An investigation of ageing-related genomic effects of resveratrol

Fatema Suliman Alatawi

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Institute for Cell and Molecular Biosciences,

Newcastle University, UK

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Declaration

I certify that this thesis is my own work, except where stated, and has not been previously submitted for a degree or any other qualification at this or any other university.

Fatema Suliman Alatawi

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Abstract

Dietary restriction (DR) increases lifespan robustly in diverse species. Effects of the dietary polyphenol resveratrol consistent with delayed ageing and/or extension of lifespan have been reported. The involvement in the longevity response to DR of the protein Sirt1, which may be activated by resveratrol and deacetylates a range of cellular substrates that includes histone proteins, identifies epigenetic processes as a pathway that may mediate effects of both DR and dietary resveratrol in delaying ageing and/or extending lifespan.

Based on a preliminary observation, the hypothesis underlying the study is that some of the beneficial effects of resveratrol on lifespan/aging are mediated through effects on histone expression that oppose changes observed in ageing. A secondary hypothesis, based on a degree of structural similarity between resveratrol and 17β-oestradiol, was that epigenetic effects of resveratrol are mediated through the estrogen receptor (ER).

The effect of resveratrol on histone protein expression was investigated in human intestinal Caco-2 cells and human MCF-7 breast cancer cells. Histone H2a, H2b, H3 and H4 expression was decreased in response to resveratrol treatment in both cell lines. In support of our hypothesis that resveratrol affects ageing through reversing ageing-associated changes in histone proteins, higher levels of H2A, H2B, and H4 expression were detected by western blotting in the small intestine of old (38 months) mice than in younger (12 months) mice. To investigate possible consequences of effects of resveratrol, including effects resulting from altered histone expression, we studied the effect of resveratrol on global gene expression in Caco-2 and MCF-7 cells to address several objectives including: (1) investigating if resveratrol has an effect similar to that of DR at the level of gene expression; (2) identifying if genes or pathways affected by resveratrol were also affected by manipulation of the expression level of Sirt1. For both cell types, the number of genes in the intersection between those affected by resveratrol and a compiled list of genes reported in other studies to respond to DR was greater than expected by chance, supporting the view that responses to resveratrol and to dietary restriction have some commonality and that resveratrol may mimic some effects of dietary restriction. We also found that there was very little overlap between genes affected by resveratrol treatment and by knockdown of *Sirt1* expression in Caco-2 cells, which adds to accumulating evidence that resveratrol does not act through effects on Sirt1.

To investigate if effects of resveratrol - in particular the reduction in histone protein expression - are mediated through the estrogen receptor (ER), Caco-2 and MCF-7 cells were treated with resveratrol in the presence or absence of the ER antagonist fulvestrant, then total cell lysate was analysed by western blotting. The reduction in histone protein (H2a, H2b, H3 and H4) expression was attenuated by fulvestrant, indicating that resveratrol reduced histone expression via an ER-dependent mechanism. For further investigation of effects of resveratrol on histone expression, Caco-2 cells were transfected with a promoter reporter construct comprising the histone H3 promoter upstream of the β-galactosidase reporter gene, and the effect on reporter gene expression of treatment with resveratrol in the presence and absence of the fulvestrant was measured. Resveratrol reduced reporter gene expression and this effect was attenuated by fulvestrant, demonstrating that resveratrol acts to reduce histone H3 expression at the level of transcription through an ER-mediated mechanism. To investigate if the response to resveratrol treatment is through interaction with estrogen response elements (EREs) in the histone H3 promoter we replaced three potential EREs within the histone H3 promoter region included in the promoter-reporter construct with random sequence. Caco-2 cells were then transfected with either original or mutated promoter-reporter construct and treated with resveratrol or the endogenous ER ligand 17-β estradiol in the presence and absence of fulvestrant. Resveratrol and 17-β estradiol both reduced reporter gene expression from both promoter reporter constructs and in all cases responses were attenuated by fulvestrant, indicating that effects of neither compound, although mediated through the ER, are on the specific sequences region we identified and replaced.

In conclusion, these data indicate that resveratrol reduces histone expression in both intestinal and breast cancer cells through an ER-mediated mechanism acting at the level of transcription and that this effect may oppose an accumulation of histone proteins (observed in mouse small intestine) that accompanies ageing. With respect to effects on gene expression, resveratrol was found to mimic some effects of dietary restriction but appeared to act through a mechanism independent of Sirt1.

Abbreviations

A260	Absorbance reading at 260nm
ac	Acetylation
Acetyl-CoA	Acetyl-coenzyme A
ar	Ribosylation
bp	Base pair
BSA	Bovine serum albumin
BSA	Bovine serum albumin
Caco-2	Colonic adenocarcinoma
cDNA	copy DNA
DMEM	Dulbecco`s modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR	Dietary restriction
E2	estrogen
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
ERE	Estrogen response element
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FCS	Foetal calf serum

FOXO	Forkhead transcription factor
GSH	Tripeptide glutathione
H	Histone
H_2O_2	Hydrogen peroxide
HDAC	Histone deacetylase enzyme
$HO^{\cdot}$	Hydroxyl radical
IGF	Insulin like growth factor
IMEM	Improved minimal essential medium
ISS	Insulin/insulin-like growth factor signalling
K	Lysine
LDL	Low density lipoprotein
LSD1	Lysine specific demethylase 1
me	Methylation
NAD	Nicotinamide adenine dinucleotide
NEAA	Nonessential amino acids
O_2^-	superoxide
PARP	Poly-ADP ribose polymers
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFP	Percentage of false positives
PGC-1α	PPAR-gamma coactivator 1 alpha
ph	Phosphorylation
PVDF	Polyvinylidene difluoride

R	Arginine
RNA	Ribonucleic acid
S	Serine
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIR	Silent information regulator
SOD	Superoxide dismutase
T	Threonine
ub	Ubiquitination
UV	Ultraviolet
αERKO	Estrogen receptor alpha knockout mice
αβERKO	Estrogen receptor alpha and beta knockout mice
βERKO	Estrogen receptor beta knockout mice

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1. Introduction

The thesis is concerned with the activity of the compound resveratrol, particularly with respect to its potential epigenetic effects relating to its ability mimic aspects of the beneficial effect of dietary restriction on lifespan. The properties of resveratrol will be considered in detail then the concept of longevity in response to dietary restriction will be introduced and a summary of epigenetic modification will be presented before outlining the specific aims of the thesis.

1.1 Resveratrol

Resveratrol (*trans* 3, 5, 4-trihydroxy-stilbene), a polyphenol that is present in red grapes and their product red wine, has been demonstrated to have a wide variety of potential health benefits. Several studies have documented its role as an anti-inflammatory (Gentilli *et al.*, 2001, Donnelly *et al.*, 2004), anti-coaggulative (Pace-Aseiak, 1995, Kirk *et al.*, 2000) and anti-oxidative (Miller *et al.*, 1995, de la Lastra and Villegas, 2007) agent. As an anti-cancer agent, resveratrol has been shown to inhibit tumour cell proliferation during three stages of cancer: initiation, promotion and progression. Moreover, it appears to induce the apoptotic death pathway in several types of tumour (Chang *et al.*, 2000; Fulda and Debatin, 2004, Rayalam *et al.*, 2008). Recently, resveratrol has received particular interest as a result of its ability to promote longevity in mammals and in lower organisms, which may be by stimulating the NAD (+) dependent deacytlases Sirt1 and Sir2 respectively, although this view is the subject of recent vigorous challenge (Pacholec *et al.*, 2010). These proteins deacetylate a large number of substrates including histones, and have an important role in the regulation of gene expression, fatty acid metabolism (Picard *et al.*, 2005) cell cycle progression, and lifespan extension (Cohen *et al.*, 2004).

The mechanisms by which resveratrol induces these biological effects are not understood fully. Resveratrol has a structure similar to that of known phytoestrogens so may mediate some of its action through its interaction with estrogen receptors ERα and/or ERβ. Some studies have reported that resveratrol may act as an ER agonist in the MCF-7 breast cancer cell line (Gehm *et al.*, 1997; Gehm *et al.*, 2004). In contrast, other studies documented apparent ER antagonist action in the same cell line (Kim *et al.*, 2004).

