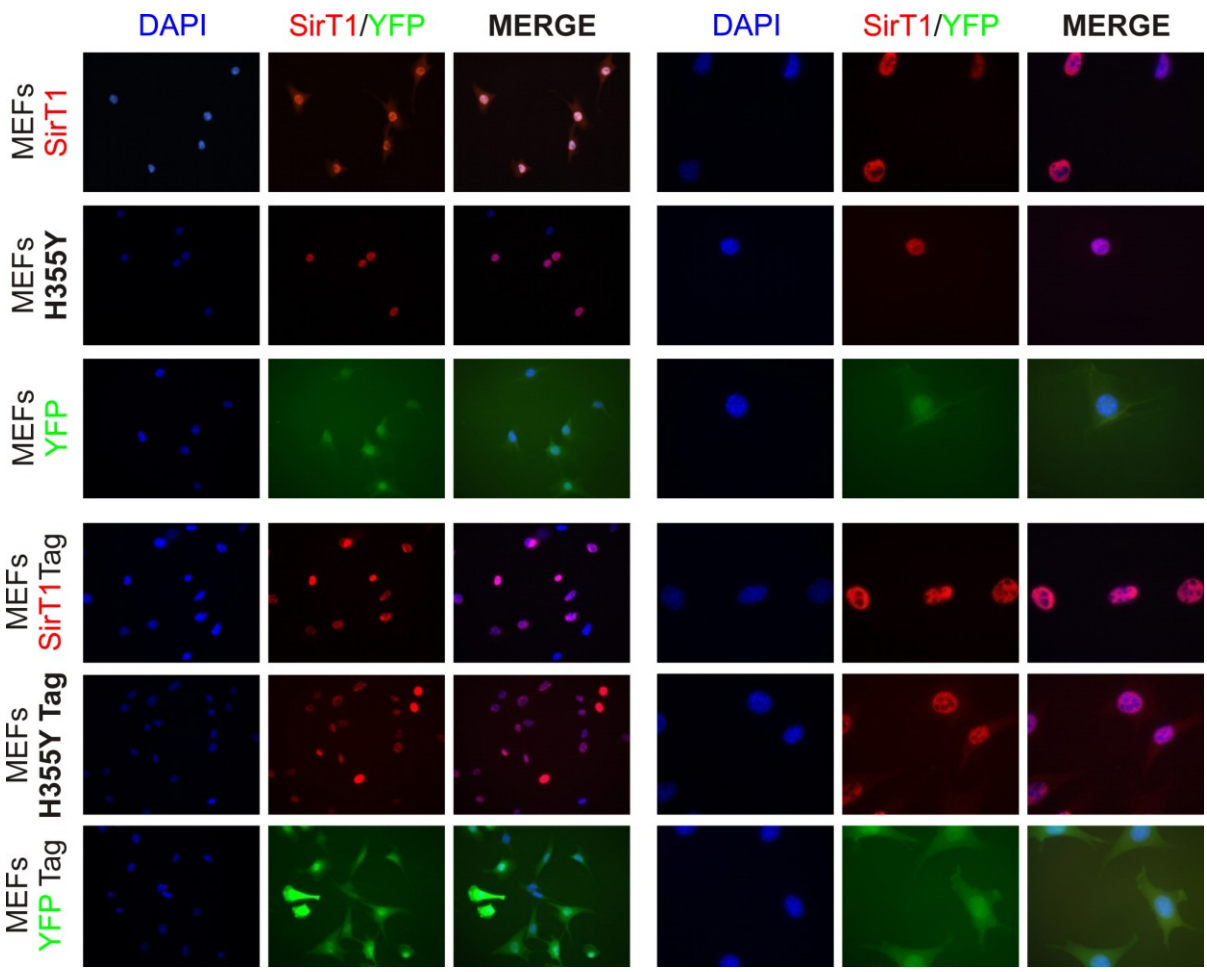

A
% Cell viability
SirT1
YFP
Resveratrol Log[μM]
B
Resveratrol LD50 [μM]
SirT1
YFP
MEFs
C
% Cell viability
SirT1 T ag
YFP T ag
Resveratrol Log[μM]
D
Resveratrol LD50[μM]
*
SirT1 T ag
YFP T ag
MEFs Tag
E
% Cell viability
SirT1 T ag
SirT1 T ag+NAM
YFP T ag
YFPSV+NAM
Resveratrol Log[μM]
F
Resveratrol LD50[μM]
*
NAM(-)
NAM(+)
NAM(-)
NAM(+)
SirT1 T ag
YFP T ag

Figure 14. SirT1 expression in the second round of infected MEFs.
MEFs SirT1 (Top, red), MEFs H355Y (Top, black), MEFs YFP (Top, green), or MEFs SirT1 Tag (Bottom, red), MEFs H355Y Tag (Bottom, black) and MEFs YFP Tag (Bottom, green) expression is confirmed through SirT1 antibody staining or YFP fluorescence following counter staining DNA with DAPI (blue). 40X magnification are represented on the left side of the panel and 100X magnification on the right side.


DAPI
SirT1/YFP
MERGE
DAPI
SirT1/YFP
MERGE
MEFs SirT1
MEFs H355Y
MEFs YFP
MEFs SirT1Tag
MEFs H355Y Tag
MEFs YFP Tag

Using these constructs, we did not observe any differences in resveratrol toxicity between SirT1, SirT1^{H355Y} and YFP in "Normal" MEFs (Fig.14A and B), but we found no difference between MEFs SirT1 Tag and MEFs YFP Tag (Fig. 15C and D). Given that generation of these cell lines required several steps and rounds of selection, we think each cell line undergoes their own evolution and that secondary mutations might explain the original difference observed in the first set of infected MEFs (Fig. 13C, D, E and F).

Resveratrol toxicity increases with passage number in MEFs.

Resveratrol is known to preferentially induce cell death in cancer cells compare to normal cells (Baarine et al., 2011; Clément et al., 1998; Dörrie et al., 2001; Lu et al., 2001). Throughout this study we have investigated resveratrol toxicity in MEFs cells at different passage (P4, P33 and P>100). While we observe an increase in sensitivity as the cells immortalized (Fig. 16), this process was not dependent on the SirT1 protein as seen with SirT1$^{+/}$ (included SirT1$^{+/+}$ and SirT1$^{+/-}$ cells) and SirT1$^{-/-}$ cells (Fig. 16A, B, C and D). Since the process of sensitization to resveratrol does not depend on SirT1, we concluded that SirT1 is not implicated in resveratrol toxicity in MEFs cells.

Figure 15. Resveratrol toxicity in MEFs infected cells with a catalytic dead mutant.
(A/C) Resveratrol dose response curves and (B/D) Lethal Dose 50. Measurements were performed across (A/B) MEFs SirT1(red), MEFs SirT1^{H355Y} (black) and MEFs YFP(green) (N=3). (C/D) MEFs SirT1 Tag(red), MEFs SirT1^{H355Y} Tag(black) and MEFs YFP Tag(green) (N=3). Linear mixed effect model did not capture a statistical difference between genotype in both normal and transformed cells.

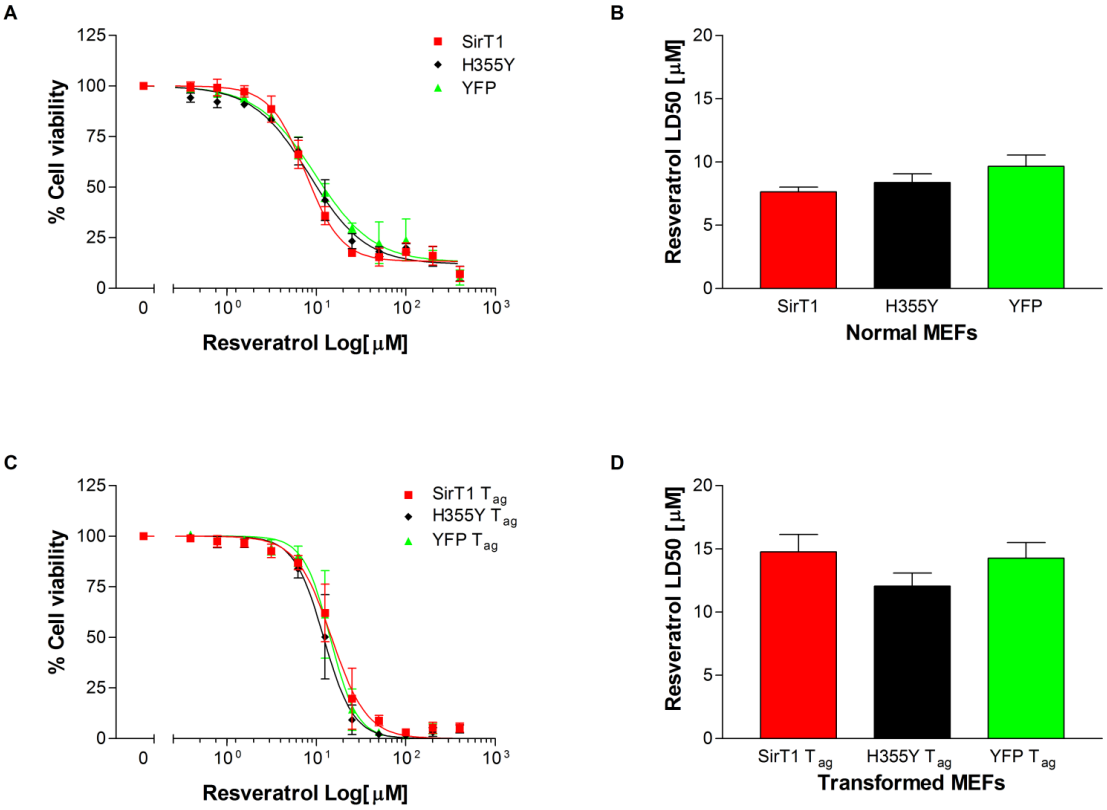
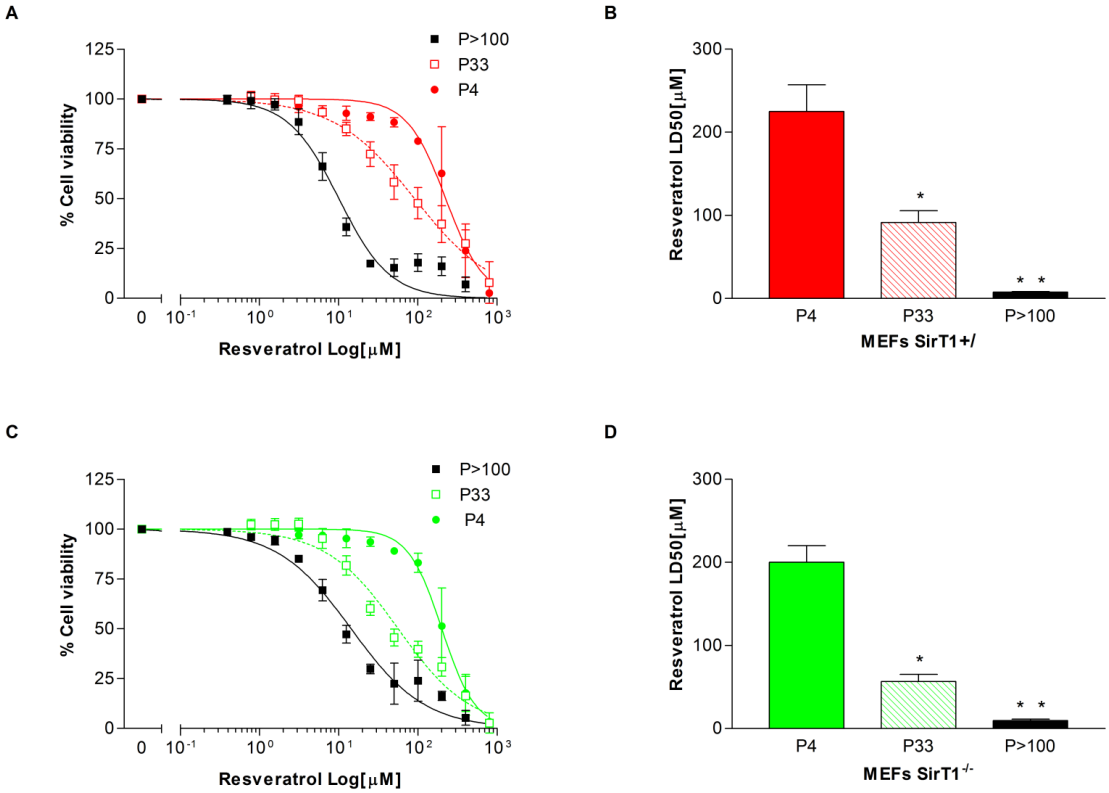

A
% Cell viability
125
100
75
50
25
0
0
10^0
10^1
10^2
10^3
Resveratrol Log[μM]
SirT1
H355Y
YFP
B
Resveratrol LD50 [μM]
20
15
10
5
0
SirT1
H355Y
YFP
Normal MEFs
C
% Cell viability
125
100
75
50
25
0
0
10^0
10^1
10^2
10^3
Resveratrol Log[μM]
SirT1 T_{ag}
H355Y T_{ag}
YFP T_{ag}
D
Resveratrol LD50 [μM]
20
15
10
5
0
SirT1 T_{ag}
H355Y T_{ag}
YFP T_{ag}
Transformed MEFs

Figure 16. Resveratrol sensitivity increase with passage number.
A) Dose response curves and B) Lethal Dose 50 in MEFs SirT1$^{+/}$ at passage P4 (red), P33 (open red circle) and P>100 (black). C) Dose response curves and D) Lethal Dose 50 in MEFs SirT1$^{-/-}$ at passage P4 (green), P33 (open green circle) and P>100 (black). ANOVA Tuckey post hoc test revealed statistical difference between passages in both MEFs SirT1$^{+/}$ and MEFs SirT1$^{-/-}$ ($P<0.01$).