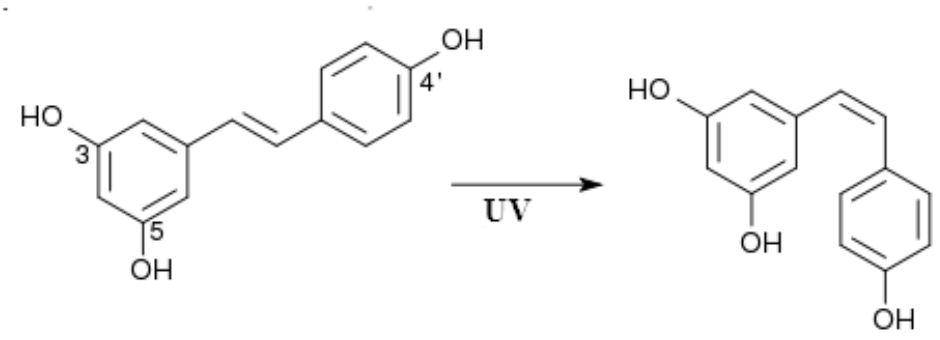
1.1.1 Sources of resveratrol

The main dietary sources of resveratrol include fruits (e.g grapes, lingberry, cranberry, jackfruit) and also peanuts. In the plant, resveratrol is also synthesised in flowers and leaves (e.g in gentum, scots pine, spruce, and butterfly orchid tree). The activity of stilbene synthase, the enzyme responsible for resveratrol synthesis, can be induced within few hours` of exposure to ultraviolet (UV) radiation, mold invasion, injury or fungal infection, with the intensity and/or duration of the challenge being related to the concentration of resveratrol in the plant (Fremont, 2000). Resveratrol occurs in different types of wine at concentrations dependent on the time of fermentation (Siemann and Creasey, 1992). Table 1.1 summarises the concentration of resveratrol found in some food sources (Mukherjee *et al.*, 2010).

1.1.2 The structure of resveratrol

Structurally, resveratrol consists of two aromatic rings connected by a styrene double bond. There are two isomers of resveratrol: *cis* and *trans*, and the *trans* isomer appears to have greater biological activity than the *cis* form (Basly *et al.*, 2000). The

trans isomer is pH and light sensitive (Trela *et al.*, 1996), and can photo-isomerise to the *cis*-form when exposed to UV (Figure 1.1) (Soleas *et al.*, 1997).



trans-resveratrol cis-resveratrol

Figure 1.1: The photo- isomerisation of trans- resveratrol into cis- resveratrol

Table 1.1: The concentration of resveratrol in natural foods. Adapted from (Mukherjee et al., 2010). The data were collected from different studies, thus different units of concentration are stated.

Source	Resveratrol concentration
100% Natural peanut butter	~0.65 μg/g
Bilberries	~16 ng/g
Blueberries	~32 ng/g
Boiled peanuts	~5.1 μg/g
Cranberry raw juice	~0.2 mg/L
Dry grape skin	~24.06 μg/g
Grapes	0.16–3.54 μg/g
Peanut butter	0.3–1.4 μg/g
Peanuts	0.02–1.92 μg/g
Pistachios	0.09–1.67 μg/g
Ports and sherries	<0.1 mg/L
Ref grape juice	~0.50 mg/L
Red wines	0.1–14.3 mg/L
Roasted peanuts	~0.055 μg/g
White grape juice	~0.05 mg/L
White wines	<0.1–2.1 mg/L

1.1.3 Resveratrol bioavailability and metabolism

Several studies, *in vivo* as well as *in vitro*, report that resveratrol is absorbed rapidly and metabolised by the small intestine. *In vitro*, Kaldas and colleagues (2002) measured the transport and metabolism of 5-40 µM resveratrol by the human intestinal Caco-2 epithelial cell line, cultured in transwells. They documented that, for all concentrations of resveratrol, transcellular absorption occurred and this absorption appeared to be concentration-dependent. Furthermore, the metabolism of resveratrol was investigated by incubating a high concentration (100 µM) of resveratrol with Caco-2 cells for 6 hours. Under these conditions resveratrol was converted into two types of conjugates: a glucuronidated conjugate (*trans*-resveratrol-3-O-glucuronide) and a sulphated conjugate (*trans*-resveratrol-3-sulfate). Under conditions where resveratrol was at physiological concentrations, sulphate conjugation was greater than glucuronidation. On the other hand, at higher concentrations of resveratrol glucuronidation became more predominant.

In studies *in vivo*, the highest plasma concentration of resveratrol metabolites [sulphate (13 µM); and glucuronide (5 µM)] was recorded in the first 15 minutes after oral administration of resveratrol (20 mg/kg of body weight) to mice. In contrast, only traces of free resveratrol were detected (Yu *et al*., 2002). These results indicate that the bioavailability of free resveratrol in plasma is very low and approximates zero soon after ingestion.

In studies in humans, the highest concentration of resveratrol metabolites (2µM) was detected within 60 minutes after oral administration of resveratrol at 25 mg to six healthy men and woman. In contrast, only traces of free resveratrol were observed indicating rapid metabolism and clearance (Waller *et al*, 2004). In a second study, oral

administration of 25 mg resveratrol per 70 kg of body weight in different foods to 12 healthy men resulted in the highest plasma concentration of resveratrol and metabolites being observed 30 minutes after administration (Goldberg *et al.*, 2003). In another study, the bioavailability of resveratrol in wine appeared not to differ when it was consumed on an empty stomach or with a meal, challenging the idea that food matrix influences resveratrol bioavailability (Vitaglione *et al.*, 2005).

Contrasting with intestinal absorption, buccal delivery was reported as a route through which free resveratrol was bioavailable. When 1mg/ml of resveratrol in 50 ml solution was held in the mouth, a peak of unconjugated resveratrol (37 ng/ml) was detected after two minutes (Asensi *et al*., 2002).

The transport of resveratrol across the small intestinal epithelium is probably through a passive diffusion mechanism (Li *et al*, 2003). In contrast, the uptake of resveratrol in hepatic cells results from the contribution of two mechanisms, a passive diffusion mechanism and a carrier-mediated pathway (Lancon *et al*, 2004). The main pathway of resveratrol excretion is through urine (Boocock *et al.*, 2007).

1.1.4 Resveratrol toxicity

There is little information about resveratrol toxicity and its target organs. To date, a small number of studies have investigated the potential toxic effects of resveratrol in animals. Juan *et al*. (2002) documented that oral administration of resveratrol at 20 mg/kg of body weight to rats for 28 days resulted in no toxic effects except limited changes in serum liver enzymes. Furthermore, a single dose of 2000 mg/kg of body weight of resveratrol did not induce any signs of toxicity in the rat.

In a second study in rats, oral administration of resveratrol at 300, 1000, and 3000 mg/kg of body weight for 28 days resulted in signs of toxicity at 3000mg/kg only. These effects included reduced food consumption and loss of body weight, along with increased kidney weight and signs of nephrotoxicity including elevated serum blood urea nitrogen and creatinine concentrations, and changes in gross renal pathology. Early death was recorded in two male rats on day 24. In these two rats, microscopic investigation of the kidneys identified lesions that were the probable cause of early death. On the other hand, administration of 1000 or 300 mg/kg of resveratrol did not result in nephrotoxic symptoms. These finding identify the kidney as a target organ for toxicity caused by resveratrol at high doses (Crowell *et al.*, 2004).

1.1.5 Biological effects of resveratrol

Resveratrol is a plant antibiotic produced in large quantities in various plants in response to stress, injury or pathyogenic infection. Thus, resveratrol has an important role in the plant's defence system, mainly against fungi (Jeandet *et al.*, 1995; Zhan *et al.*, 2010). Traditionally, roots of *polygonum cuspdatum*, the richest sources of resveratrol, have been used in traditional Asian medicine to treat a wide range of diseases, including fungal infection, skin inflammation, and disease of the liver, heart, and blood vessels (Arichi *et al.*, 1982).