A
P>100
P33
P4
% Cell viability
Resveratrol Log[μM]
B
Resveratrol LD50[μM]
P4
P33
P>100
MEFs SirT1+/
C
P>100
P33
P4
% Cell viability
Resveratrol Log[μM]
D
Resveratrol LD50[μM]
P4
P33
P>100
MEFs SirT1-/-

Chapter 4: Discussion

In the literature, it has been demonstrated that Sirtuins from unicellular organisms require a conserved histidine residue in their catalytic domain in order to perform their deacetylation reaction (Hoff et al., 2006; Jackson et al., 2003; Min et al., 2001; Smith and Denu, 2006). While this hypothesis is well supported in lower kingdoms, the impact of this mutation has not been adequately tested in higher organisms. We therefore tested the deacetylase activity of the mammalian SirT1 histidine 355 to tyrosine mutation. Our results, based on the deacetylation of lysine 382 of a p53 derived peptide, clearly shows that the deacetylase activity is abrogated by the mutation. This result is consistent with the results generated by Luo J et al. who have tested the level of p53 acetylation in transfected H1299 cells with a mouse SirT1 histidine to alanine mutation (Luo et al., 2001). To ensure that the mutation does not impact post-translational modifications or protein processing, we have expressed the protein in both mammalian and bacterial systems. Given that we have observed the same phenomena between both systems, this result clearly indicates that processing or post-translational modifications do not account for the loss of catalytic activity observed in the mutant. While the histidine residue has not been implicated in substrate binding, but only in the resolution of the intermediate product during catalysis (Hawse et al., 2008; Hoff et al., 2006), our result indicates that the histidine mutation is a general mechanism responsible for Sirtuins activity. We believe that this loss-of-function mutation likely abrogates SirT1 activity on every substrate.

Resveratrol given at a high concentration is known to induce cell death by different mechanisms such as apoptosis, autophagy and necrosis (Pervaiz and Holme, 2009).

Interestingly, many have reported increased toxicity specifically towards cancer cells (Baarine et al., 2011; Clément et al., 1998; Dörrie et al., 2001; Lu et al., 2001). Resveratrol beneficial effects have been attributed to its activation of the deacetylase SirT1(Howitz et al., 2003b), a protein that has been reported to be toxic when heavily overexpressed (Alcendor et al., 2007; Kakefuda et al., 2009). As such, we have investigated the role of SirT1 in resveratrol toxicity across both normal and neoplastic transformed cells since SirT1 may also have a function in cancer. We were expecting to see a SirT1 dependent effect based on the model where hyper-activation of SirT1 by resveratrol cause cytotoxicity.

We first evaluated resveratrol toxicity in ES cells. This model was selected as readily available and has been previously utilized to study the function of SirT1 in our laboratory (McBurney et al., 2003a). Using this cell line we have found that SirT1 positive cells are more sensitive to resveratrol than SirT1 negative cells. Surprisingly, this phenomenon was not dependent upon SirT1 deacetylase activity. In corroboration, some papers have reported deacetylase independent function of SirT1 (Campagna et al., 2011; Pfister et al., 2008; Vaitiekunaite et al., 2007). Furthermore, we have generated a mouse strain that carry a point mutation in the catalytic core domain of SIRT1 gene. This point mutant encodes for a catalytic dead SirT1 protein (H355Y). These mice possess most but not all abnormalities of the SirT1 knockout mice suggesting that SirT1 has deacetylase independent functions (Seifert et al., 2011). That might represent a novel function of SirT1 protein not associated with its NAD-dependant catalytic activity.

Recently, two groups have investigated the role of SirT1 in resveratrol toxicity in cancer cells (Pizarro et al., 2011; Singh et al., 2011). Depending on the methodology and cell line used to assess the implication of SirT1, they have reached different conclusion.

Pharmacological inhibition of SirT1 with sirtinol or nicotinamide did not reverse apoptosis in neuroblastoma cells and the authors concluded that SirT1 was not implicated in resveratrol toxicity (Pizarro et al., 2011). In contrast, siRNA knockdown reduce the severity of apoptosis observed in lymphoma cells which leads to the conclusion that SirT1 was in part responsible for resveratrol toxicity through inhibition of NF-κB (Singh et al., 2011). Reconciliation between their interpretations is possible if we consider that SirT1 role in resveratrol toxicity is independent of is deacetylase activity.

Since resveratrol toxicity is dependent on the cancerous status of the cells (Baarine et al., 2011; Clément et al., 1998; Dörrie et al., 2001; Lu et al., 2001), we have analyzed non cancerous primary and low passages MEFs cells. Interestingly, we have found that SirT1 did not modify resveratrol toxicity in these cells. Mader I et al. equally did not observed an implication of SirT1 in resveratrol mediated apoptosis in preadipocytes, a resveratrol sensitive normal cell line, which support our findings (Mader et al., 2010). These results suggest a cancer specific role of SirT1 in resveratrol toxicity. SirT1 function in cancer is currently highly dispute in the literature. Based on *in vivo* data, SirT1 is not required for the process of carcinogenesis (Boily et al., 2009a). However, SirT1 has pro-survival function under stress and it is conceivable that some cancer cells depend on SirT1 for their survival. For example, cancerous cells within the core of the tumor often have to deal with reduce oxygen level. SirT1 is a redox sensitive protein that might get activate or over-expressed under these conditions. That will be consistent with the pro-survival or oncogenic function attribute to SirT1 in cancer cells and with SirT1 serving as the nexus between resveratrol and

its chemotherapeutic functions. However, this phenomenon might depend on the specific events that lead to cancer cells adaptation.

In order to test the cancer specific effect of SirT1 in the same genetic background we have also re-introduced by retro-viral infection SirT1 gene in MEFs SirT1$^{-/-}$ cells transformed or not with the large T-antigen (Tag). By comparing these results to those in the untransformed MEFs, we sought to demonstrate that the process of transformation increased sensitivity towards resveratrol in a SirT1 dependant manner. We performed the retro-viral infection procedure twice with and without a catalytic dead mutant (SirT1^{H355Y}) and we confirmed that SirT1 is not implicated in resveratrol toxicity in normal MEFs. However, we have obtained contradictory results as to whether or not SirT1 is implicated in resveratrol toxicity in infected MEFs Tag cells. Results from our first set of infected MEFs Tag first suggested that SirT1 conferred sensitivity to transformed cells but this phenomenon was not reversed by nicotinamide treatment. This conclusion is identical to that previously obtained using ES cells. To further confirm this result, we repeated our analysis using our catalytic dead mutant SirT1^{H355Y}. Results from our second set of infected MEFs Tag did not show any difference between MEFs SirT1 Tag, a catalytic dead mutant SirT1^{H355Y} Tag and YFP Tag. The fact that we did not reproduce the difference between MEFs SirT1 Tag and MEFs YFP Tag suggests that SirT1 is not implicated in resveratrol toxicity. As such, secondary mutations might explain the previous difference observed. Indeed, upon further investigation, we observed that the dose response curves performed in MEFs$^{-/-}$ and MEFs$^{-/-}$ Tag before they have been infected with the SirT1, or YFP constructs, indicate that MEFs YFP Tag LD50 double compare to the original cells (Fig 10 C and D and Fig S8 A, B). This result inferred that MEFs YFP Tag possess a secondary mutation that confers on them increased resistance

to resveratrol, and that the difference observed in the first set of infected cells was not due to SirT1. Since we were not able to reproduce the phenomenon observed in ES cells with our isogenic model of infected MEFs, we concluded that the implication of SirT1 in resveratrol toxicity is not a general mechanism and might represent a cell type specific event. However, it is important to keep in mind that generation of B6 cell line required the knockout of each SirT1 allele one by one. This process required that B6 cells undergo two rounds of selection. It is conceivable that the effect seen is an artifact of B6 cell line.

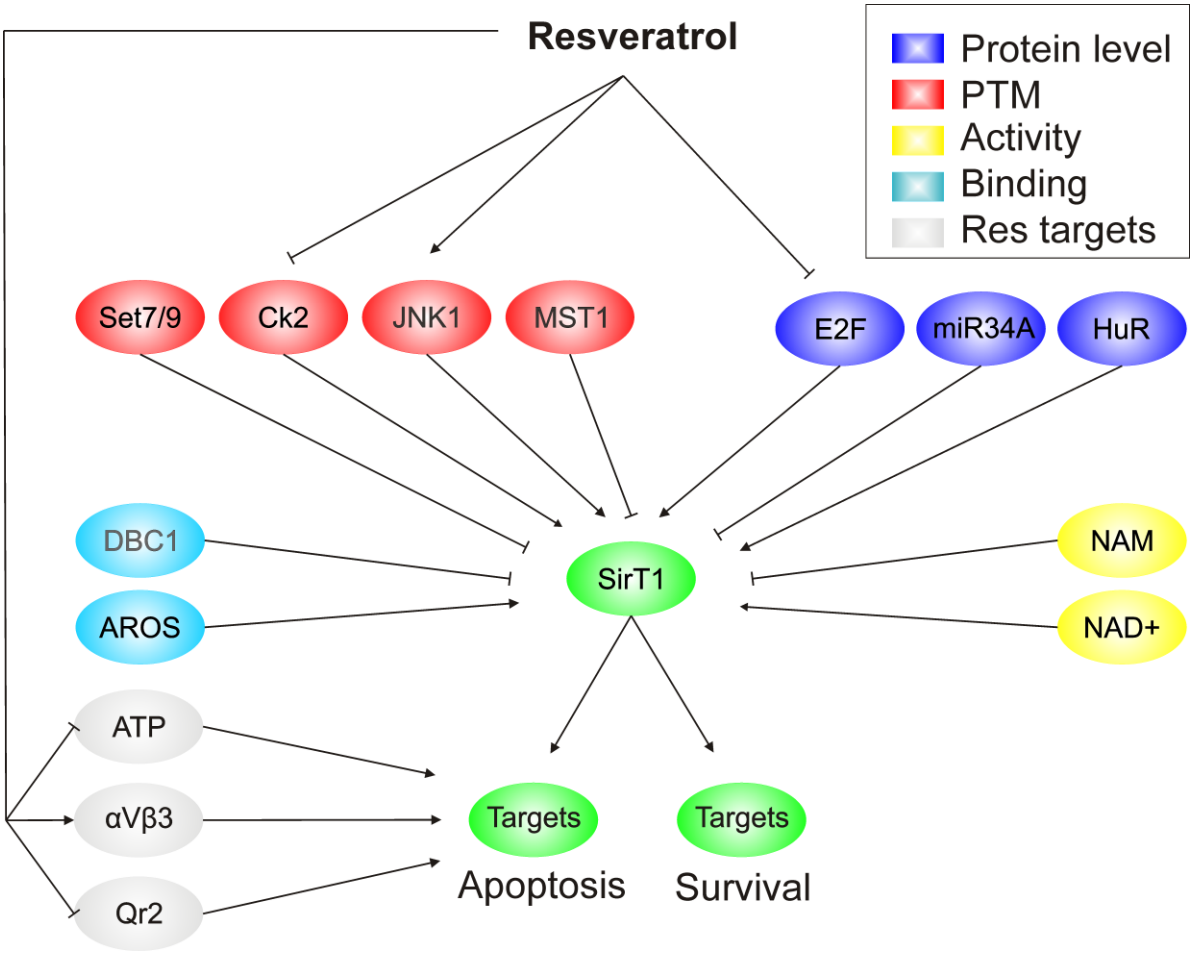
With our model of infected cells we did not observe a differential sensitivity between MEFs "normal" and MEFs Tag, however the MEFs$^{-/-}$ used for this experiment has been immortalized and extensively sub-culture. It is in turn possible that genetic alteration had happen in these cells and that the presence of the large T antigen did not confer any further transformation. When we compare MEFs sensitivity as a function of cell passage number, we observe that the process of immortalization and accumulation of genetic alterations does indeed increase sensitivity towards resveratrol. This process was independent of SirT1. If SirT1 is the primary target of resveratrol toxicity in cancer cells, the process of immortalization should not display increased sensitivity towards resveratrol treatment in absence of SirT1. We in turn rejected the hypothesis that SirT1 is implicated in resveratrol mediated toxicity in both normal and transformed cells.

Interestingly, some of the beneficial effects attributed to resveratrol depends on SirT1 however, our results suggest that SirT1 is not involved in its toxicity (Boily et al., 2009a; Csiszar et al., 2008; Ungvari et al., 2009; Lee et al., 2009; Sun et al., 2007). This apparent discrepancy might be explained by the pleiotropic nature of resveratrol. Firstly, it was clearly demonstrated that SirT1 is not directly activated by resveratrol. The apparent

activation obtained by Howitz et al. was dependent on the attached fluorophore to the peptide assessed in their *in vitro* deacetylation assay (Howitz et al., 2003a ; Beher et al., 2009; Borra et al., 2005; Kaeberlein et al., 2005; Pacholec et al., 2010). It is conceivable that low dose of resveratrol modulates SirT1 level or activity indirectly through its action on modulators of SirT1 and provide its health beneficial effects (Fig. 17). However, resveratrol toxicity observed at high dose might be mediated through others proteins that have been shown to bind to this agent such as: quinone reductase 2 (QR2), ATP synthase (ATP) and integrin ($\alpha V\beta 3$) (Fig. 17).