Numerous studies have reported resveratrol as being protective against cardiovascular disease, having anti-oxidant functions (Miller *et al.*, 1995; de Lastra and Villegas, 2007), anti-inflammatory action (Gentilli *et al.*, 2001), reducing blood-glucose, having estrogenic action (Deng *et al.*, 2008), and protecting against cancer (Shankar *et al.*, 2011).

The mechanisms by which resveratrol induces these biological effects are still unclear. A possible mechanism is that the pharmacological actions of resveratrol may result from its effect as antioxidant and/or its estrogenic properties (Gehm *et al.*, 1997, Runqing and Ginette, 1999). The potential roles of resveratrol as antioxidant factor and its property as phytoestrogen are discussed in section 1.1.5.1 and section 1.1.5.2 respectively.

1.1.5.1 Resveratrol as an antioxidant

As a consequence of metabolism in normal living cells, free radical molecules with unpaired electrons are formed. These compounds are considered to be highly reactive molecules that induce considerable damage to cell contents such as DNA and membranes (proteins and lipids). Furthermore, the presence of free radicals can generate highly reactive molecules known as reactive oxygen intermediates (ROI). These compounds contain an oxygen atom and include superoxide (O_2^-), the hydroxyl radical ($OH\cdot$) and hydrogen peroxide (H_2O_2) (Pervaiz, 2003). Under normal physiological conditions, excessive accumulation of ROI is controlled by the cellular antioxidant defence system. This system comprises several enzymes including catalase and superoxide dismutase (SOD), along with the tripeptide glutathione (GSH) (Pervaiz, 2003; Vermerris, 2006).

A defect in the ability of cells to control the production of ROI results in their excessive accumulation, a state that leads to oxidative stress. Exposure of macromolecules such as lipids, proteins, and nucleic acids to ROI result in their deleterious oxidation. For example, the oxidative modification of low density lipoproteins (LDL) is associated with increased the incidence of cardiovascular diseases such as atherosclerosis. The oxidized form of LDL attaches strongly to a

non-regulated scavenger receptor system causing LDL to accumulate excessively in monocytic subendothelial cells (Pervaiz, 2003).

In laboratory studies, antioxidant action of resveratrol has been reported in various pathways. Firstly, resveratrol inhibited oxidation of polyunsaturated fatty acids found in LDL. This inhibitory effect results from the ability of resveratrol to prevent copper-catalyzed oxidation (LDL has a high affinity for copper) (Frémont *et al.*, 1999). Secondly, resveratrol has been reported to inhibit the membrane lipid peroxidation by reducing the toxicity of the ROI in living cells. In *in vitro*, administration of resveratrol to rat adrenal pheochromocytoma cells (PC12) which had been exposed to ethanol-induced oxidation, resulted in protection against cell death (Sun *et al*., 1997; Chan and Chang, 2006). This effect results from the ability of resveratrol to block internalization of oxidized lipoproteins. Furthermore, resveratrol has been shown to have an inhibitory effect on oxidised lipoprotein-induced neuronal cell death (Draczynska-Lusiak *et al.*, 1998; Zhang *et al.*, 2010).

1.1.5.2 Resveratrol as a phytoestrogen

Phytoestrogens are "nonsteroidal compounds of plant origin that can bind to estrogen receptors as agonists and consequently mimic biological actions of endogeneous estrogens" (Grynkiewicz and Opolski, 2005). These compounds have a chemical structure similar to 17β-estradiol (Kuiper *et al.*, 1997), and have an ability to bind to estrogen receptors (ER) to induce estrogenic and/or antiestrogenic effects (Gehm *et al*, 1997, Bowers *et al*, 2000). Although the affinities of these compounds for the ER are at least 1000-10,000 times lower than estradiol, they are unquestionably able to induce endocrine effects. For example, phytoestrogens have been reported to have

beneficial effects on the cardiovascular system, relieve menopausal symptoms, and decrease the risk of breast cancer and osteoporosis. Furthermore, phytoestrogens have been reported to decrease cholesterol levels without inducing hypertriglyceridemia. These data indicate that phytoestrogens, including resveratrol, may be an alternative hormone therapy for postmenopausal women (Wuttke *et al*, 2003, Beck *et al*, 2005).

Resveratrol has been reported to bind to ERα and ERβ, with high affinity to ERβ (Bowers *et al.*, 2000). Resveratrol has been shown to have a mixture of agonist and antagonist effects at ERs. For example, resveratrol has been shown to have agonist activity when it binds with ERα. In *in vivo* studies, resveratrol treatment of stroke-prone spontaneously hypertensive rats demonstrated an estrogen-like effect, resulting in endothelium-dependent vascular relaxation induced by acetylcholine, and protection from a reduction in strength of the femoral bone caused by ovariectomy (Bhat *et al*., 2001). However, in this study it was not possible to dissociate effects mediated through ERα with those mediated through ERβ. Furthermore, treatment of MCF-7 cells with resveratrol stimulated gene expression and promoted cell proliferation (Gehm *et al.*, 1997; Gehm *et al.*, 2004) in a manner similar to estrogen. In contrast, an antagonist effect that contrasts with the response to estrogen has been observed. For example, Kim *et al.* (2004) reported that treatment of the MCF-7 cell line with resveratrol resulted in inhibition of cell growth.

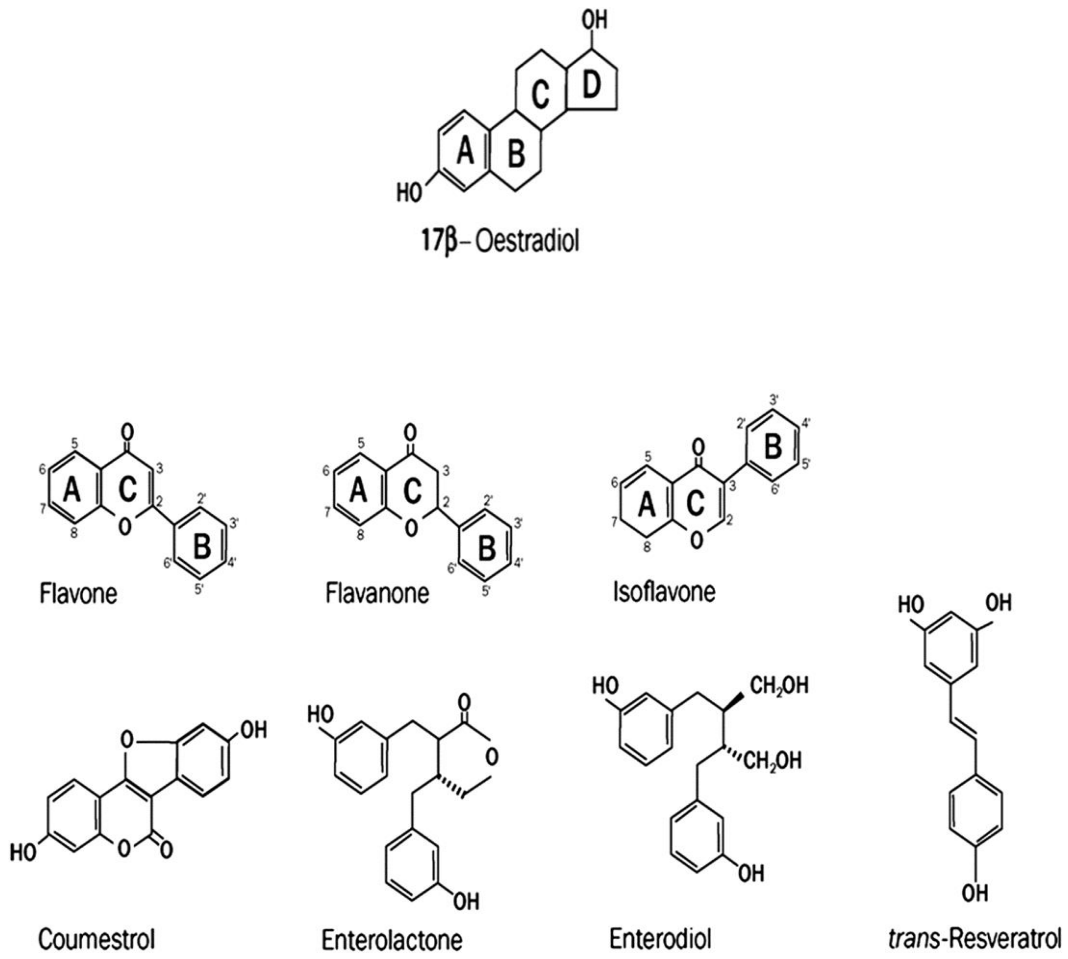


Figure 1.2: Chemical structures of selected phytoestrogens and 17βestradol. Adapted from (Rice and Whitehead, 2006)