SirT1 has been implicated in many cellular functions and we cannot completely rule out the possibility that it might be involve in resveratrol toxicity. Resveratrol is known to induce cell death by apoptosis and/or autophagy. It is now recognized that there is interplay between the two pathways (Eisenberg-Lerner et al., 2009). Autophagy can cooperate, antagonize or enable apoptosis mediated cell death. It was recently shown that resveratrol toxicity in glioblastoma cells is mediated by both apoptosis and autophagy (Filippi-Chiela et al., 2011). In a model where SirT1 serves as a factor regulating the balance between these two pathways, it is conceivable that we may have missed the implication of SirT1 in resveratrol mediated cytotoxicity. Since we have measured cell viability and not directly apoptosis or autophagy it would in turn be interesting to investigate the role of SirT1 in resveratrol mediated autophagy or apoptosis with specific markers. This phenomenon is currently under investigation in our laboratory.

Figure 17. Possible scenarios for indirect activation of SirT1 by resveratrol.
Resveratrol can potentially activate or inhibit SirT1 modulators and this could lead to differential SirT1 substrates preference and induce either cell death or cell survival. SirT1 known modulators comprise proteins that regulate SirT1 function by post translational modification (PTM) (Red), protein level (Blue), enzymatic activity (Yellow) or by direct binding (Aqua). However, our data support the model where resveratrol induces cell death independently of SirT1 probably by is action on other known substrates such as ATP synthase (ATP), Quinone reductase 2(QR2) or integrin ($\alpha V\beta 3$) (Grey). Current known resveratrol interactions are depicted by a line.


Resveratrol
Protein level
PTM
Activity
Binding
Res targets
Set7/9
Ck2
JNK1
MST1
E2F
miR34A
HuR
DBC1
AROS
SirT1
NAM
NAD+
ATP
αVβ3
Qr2
Targets
Targets
Apoptosis
Survival

We have tested our hypothesis on a specific cancer cell line model that utilized large T antigen for the process of transformation. To clarify the role of SirT1 in resveratrol mediated cytotoxicity it might be required to performed *in vivo* study. We are currently in the process of generating transgenic mice that harbor SirT1 catalytic dead mutant ($SirT1^{Y/Y}$) with two well characterized hormone dependent cancer models; the PTEN flox prostate cancer (Ma et al., 2005) and the MMTV-PyMT (Lin et al., 2003) breast cancer model. We are planning to compare the chemopreventive and chemotherapeutic effect of SirT1 and resveratrol in these mice. These *in vivo* models will be a useful tool to clarify the role of SirT1 in resveratrol mediated toxicity.

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Contributions of collaborators

Primary Mouse embryonic fibroblasts were isolated by Xiao Hong He and Dr. Michael McBurney. MEFs transformation with large T antigen was performed by Jiahu Wang. Genotyping and plasmid constructs were done by Karen Jardine.

Appendices

Figure S 1. AlamarBlue linearity domain.
Different amount of MEFs cells ranging from 500-200,000 cells per well (N=4) were plated and incubated with alamarBlue reagent for 4hours. Fluorescence signal (RFU) was measured at 595nm with Fluoroskan Ascent FL plate reader (Thermo Scientific). Linear regression was performed with GaphPad prism in order to determine the linear coefficient (R^2= 0.9941).

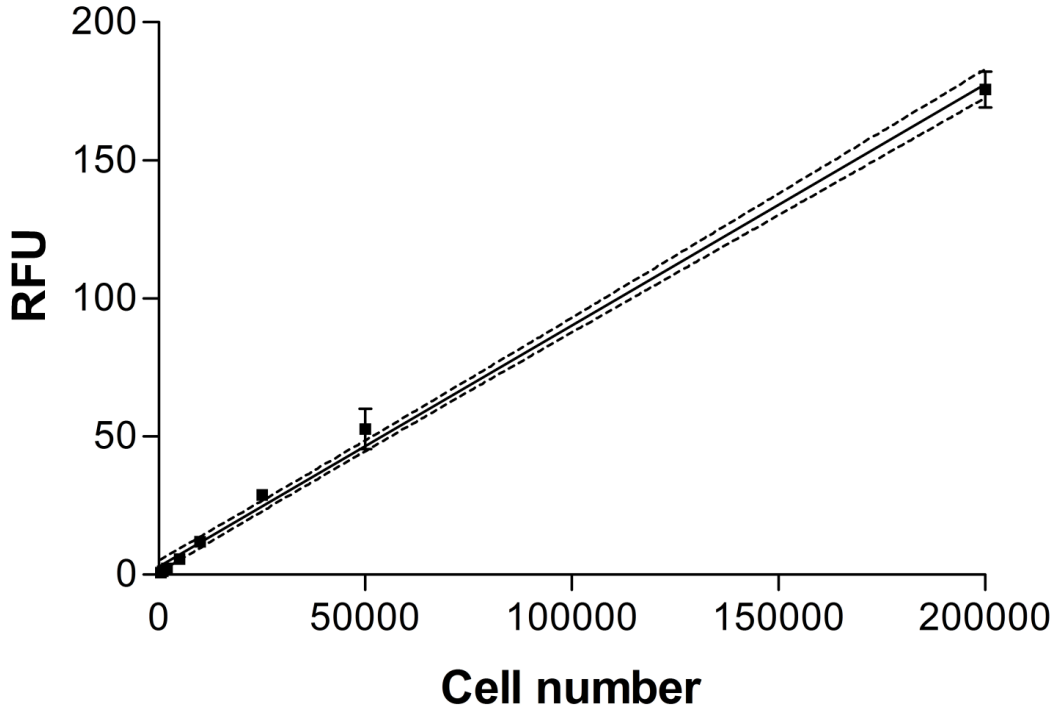
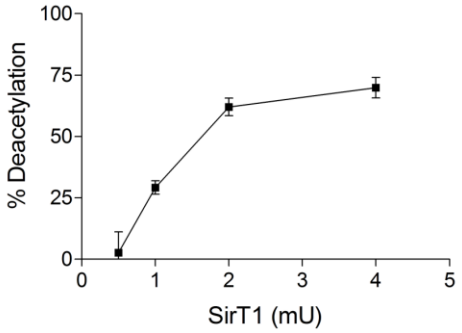

200
150
100
50
0
RFU
0
50000
100000
150000
200000
Cell number

Figure S 2. Enzymatic assay linearity domain.
A) Percentage of deacetylation for different concentration of purified SirT1 from Enzo Life Sciences ranging from 0.5-4mU per well (N=3). Deacetylation levels were assessed with the SirT1 HTRF deacetylase assay from Cisbio bioassay. Fluorescence signal was measured at 620 and 665nm with Synergy 2 reader (BioTek). B) Linear regression with data point 0.5, 1 and 2mU was performed with GraphPad Prism in order to determine the linear coefficient (R^2= 0.9507).

A


100
75
50
25
0
% Deacetylation
0
1
2
3
4
5
SirT1 (mU)

B

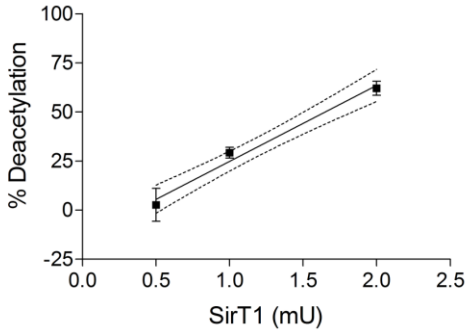

100
75
50
25
0
-25
% Deacetylation
0.0
0.5
1.0
1.5
2.0
2.5
SirT1 (mU)

Figure S 3. SirT1 protein is not present in cell line derived from SirT1-/- mouse embryo.

Blots were probed against SirT1, β-actin or large T antigen (Tag). A) Mouse embryonic stem cells from the R1 ($SirT1^{+/+}$) or B6 ($SirT1^{-/-}$) cell lines. B) Immortalized mouse embryonic fibroblast (in the presence or absence of the large T antigen) are used to perform retroviral infection. Positive controls are SirT1 purified from Biomol Research Laboratories (SirT1) and immortalized $MEFs^{wt}$. C) Primary mouse embryonic fibroblast derived from heterozygous or knockout animals.

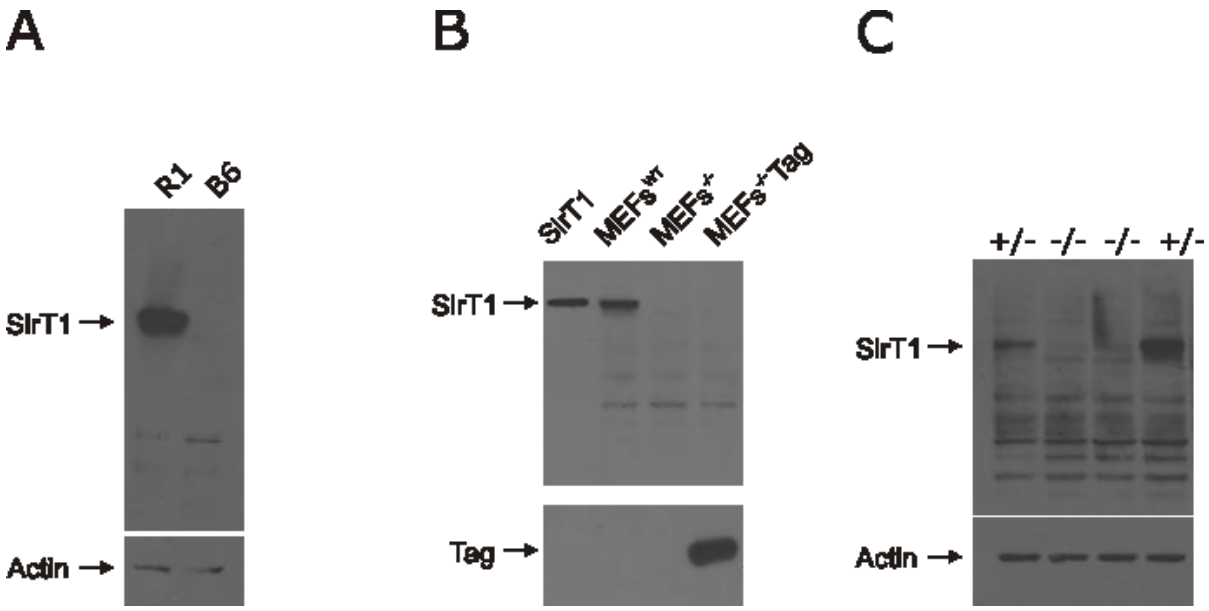
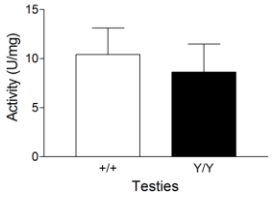
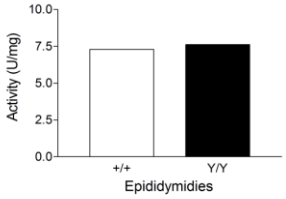

A
R1
B6
SirT1
Actin
B
SirT1
MEFs^wt
MEFs^+/-
MEFs^+/-Tag
SirT1
Tag
C
+/-
-/-
-/-
+/-
SirT1
Actin

Figure S 4. Endogenous sirtuins activity in mice tissues.
A) Adult mice testis from $SirT1^{+/+}$ (N=2) and $SirT1^{Y/Y}$ (N=3) animals. B) Adult mice epididymides from $SirT1^{+/+}$ (N=1) and $SirT1^{Y/Y}$ (N=1) animals. C) Embryonic liver from $SirT1^{+/+}$ (N=1), $SirT1^{+/Y}$ (N=2) and $SirT1^{Y/Y}$ (N=1) animals. No difference was observed between genotypes.

A


Activity (U/mg)
15
10
5
0
+/+
Y/Y
Testies

B


Activity (U/mg)
10.0
7.5
5.0
2.5
0.0
+/+
Y/Y
Epididymidies

C

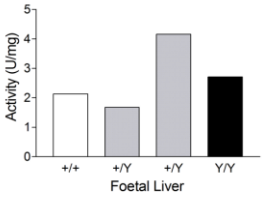
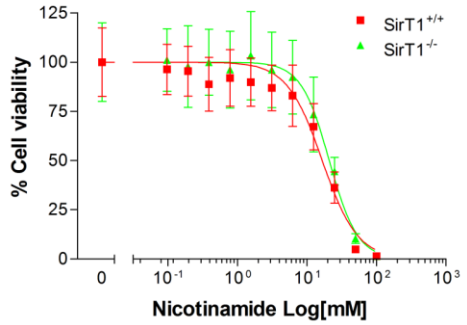

Activity (U/mg)
5
4
3
2
1
0
+/+
+/Y
+/Y
Y/Y
Foetal Liver

Figure S 5. Nicotinamide dose response curves in embryonic stem cells.
A) Dose response curves and B) Lethal Dose 50 of embryonic stem cells SirT1$^{+/+}$ (red) and SirT1$^{-/-}$(green). No statistical difference was obtained by student t-test.

A


125
100
75
50
25
0
% Cell viability
SirT1+/+
SirT1-/-
0
10-1
100
101
102
103
Nicotinamide Log[mM]

B

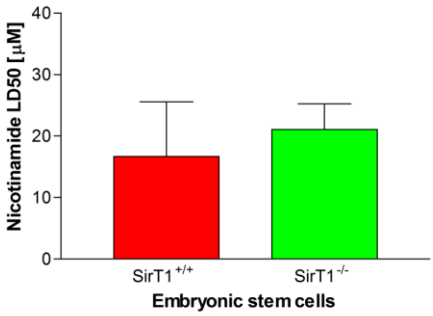

40
30
20
10
0
Nicotinamide LD50 [µM]
SirT1+/+
SirT1-/-
Embryonic stem cells

Figure S 6. SirT1 immunostaining in immortalized MEFs.
Immunostaining against SirT1 (red) followed by counter staining DNA with DAPI (blue) was performed on MEFs SirT1$^{+/+}$ (Top, red) and MEFs SirT1$^{-/-}$ (Bottom, green). Merged images of 40X and 100X magnification confirm nuclear localization of SirT1. Non specific binding of the antibody is observed in the cytoplasm of SirT1$^{-/-}$ cells.