1.1.5.3 Resveratrol and age-related diseases

Several studies have demonstrated that resveratrol may be able to play a role in reducing the incidence of age-related disease such as cardiovascular disease (Petrovski and Gurusemy, 2011) and cancer (Sun et al., 2006, Raylam *et al.*, 2008; Dhar *et al.*, 2011), as well as Alzheimer disease (Karuppagounder, 2008; Vingtdeux *et al.*,2008). The potential roles of resveratrol in delaying common age-related diseases are discussed in the following sections.

1.1.5.3.1 Resveratrol as an anti-cancer factor

Carcinogenesis is a process that involves unlimited growth of the cells such that the tumour expands locally by invasion and spreads systemically by metastasis. The carcinogenesis process consists of three different phases: initiation, promotion and progression. The first phase, initiation, involves a mutation of DNA in normal cells. Mutations may be induced by exposure to procarcinogenic agents such as polycyclic aromatic hydrocarbone (PAH), or nitrosamines (Grynkiewicz and Opolski, 2005).

The second stage is promotion, which results from expression of the mutated DNA. Proliferation of the initiated cell results, followed by further progression through the carcinogenesis pathway. The third phase is advancement and invasion. This stage is associated with the growth of the initiated cells into a biologically malignant tissue. True cancer develops in this stage, when a portion of the tumour cells is transformed into malignant forms. In this case chemopreventive intervention is ineffective due to the advanced state of the tumour. In the final stage of invasion, tumour cells can invade the other tissues that are distant from their original site to start new clones of growth (Grynkiewicz and Opolski, 2005).

Resveratrol, as anti-cancer agent, has been shown to inhibit tumour cell proliferation during all three stages of cancer- initiation, promotion and progression- in several types of tumour including breast cancer, prostate cancer (Fang *et al.*, 2012), colon cancer and pancreatic cancer (Shanker *et al.*, 2011). Studies *in vivo* using animal models, recorded that the oral administration of resveratrol inhibited the development of different types of tumours including espophageal cancer (Li *et al.*, 2002), intestinal cancer (Tessitone *et al.*, 2000) and breast cancer (Bhat *et al.*, 2001; Banerjee *et al.*, 2002). Furthermore, oral administration of resveratrol has been reported to protect against colon cancer in rats exposed to carcinogenic compounds such as 1,2-dimethylhydrazine (Sengottuvelan *et al.*, 2006 a; Sengottuvelan *et al.*, 2006 b).

There are several mechanisms by which resveratrol may suppress the cancer process. For example, resveratrol may suppress the initiation stage of tumorigenesis by inhibiting the phase I cytochrome P450 enzymes such as CYP1A1, CYP1B1, which are overexpressed in various human tumours including breast cancer, lung, liver and colon cancer (Chang *et al*., 2000; Chan and Delucchi, 2000). Oxidation by these enzymes involves either unmasking or adding a polar group such as hydroxyl (-OH), amino (-NH2) or sulphydryl (-SH) to promote degradation and elimination of xenobiotics (pro-carcinogens and drugs) by making them water-soluble (Sheweita and Tilmisany, 2003; Jancova *et al.*, 2010).

Moreover, resveratrol can stimulate phase II drug metabolising enzymes such as glutathione S-transferase, UDP-glucuronosyl transferase, and menadione oxido-reductase, which have a fundamental role in reducing DNA damage (Savouret and Quesne, 2002). Conjugation by these phase-II enzymes with large polar groups such

as glucuronide, glutathione or sulphate plays a critical role in increasing solubility and clearance (Sheweita and Tilmisany, 2003; Jancova *et al.*, 2010).

Depending on the relative carcinogenicity of the parent molecule and metabolite, phase- I and II enzyme action may either be beneficial or detrimental with respect to cancers-protection, hence inhibition by resveratrol may equally be of benefit or harm. Effective action of these enzymes may have a protective effect as a result of catalysing the conversion of carcinogenic xenobiotics compounds to less active and/or more rapidly eliminated metabolites (Sheweita and Tilmisany, 2003). However, other actions of these enzyme families can convert pro-carcinogens to carcinogens, thus the interactions are complex.

A third mechanism through which resveratrol can be chemopreventive is by stimulation of DNA repair systems. Excessive exposure to UV irradiation and xenobiotics is associated with DNA damage. Failure of cellular system to repair this damage leads to carcinogenesis. For protecting cellular DNA against the effects of mutational damage, several genes such as p53 survey the genome for damage and / or to repair this damage.

P53, a tumor suppressor protein, has many biological functions including cell-cycle regulation, DNA damage repair, induction of apoptosis, development, differentiation, and cellular senescence (Bai and Zhu, 2006). As a tumor suppressor, p53 is essential in protecting DNA against mutagenic damage in addition to its role in preventing inappropriate cell growth. DNA damage has been reported to activate p53 through a mechanism depending on phosphorylation at Ser 15 (She *et al.*, 2001). This activation of p53 promotes the transcriptional activity of several genes involved in the DNA repair system and induces cell cycle arrest in the G1, G2 and S phases to provide

additional time for the cell to repair DNA damage (Bai and Zhu, 2006; She *et al.*, 2001). Mutation or loss function of the p53 gene has been shown to increased susceptibility to cancer by 50% (Olivier *et al.*, 2010).

Resveratrol is able to stimulate DNA repair by increasing the activity of p53 in various cell lines. Resveratrol also inhibits tumour promotion by modulation of the cell cycle and by the induction of apoptosis (Benitez *et al.*, 2006). Resveratrol may also induce apoptosis by activating MAP kinases such as ERKs and p38 kinase, which play essential roles in stimulating p53 phosphorylation at Ser 15 (She *et al.*, 2001).

1.1.5.3.2 Resveratrol as an anti-cardiovascular disease factor

The protective role of resveratrol against coronary disease appears to result from several features of its activity. Firstly, resveratrol has been documented to reduce the production of nitric oxide (NO) from vascular endothelium, which is involved in the inflammatory responses (Tsai *et al.*, 1999, Donnelly *et al.*, 2004). Increased levels of NO can induce vascular damage, which is increased by development of atheromatous plaques (Orsini *et al.*, 1997). Secondly, a potential effect of resveratrol is to inhibit oxidation of LDL, which plays a critical role in atherosclerosis (Fan *et al.*, 2008). Thirdly, resveratrol has been shown to inhibit platelet aggregation, a critical factor associated with atherosclerosis process (Olas *et al.*, 2001, Fraczek *et al.*, 2012). This process of thrombus formation is induced when platelets attach to the endothelial surface of blood vessels. This aggregation of platelets sets into motion the process of vascular occlusion. A dose-dependent decline in platelet aggregation has been documented with resveratrol, providing further evidence of its protective properties

against coronary artery disease. This effect has been associated with the ability of resveratrol to suppress eicosanoid synthesis (Fremont, 2000, Stocco *et al.*, 2012).

1.1.5.4 Resveratrol and longevity

Dietary restriction is known to delay the aging process in short- lived organisms including yeast, flies, worms and mice. Ongoing studies in primates, including humans, are indicating that dietary restriction induces metabolic and physiological changes consistent with longevity in these longer-lived organisms also (Messaoudi *et al*, 2006). A possible mechanism through which dietary restriction may enhance longevity – additional to protection against specific age-related diseases- includes reduced generation of free radical molecules by mitochondria that cause oxidative damage of macromolecules (protein, lipid, and DNA). Moreover, dietary restriction (DR) has been reported to improve body energy efficiency by inducing the biogenesis of mitochondria that produce fewer free radical molecules and consume less oxygen (Civitarese *et al.*, 2007). In *vivo*, DR has been demonstrated to increase the size of the T-cell population, which has an important role in protecting against infection and cancer, in both mice and rhesus monkeys (Messaoudi *et al*, 2006). In rhesus monkeys, DR delayed the incidence of age-related diseases, decreased body weight, improved immune functions, decreased the level of lipid in blood, and reduced blood pressure, but an effect to prolong life was not observed (Mattison *et al.*, 2003, Mattison *et al.*, 2012)

Resveratrol has been reported to mimic to DR and extend lifespan in diverse species (Howitz *et al*., 2003; Wood *et al*, 2004; Baur *et al.*, 2006) through mechanisms that

may be dependent on activation of Sirt1 (Baur *et al.*, 2006). A further overview of Sirt1 function and its possible role in the response to DR is provided in section 1.4.

In lower organisms, such as the yeast *Saccharomyces cerevisiae*, resveratrol significantly increased life span through a mechanism dependent on Sirt1 (Howitz *et al.*, 2003). Resveratrol was also reported to promote extended lifespan in worms, fruit flies (Wood *et al.*, 2004; Bass *et al.*, 2007) and vertebrate fish (Valenzano *et al.*, 2006) by similar mechanisms. In mammals, resveratrol mimics effects of dietary restriction in improving health. Effects of resveratrol consistent with lifespan extension include reduced insulin-like growth factor-1 (IGF-I) levels, increased insulin sensitivity, increased mitochondrial number, and improved motor function. In mice, resveratrol was reported to oppose the effects of a high-fat diet (Baur *et al.*, 2006; Lagouge *et al.*, 2006; Brasnyo *et al.*, 2011) and long term consumption of low doses (4.9 mg/kg/day) induced expression of genes in brain, skeletal muscle and heart in pattern similar to DR (Barger *et al*, 2008a; Barger *et al*, 2008b). For example, uncoupling protein 3 (upc3) increased in response to DR (2.8 fold, P=0.01) and resveratrol (2 fold, P=0.018) treatment. Chromodomain-helicase-DNA-binding protein 1 (CHD1) and DNA (cytosine-5)-methyltransferase 3A (DNMT3A) were also increased with both DR and resveratrol treatment. Sirt5 was significantly decreased in response to DR and resveratrol treatment (Barger *et al*, 2008a; Barger *et al*, 2008b).

1.2 Estrogen receptors

Estrogen receptors are ligand-activated transcription factors belong to the large superfamily of receptors known as nuclear receptors located inside the target cells of estrogen action. There are two functional forms of estrogen receptors, ERα and ERβ, which are encoded by different genes located on separate chromosomes. The ERα

gene (*ESR1*) is located on chromosome 6q25.1, whereas the ERβ gene (*ESR2*) is located on chromosome 14q23.2 *(*Li, 2003). These receptors mediate the biological functions of the hormone 17-β estradiol. Although ERα and ERβ bind with high affinity to the same ligand, they respond differently and induce opposite effects *(*Dahlman-Wright, 2006).

1.2.1 Estrogen receptor structure

Estrogen receptors are composed of six structural domains designed A through to F. The amino- terminal region, composing domain A and B, includes the transactivation region, known as activation function 1 (AF-1). This region is able to enhance transcription regulatory activity of the receptor in the absence of bound hormone (Felig *et al.*, 1995; Kong et al., 2003; Kumar, 2011). Also, the A/B domain contains a coregulator region, which allows coactivators and corepressors to bind and modulate the transcriptional activity of the ER. The DNA-binding domain, comprising to C region, has two zinc finger motifs that play an essential role to recognize and bind to specific sequences known as estrogen response elements (EREs) within the promoter of the target gene. The ER dimerization and hinge region, the D domain, is required for ER dimerization and also for binding to the ERE of the target gene. The E domain is the hormone binding domain and has a binding cavity for estrogen and is also binding site for agonist/antagonist compounds. The C-terminal domain – the F domain- also contains a transcriptional activation domain known as activation function 2 (AF-2), which is a ligand-dependent region (Kumar, 2011).

Structurally, ERα and ERβ show a considerable homology in the DNA-binding domain (96%) and dimerization /ligand-binding domain (60%) but the A/B domain

and dimerization and hinge (D) domain are not well conserved between ER α and ERβ (Figure 1.3) (Kumar, 2011).

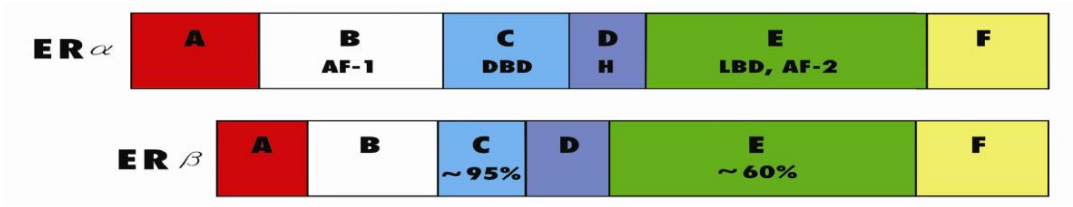


Figure 1.3: Estrogen receptor structure. Estrogen receptors are composed of six structural domains: A and B domain, DNA binding domain (C), hinge domain (D), ligand binding domain (E), and F domain. ERα and ERβ are homologous in the DNA-binding domain (96%) and ligand-binding domain (60%). Adapted from Akingbemi et al., 2005)

1.2.2 Tissue distribution of estrogen receptor

Estrogen receptors are expressed in many tissues. ERα and ERβ have been detected in breast and ovarian tissues, heart, bone, urogenital tract and the hypothalamus (Yaghmaie *et al.*, 2005). The liver and the epithelium of the reproductive duct in male were reported to express ERα exclusion (Gustafsson, 1999, Hess, 2003). In contrast, only ERβ was detected in the gastrointestinal tract (Langen *et al.*, 2011).

1.2.3 Estrogen receptor activation and action

Estrogen is a hydrophobic compound that is able to diffuse through the phospholipid membrane of the cell to bind and to activate intracellular ER. Two major mechanisms have been described to explain the action of the ligand-ER complex: the classic

pathway, known as the genomic pathway, and the nongenomic pathway (Kong *et al.*, 2003).

1.2.3.1 Genomic pathway

The genomic pathway, also known as the classical pathway, involves the direct binding of estrogen with the ER located in the cytoplasm. This binding can release the ER from heat shock proteins (HSPs). Subsequently, the ER-ligand complex migrates from the cytoplasm into the nucleus where homodimers form, then bind to the estrogen response element (ERE) in the DNA to activate gene transcription (Bjornstrom and Sjoberg, 2005; Mason *et al.*, 2010). Activation of this pathway involves interaction of ER-ligand complex with other transcription factors including nuclear factor-KappB 1 (NF-KappaB1), activator protein 1, and specific protein 1 (Sp-1) to influence gene transcription (Levin, 2005).

1.2.3.2 Nongenomic pathway

In addition to the ERs located in the nucleus and cytoplasm, it has been reported that some are associated with cell surface membrane and are rapidly activated by binding to estrogen. This binding stimulates various intracellular signaling cascades to recruit second messengers including physphatidylinosinol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPKs), which regulate transcription of specific genes (Bjornstrom *et al.*, 2005).