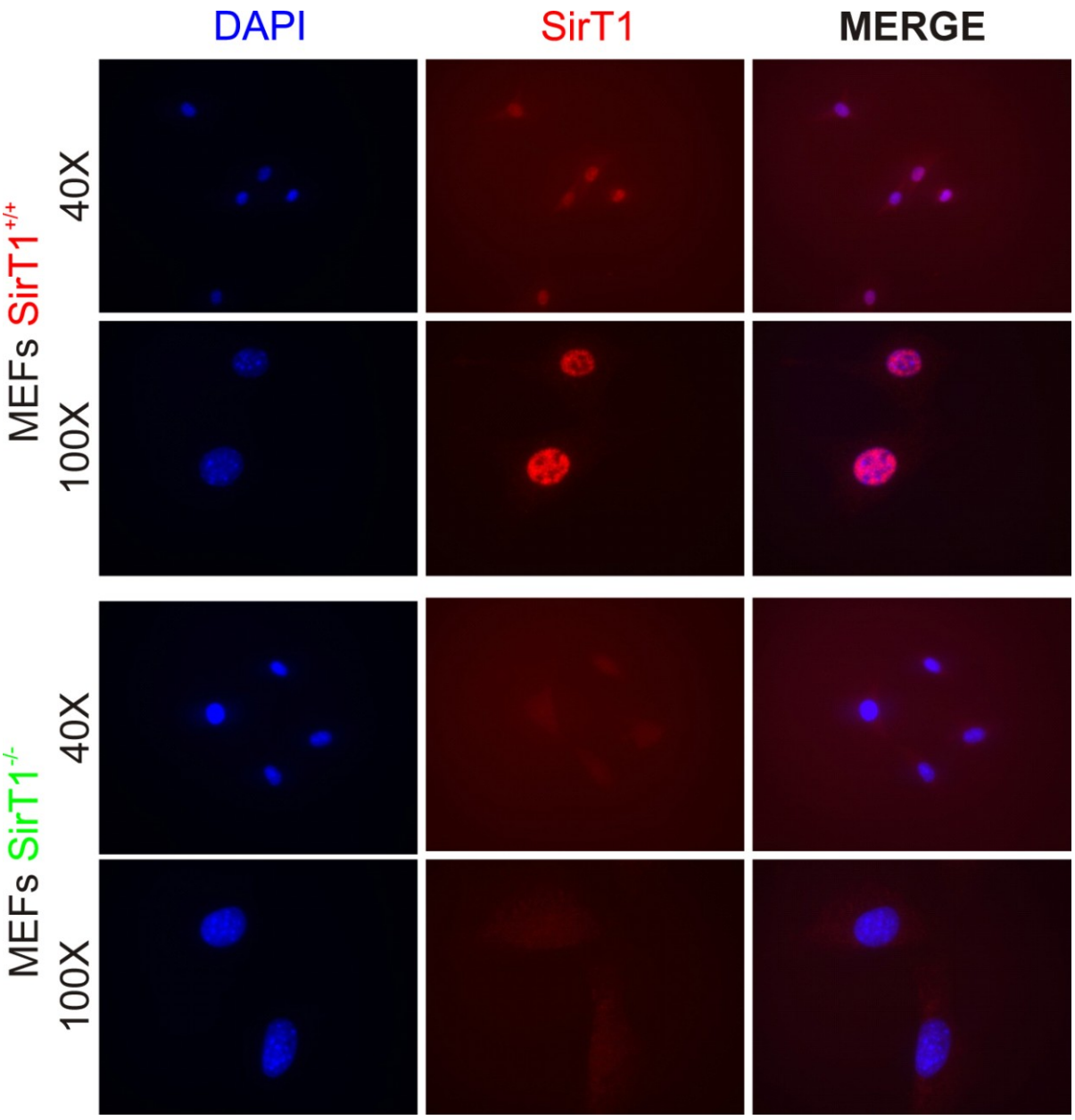
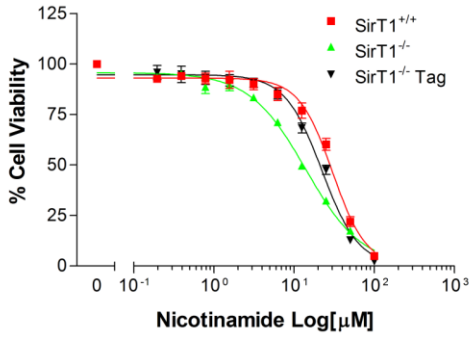

DAPI
SirT1
MERGE
MEFs SirT1+/+
40X
100X
MEFs SirT1-/-
40X
100X

Figure S 7. Nicotinamide dose response curves in immortalized MEFs.
A) Dose response curves and B) Lethal Dose 50 of MEFs cells SirT1$^{+/+}$(red), SirT1$^{-/-}$(green) and SirT1$^{-/-}$ Tag (black).

A


% Cell Viability
125
100
75
50
25
0
0
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Nicotinamide Log[μM]
SirT1+/+
SirT1-/-
SirT1-/- Tag

B

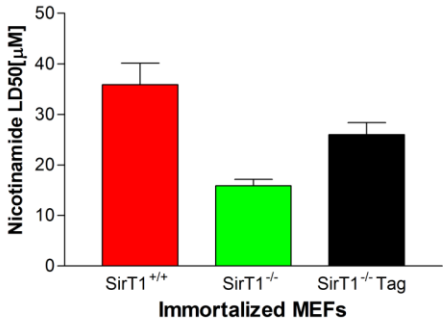
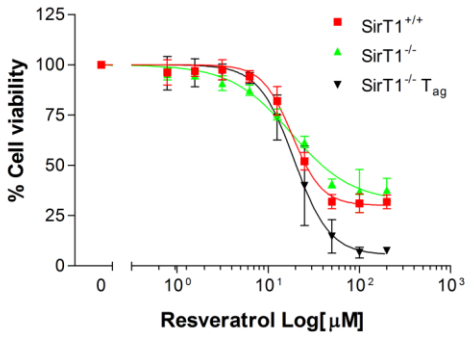
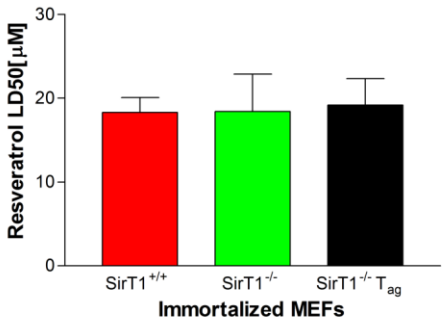

Nicotinamide LD50[μM]
50
40
30
20
10
0
SirT1+/+
SirT1-/-
SirT1-/- Tag
Immortalized MEFs

Figure S 8. Resveratrol toxicity in immortalized MEFs cells.
A) Dose response curves and B) Lethal Dose 50 of MEFs $SirT1^{+/+}$(red), MEFs $SirT1^{-/-}$ (green) and MEFs $SirT1^{-/-}$ Tag(black) (N=5). Linear mixed effect model did not capture a statistical difference between $SirT1^{+/+}$, $SirT1^{-/-}$ and $SirT1^{-/-}$ Tag.