1.2.4 Estrogen receptors and reproduction

Estrogen receptor knockout (KO) mice have revealed functions of the estrogen receptors. Estrogen receptor α knockout mice (αERKO), estrogen receptor β

knockout mice (βERKO) and estrogen receptors α and β knockout mice (α βERKO) reveal further roles of the specific estrogen receptors (α and β). Uses of αERKO mice demonstrated the role of ERα in sexual maturation and fertility. For example, female αERKO mice have estrogen insensitivity associated with defects in the reproductive tract, including hypergonadism and haemorrhagic (blood-filled) ovarian cysts, plus abnormality in pubertal mammary gland development. Males develop phenotypes including testicular degeneration, lack of spermatogenesis and inactive sperm. Consequently, both sexes of these mice are sterile and show severe defects in sexual behaviour (Emmen and Korach, 2003; Hewitt and Korach, 2003). With respect to reproductive function, lack of ERβ has much more minor consequences, βERKO female mice have compromised fertility, a reduction in the corpora lutea associated with defective follicle development and ovulation. Interestingly, these mice show normal reproductive behaviour, a normal response to the estrogen and normal mammary gland development. No effect was observed on the fertility and sexual behaviour of male mice (Korach, 1994). Taken together, these studies suggest that the action of ERα and ERβ are independent and gender- specific.

1.2.5 Estrogen receptors and ageing

Various lines of evidence point towards a decline in ER expression with ageing, with ageing-related phenotypic consequences. For example, a study in humans using retinal tissue extracted from three females at 35, 49 and 74 years indicated that the expression of estrogen receptor ERα reduced with age, suggesting that alterations in estrogen receptor expression may be involved in pathologies associated with age, such as reduction of visual function, cataract, glaucoma, and dry eye (Ogueta *et al.*, 1999).

In female rats, the number of cells expressing estrogen receptor ERβ in the brain nuclei (AVPV region) dramatically decreased in old animals (24-26 months) compared with young animals (3-4 months), whereas an age-related increase in the number of cells expressing ERα was observed. The AVPV region plays an important role in reproductive physiology and behaviour, suggesting the importance of ERβ signalling in mediating reproductive behaviour (Chakraborty *et al.*, 2003). In a study with the aim to investigate the effect of age on estrogen receptor expression and whether or not this effect was opposed in response to DR, male rats (at 2-18 months old) were subjected to DR and estrogen receptor expression was measured in the testicular tissues. An age-related reduction in ERα and ERβ was observed, which may explain the functional deficit of the testicular cells observed with age. In contrast, ER expression was maintained at higher levels under conditions of DR, indicative of a protective effect (Hamden *et al.*, 2008).

1.2.6 Estrogen receptors and obesity

A growing body of evidence using estrogen receptor knockout (KO) mouse models indicates that estrogen receptors may be involved in the regulation of fat deposition. Heine *et al* (2000) reported that knockout of ERα in mice was associated with different phenotypes including obesity, insulin resistance, increased plasma leptin and decreased plasma adiponectin associated with a reduction in energy expenditure. Recently, further study using ERα KO mice provided evidence for a contribution of ERα to the regulation of fat deposition and insulin resistance (Manrique *et al.*, 2012).

1.2.7 Estrogen receptors and cancer

Several studies have demonstrated that changes in estrogen receptor expression can be associated with cancer development in different tissues including breast (Dotzlaw *et al.*, 1999), ovarian (Rutherford *et al.*, 2000), and colon (Foley *et al.*, 2000) tissues.

In ER-positive cancer such as breast cancer, overexpression of estrogen receptor ERα and reduced expression of ERβ has been reported, suggesting that the ratio of ERα: ERβ may determine the susceptibility of the tissue to tumorgensis (Roodi *et al.*, 1995; Leygue et al., 1998; Iwao *et al.*, 2000).

Estrogen receptors have been identified as a target for cancer treatment using endocrine therapy, selective estrogen receptor modulators (SERMS) compounds with the ability to bind and to modulate estrogen receptor activity. These compounds include tamoxifen, which acts as an ER antagonist in breast cancer (Osborne, 1998, Sestak and Cuzick, 2012) and fulverstrant, which acts as a potential antagonist and promotes estrogen receptor degradation (Wakeling *et al.*, 2000, Buzdar, 2008; Larsen *et al.*, 2012).

1.3 Chromatin and histone proteins

In the nucleus of eukaryotic cells, DNA is packed as chromatin, consisting of repeated nucleosome unites. Typically, a nuclosome consists of genomic DNA (147 base pairs) wrapped around an octomer of the core histone proteins H2A, H2B, H3, and H4 (Figure 1.4) (Luger *et al.*, 1997, Izzo and Schneider, 2011). Chromatin exists in two major forms heterochromatin, which is transcriptionally silent, and euchromatin, which is transcriptionally active (Grewal and Moazed, 2003).

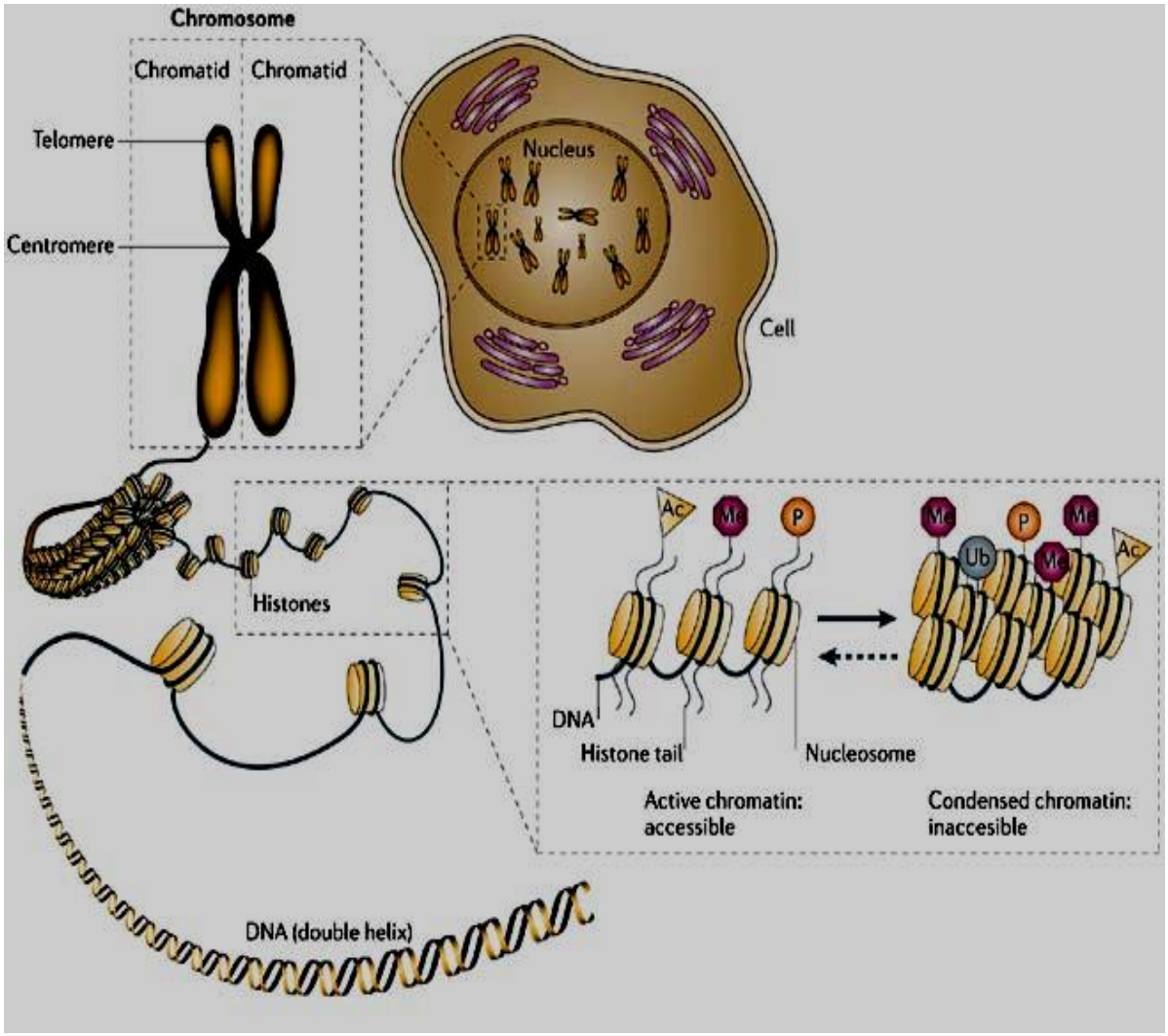


Figure 1.4: Chromatin structure. Adapted from (Sparmann and Lohuizen, 2006). *In the nucleus of eukaryotic cells, DNA is wrapped around an octomer of the core histone proteins H2A, H2B, H3 and H4 to form a nuclosome. Repeated nucleosome unites are packed to form chromatin. Chromatin is then folded to form chromosomes.*

1.3.1 Histone protein features

There are two types of histone proteins- core histone proteins- including H2A, H2B, H3 and H4, and a linker histone known as H1. These proteins are rich in the positively charged amino acids which bind tightly to the negative charge of DNA. Each of core histone proteins has a flexible N-terminal tail extending outward. In addition, histones H2A and H2B have a C-terminal tail that also extends outward from the nucleosome (Izzo and Schneider, 2011).

1.3.1.1 Histone genes

Several subtypes of each histone protein are encoded by different genes. These genes are divided, based on their expression, into three major groups: (1) replication-dependent histone genes, whose expression is tightly coupled with the S-phase of the cell cycle. In total, 58 histone genes have been identified as replication-dependent histone genes. These genes lack introns and encode an mRNA that lacks a poly (A) tail. (2) Replication- independent histone genes, which encode replacement histones. These variant histone proteins are synthesized from polyadenylated mRNAs that are expressed throughout the cell cycle and in non-dividing differentiated cells. (3) Genes expressed in specific tissues such as H1t and H3t genes, which are expressed in testicular tissues (Albig *et al.*, 1997; Marzluff *et al.*, 2002).

Approximately 80% of the histone genes are clustered on the short arm of chromosome 6 (6p21.3-22). Several genes encoding core histone proteins (H2a, H2b, H3 and H4) are clustered on long arm of chromosome 1 (1q21) (Albig *et al.*, 1997; Marzluff *et al.*, 2002).

1.3.1.2 Regulation of histone gene expression

The replication-dependent histone mRNAs are tightly cell-cycle regulated and their expression is dependent on the process of DNA replication. Three major pathways have been reported to regulate the levels of histone mRNAs synthesis during the cell cycle. The first pathway regulates histone mRNA transcription at G1/S phase transition. Histone gene transcription increases three to five fold during G1/S phase transition and then decreased to baseline level at the end of S phase (Marzluff and Duronio, 2002). The two remaining pathways regulate histone mRNA production at a posttranscriptional level. The first pathway of posttranscriptional regulation occurs in the cytoplasm to govern the half-life of histone mRNAs through their degradation when DNA synthesis is inhibited. For example, the half-life of histone mRNAs in S-phase dropped from 30-60 minutes to 10-15 minutes when chromosome replication is blocked with inhibitors of DNA chain elongation (Marzluff and Duronio, 2002; Gunjan *et al.*, 2005). The second pathway of posttranscriptional regulation occurs in the nucleus to produce mature histone mRNA. Since histone genes lack introns, the formation of mature histone mRNA requires only one RNA- processing reaction, which involves an endonucleolytic cleavage to form the 3` end of the mRNA. This reaction is directed by a purine-rich sequence, termed the histone downstearm element, that is complementary to the 5` end of U7 snRNA. Additionally, the 3` end of the histone mRNA tail contains a stem-loop that interacts with a specific protein termed the stem-loop binding protein (SLBP). This protein participates in many steps of histone mRNA metabolism in both the nucleus and cytoplasm. SLBP remains associated with mature histone mRNA to form a complex to protect histone mRNA from degradation during S-phase. At the end of S-phase, the histone mRNA-SLBP

complex disassociates, allowing rapid degradation of both histone mRNA and SLBP (Marzluff and Duronio, 2002; Gunjan *et al.*, 2005).

Histone protein synthesis and DNA replication are considered to be interdependent process. For example, DNA replication is affected by the rate of histone protein synthesis. Overexpression of the HIRA protein, which represses transcription of all the replication-dependent histone genes, arrests cells in S-phase (Marzluff and Duronio, 2002; Gunjan *et al.*, 2005).

1.3.2 Histone modification

The core histone proteins and their tails (N-terminal and C-terminal) undergo a variety of post-translational modifications such as acetylation (ac) of lysine residues, methylation (me) of lysine and arginine residues, phosphorylation (ph) of serine, threonine and tyrosine (Y) residues, mono-ubiquitination (ub) of lysine residues, SUMOylation of lysine residues, and ADP-ribosylation (ar) of lysine residues (reviewed in Perterson, 2004). These modifications are highly specific, occurring in specific amino residues within specific histone proteins (Bartova *et al.*, 2008; Izzo and Schneider, 2011) and mostly occurring in amino terminal tails (Figure 1.5). Among these modifications of histone, histone acetylation is a highly dynamic process. Histone H3 and histone H4 proteins in particular are subject to extensive modifications (Cosgrove *et al.*, 2004).

Histone modifications play fundamental roles in gene expression, DNA repair, apoptosis, DNA replication and chromosome condensation (Cheung *et al.*, 2000). In

addition, histone acetylation and histone methylation are involved in epigenetic regulation of chromatin (Bartova *et al.*, 2009).

1.3.2.1 Histone acetylation

Histone acetylation is a highly dynamic process involving the addition of acetyl groups to lysine amino groups on the N-terminal tails of the core histones. This reaction is catalysed by histone acetyltransferases (HATs) and requires acetyl coenzyme A (acetyl co-A) as an acetyl group donor. Different lysine (K) residues are known to be targets for acetylation within histones. These sites are H3K9, H3K14, H3K18, H3K23, and H3K27 at histone H3 and H4K5, H4K8, H4K12, H4K16 at histone H4. In addition, H2AK5 at histone H2A and H2BK5, H2BK12, H2BK15 and H2BK20 at histone H2B are recognised to be acetylation sites (Bartova *et al.*, 2008). The hyperacetylation of lysine residues is associated with activation of gene expression by neutralising the positively charged lysine residues in the histone tails, which decreases their affinity for DNA. This process influences nuclosome unfolding and thereby facilities binding of transcription factors to the promoter of the target gene (Jacobson *et al.*, 2000).

Histone acetylation is a reversible process in which the acetyl group is removed from acetylated lysine residues by histone deacetylase enzymes (HDACs). These enzymes are subgroubed into three classes. Class I histone deacetylase enzymes comprise small proteins (377-488aa) and include HDAC-1, HDAC-2, HDAC-3 and HDAC-8 (Bjerling *et al.*, 2002). Class II HADCs are larger proteins (669-1215aa) and include HDAC-4, HDAC-5, HDAC-6, HDAC-7 and HDAC-9 (Fischle *et al.*, 2002). Class III HADCs are NAD-dependent proteins, and include the mammalian sirtuin (Sirt1-7)

family, and its homolog Sirt2 in yeast (Delage and Dashwood, 2008). Class I and class II histone deacetylases require zinc as a co-factor (Hernick and Fierke, 2005). Class III HADCs require one molecule of NAD+ for each acetyl group removed from the target protein (Blander and Guarenet, 2004). Generally, histone deacetylation has been associated with transcriptional repression.

Links between histone acetylation and ageing have been reported. For example there was a significant decrease in the level of histone H3 acetylation at K9 in liver in old (30 months) rats compared with young (15 months) rats (Kawakami *et al.*, 2009). Two possible mechanisms were suggested to explain this reduction of histone acetylation with age: (1) upregulation of Sirt 1, which catalyses the deacetylation of histone proteins and downregulates on of histone acetyltransferase with age, and (2) a histone turnover, where acetylated histone is replaced by newly synthesize protein (Kawakami *et al.*, 2009).