A


125
100
75
50
25
0
% Cell viability
0
10^0
10^1
10^2
10^3
Resveratrol Log[μM]
SirT1$^{+/+}$
SirT1$^{-/-}$
SirT1$^{-/-}$ T$_{ag}$

B


30
20
10
0
Resveratrol LD50[μM]
SirT1$^{+/+}$
SirT1$^{-/-}$
SirT1$^{-/-}$ T$_{ag}$
Immortalized MEFs

RESUME

Publications

1. Seifert E., Caron A., **Morin K**., Coulombe J., He H. X., Jardine K., Dewar-Darch D., Boekelheide K., Harper E. M., McBurney W. M. SirT1 catalytic activity is required for male fertility and metabolic homeostasis in mice. FASEB (2011) e193979.

2. Phenix H., **Morin K**., Batenchuk C., Parker J., Abedi V., Yang L., Tepliakova L., Perkins TJ., Kaern M. Quantitative pathway analysis and pathway inference from genetic interaction data. PLoS Computational Biology (2011) 7(5) e1002048.

3. Butcher, J., Abdou, H., **Morin, K**., Liu, Y. Micromanaging oligodendrocyte differentiation by noncoding RNA: Toward a better understanding of the lineage commitment process. Journal of neuroscience (2009) 29(17):5365-5366

4. Kapoor, A., Gould, W.D., Bédard, P., **Morin, K**. Application of biotechnology for treatment of nitrogen compounds in gold mill effluents. CIM Bulletin (2004) 97(1081):84-90

Presentations

1. Embo Practical course on Networks in Biology analysis, modeling and reverse engineering, 2009, Bologna, Italy

   Poster Title: Quantitative epistasis analysis and pathway inference by gene expression profiling
   Authors: Phenix, H., **Morin, K**., Batenchuk, C., Tepliakova, L., Kaern, M.

2. Progress in Systems Biology: the Brain and Mind, 2009, Ottawa, Canada

   Poster Title: Cyclic Fluctuations in Glycerophoshocholine Metabolism over a 24 h Period in the Murine Temporal Cortex
   Authors: **Morin, K**., Swayne, L.A., Bennett, S.A.L., Figeys, D.

3. The international Genetically Engineered Machine competition (iGEM), 2008, Boston, USA

   Poster/Oral: The Repulsalator: A Synthetic Pulse Generator in Yeast for Sustainable Expression of Recombinant Proteins
   Authors: Batenchuk, C., Jedrysiak, D., Abdennur, N., Orton, M., Tye, T., Jolin-Dahel, K., **Morin, K**., Adiga, S., Euler, C., Zinoviev, R., M. Kaern

External scholarships and Awards

1. **Best teacher assistant award in biochemistry**, Faculty of Science, University of Ottawa, 2010-2011

2. **Doctoral research scholarship**, Fonds québécois de recherche sur la nature et les technologies (FQRNT), 2010-2013 (refused)

3. **Graduate scholarship**, Natural Sciences and Engineering Research Council (NSERC), 2009-2010

4. **Alexander Graham Bell Canada graduate scholarship**, Natural Sciences and Engineering Research Council (NSERC), 2008-2009

Work experience

University of Ottawa

Master degree (May 2009-December 2011)
Ottawa Hospital Research Institute, Ottawa, Ontario

- Undergraduate student supervision
- Mouse embryonic stem cells and fibroblast culture and diverse cancer cells culture
- Cell viability and enzymatic assay
- Drug dose response curve analysis
- Immunofluorescence
- Cell cycle analysis by flow cytometry
- Retroviral and calcium phosphate transfection
- Batch binding and FPLC his-tag protein purification
- Western blot analysis
- Mouse hippocampus lipid extraction and phosphatidylcholine determination by HPLC-MS
- Troubleshooting FPLC, HPLC, mass spectrometer and Western blot
- Mouse handling and brain dissection
- High-throughput fitness profiling in yeast

Let's Talk Science and Science Travel (May 2011)
University of Ottawa, Ottawa, Ontario

- Outreach to high school student to science and technology
- Prepared power point presentation
- Unassisted presentation in French and English in diverse science subject including robotics, chemistry, electrochemistry, DNA and evolution
- Delivered hands-on science activities
- Provided information about career in science

Teacher assistant (January 2008-April 2011)
Faculty of Science, Ottawa, Ontario

- Taught the theoretical aspects regarding various biological techniques
- Supervised students in the laboratory
- Corrected exams and laboratory reports in Molecular biology, Biochemistry, Macromolecules, and Molecular evolution

Honours research project (September 2007-April 2008)
Ottawa Hospital Research Institute, Ottawa, Ontario

- Mammalian cell culture
- Protein immune-purification (HA/MYC/GST)
- *In vitro* Kinase assays
- Western blot analysis
- Sub-Cloning

Canadian Food Inspection Agency

Analytical technologist (May to August 2006 & 2007)
Feed and Fertilizer group, Ottawa, Ontario

- Ground fertilizer sample
- Sample preparation for ICP analysis
- Titrimetric determination of soluble potash contain in fertilizer
- Gravimetric determination of phosphorous contain in fertilizer
- Organisation and management of the work load

Natural Resources Canada

Analytical technologist (2002-2004)
Environmental group, Ottawa, Ontario

- Implemented an automated High Pressure Liquid Chromatography system
- Colorimetric analysis of ammonia, nitrate, nitrite, thiocyanate and cyanates
- Bacterial enumerations in aerobic and anaerobic conditions
- Bacterial growth on Petri dish for isolation and identification
- Field work to collect and analyze sample from Mining waste

Analytical technologist (2001-2002)
CANMET/MMSL Laboratories, Ottawa, Ontario

- Sample preparation by four acids, microwave and fusion digestion
- Anions and sulphur species analysis by chromatography
- Electrochemical (ISE) determination of ammonium and cyanates
- Titrimetric and spectrophotometric determination of ferrous iron
- Determination of total, inorganic and organic carbon
- Titrimetric determination of total thiosalts

Aur Resource Inc

Analytical technologist (May to August 1999 & 2000)
Louvicourt Mine, Val d'Or, Québec

- Ground and pulverize mining sample
- Sample preparations by various acid digestion techniques
- Determination of zinc contain by EDTA titration
- Determination of cupper contain by electroplating analysis
- Atomic absorption analysis of metals

Education

Master degree in Biochemistry 2008-2011
Faculty of Medicine, Biochemistry, Microbiology and Immunology department, University of Ottawa, Ottawa, ON

Honours Bachelor Science degree in Biochemistry 2003-2008
University of Ottawa, Ottawa, ON
Graduate with honourable mention Summa Cum Laude

College degree in Analytical Chemistry 1998-2001
Cégep de l'Outaouais, Gatineau, Qc