In contrast, a study in yeast reported an increase in the level of histone 4 acetylation (H4K16ac). This increase in histone acetylation was associated with a progressive decrease in the level of the Sir2 deacetylase protein with age (Dang *et al.*, 2009).

1.3.2.2 Histone methylation

Lysine, arginine, and histidine residues in the histone proteins can be substrates for methylation (Kouzarides, 2007). Lysine residues can be monomethylated, dimethylated or trimethylated (Sims *et al.*, 2003) with specific targets being H3K4, H3K9, H3K27, H3K36, H3K79 and H4K20 (Garcia *et al.*, 2004). Lysine methylation of histone proteins is site and state-specific and is catalyzed by lysine methyltransferases (KMT) (Dillon *et al.*, 2005). This process is reversed by histone

demethylase enzymes such as lysine specific demethylase 1 (LSD1), which is specific for mono-methylated and di-methylated residues (Shi *et al.*, 2004).

Arginine (R) residues can be mono-methylated or di-methylated. The reaction is catalysed by enzymes of the protein arginine methyl transferase family (PRMT). The reverse reaction is catalysed by peptidylarginine deiminase 4 (PAD4) (Cuthbert *et al.*, 2004; Wang *et al.*, 2004). Three sites are known to be targets for arginine methylation in histone H3 (R2, R17, and R26) whereas one site (R3) in H4 has been reported to be methylated (Izzo and Schneider, 2011).

Histone methylation plays essential role in the regulation of many biological processes such as, development, differentiation, cell-cycle, and DNA damage. The functions of histone methylation depend mainly on the methylation status and the genomic location. For example, For example, H3K9 methylation is associated with heterocromatin formation and euchromatic gene silencing, whereas, H3K27 methylation plays an essential role in HOX genes suppression and in X chromosome inactivation and imprinting during development (Greer and Shi, 2012).

Alteration in the methylation of specific histones has been reported to be associated with disease and ageing (Greer and Shi, 2012). Histone methyltransferase (EZH2), which catalyzes the trimethylation of histone 3 on lysine 27 (H3K27me3), is upregulated in different types of cancer such as breast cancer, prostate cancer, and lymphoma (review in Greer and Shi, 2012). The resulting effect to reduce H3K27me3 may be in part, associated with the cancer process.

In rat liver, the level of trimethylation of histone H4 at lysine 20 (H4K20me3) was seen to increase with age (Sarg *et al.*, 2002).

Histone methylation may be linked also to lifespan. Knockdown of the ASH-2 and WDR-5, members of an H3K4 trimethylation (H3K4me3) complex, or the regulator protein SET-2 extends lifespan of worms. All these proteins catalyse H3K4 trimethylation in both the developing and the adult germline in the worm. In line with this finding, overexpression of RBR-2, the H3K4me3 demethylase in the worm, extends lifespan, whereas knockdown of RBR-2 reverses this effect (Greer *et al.*, 2010).

1.3.2.3 Histone phosphorylation

All histone proteins are subject to phosphorylation at serine (S), threonine (T) and tyrosine (Y) residues. This modification is catalyzed by several distinct kinases, and is also dependent on phosphatise activity (Singh and Gunjan, 2011). Histone phosphorylation has important roles in regulating gene expression, chromatin condensation, the DNA damage response and apoptosis (Nowak and Corces, 2004). For example, phosphorylation of H2AX at S139 is rapidly increased in response to DNA damage causing activation of DNA repair genes and a delay of the cell cycle. Defects in H2AX phosphorylation have been associated with cancers (Singh and Gunjan, 2011).

Phosphorylation of H3 at S10 (H3S10) has been reported to promote gene activity through cross-talk with other histone modifications. For example, H3S10 phosphorylation can supporting an active transcriptional state by stimulating acetylation of histone H3K14, inhibiting acetylation of histone H3K9, and modulating methylation of histone H3K9. Furthermore, phosphorylation of H3T11 activates transcription by enhancing demethylation of H3K9. Phosphorylation of H3S10 along with H3T45 and H2BS14 is implicated in apoptosis (Cohen *et al.*, 2011).

There is very limited published data on the effect of age on histone phosphorylation, however the link was studied in the rat liver. This study reported an increase in H3 phosphorylation at S10 in 30 month-old rats compared with 15 month-old animals (Kawakami *et al.*, 2009).

1.3.2.4 Histone ubiquitinylation

Ubiquitin is a small protein that can be covalently attached to specific lysine residues in proteins including the histone proteins (Peterson and Laniel, 2004). Ubiquitination occurs on specific lysine residues in the C-terminal region of histone H2A and histone H2B (Sun and Allis, 2002). A single molecule of ubiquitin is covalently attached to both H2A at Lys 119 (ubH2A) and H2B at Lys 120 (ubH2B) (Cao and Yan, 2012).

The monoubiquitination of H2A and H2B has been reported to alter chromatin dynamics and regulate gene expression. H2A ubiquitination is associated with gene silencing, whereas ubiquitination of H2B is associated with active transcription (Cao and Yan, 2012). Crosstalk between H2B ubiquitination and histone H3 methylation has been reported. H2B ubiquitination is required for H3K4 and H3K79 methylation. This histone crosstalk seems to function unidirectionally. Mutations affecting H2B ubiquitination, reduced level of H3 methylation. In contrast, mutation of the H3 methylation sites or methyltransferases has no effect on H2B ubiquitination (He and Lehming, 2003).

Similar to H2B, crosstalk between H2Aub and histone H3K4 methylation has been observed. For example, H2Aub inhibits MLL3, which mediates di- and trimethylation of histone H3 at Lys-4 causing transcriptional repression (Nakagawa *et al.*, 2008).

A link between histone ubiquitination and ageing was observed in mouse brain where the level of histone ubiquitination was increased by 30% in old mice compared with young animals (Morimoto *et al.*, 1993).

1.3.2.5 Histone SUMOylation

SUMO is a small ubiquitin-related modifier that can be attached covalently to histone proteins at specific lysine residues and is associated with transcriptional repression of the associated region of the genome (Shiio and Eisenman, 2003). Several sites are recognised to be targets for SUMOylation including K6 and K7 in H2B, K126 in H2A, K16/17 in H2B and all five lysines in the N terminal region of H4. This modification is catalysed by dedicated E1 activating (SAE1/SAE2) and E2 conjugating (UBC9) enzymes to form a peptide bond between the C-terminus of SUMO and the amino group of the target lysine (Iñiguez-Lluhí, 2006).

In addition to direct effects of SUMOylation on histone proteins, chromatin structure is affected indirectly through effects on histone-modifying enzymes. For example, SUMOylation reduces the activity of histone deacetylases. Additionally, the activity of histone demethylase LSD1, which catalyses removal of methyl groups from mono- or di-methylated histone H3 at lysine 4 (H3K4), is repressed by SUMO. Loss of SUMOylation thus promotes transcriptional repression by stimulating both histone acetylation and histone demethylation (Iñiguez-Lluhí, 2006; Ouyang and Gill, 2009).

SUMO plays critical role in the regulation many biological processes such as cell cycle progression, genomic stability, and transcription by modifying various enzymes

and cofactors that are important for regulated gene expression, as well as coordinating chromatin structure and histone modifications (Ouyang and Gill, 2009).

1.3.2.6 Histone ADP- ribosylation

This modification involves the addition of (anionic) ADP ribose polymers to histone proteins by the action of PARPs (poly-ADP ribose polymers), predominating at lysine residues in the tails of histone H4 (K13), H2B (K30), H3 (K27 and K37) and H4 (K16). These modifications take place during or after histone synthesis. ADP-ribosylation has important roles in various biological processes such as DNA repair, cell cycle regulation replication or transcription (Hottiger, 2011). The recognized sites of ADP-ribosylation are also targets for other histone modifications, such as acetylation, methylation, and phosphorlation, thus ADP-ribosylation may interact with these other histone modifications. For example, acetylation of H4 at K16 inhibits ADP-ribosylation of H4. Furthermore, ADP-ribosylation of histones reduces their phosphorylation and prevents demethylation of H3K4me3 (Messner and Hottiger, 2011).

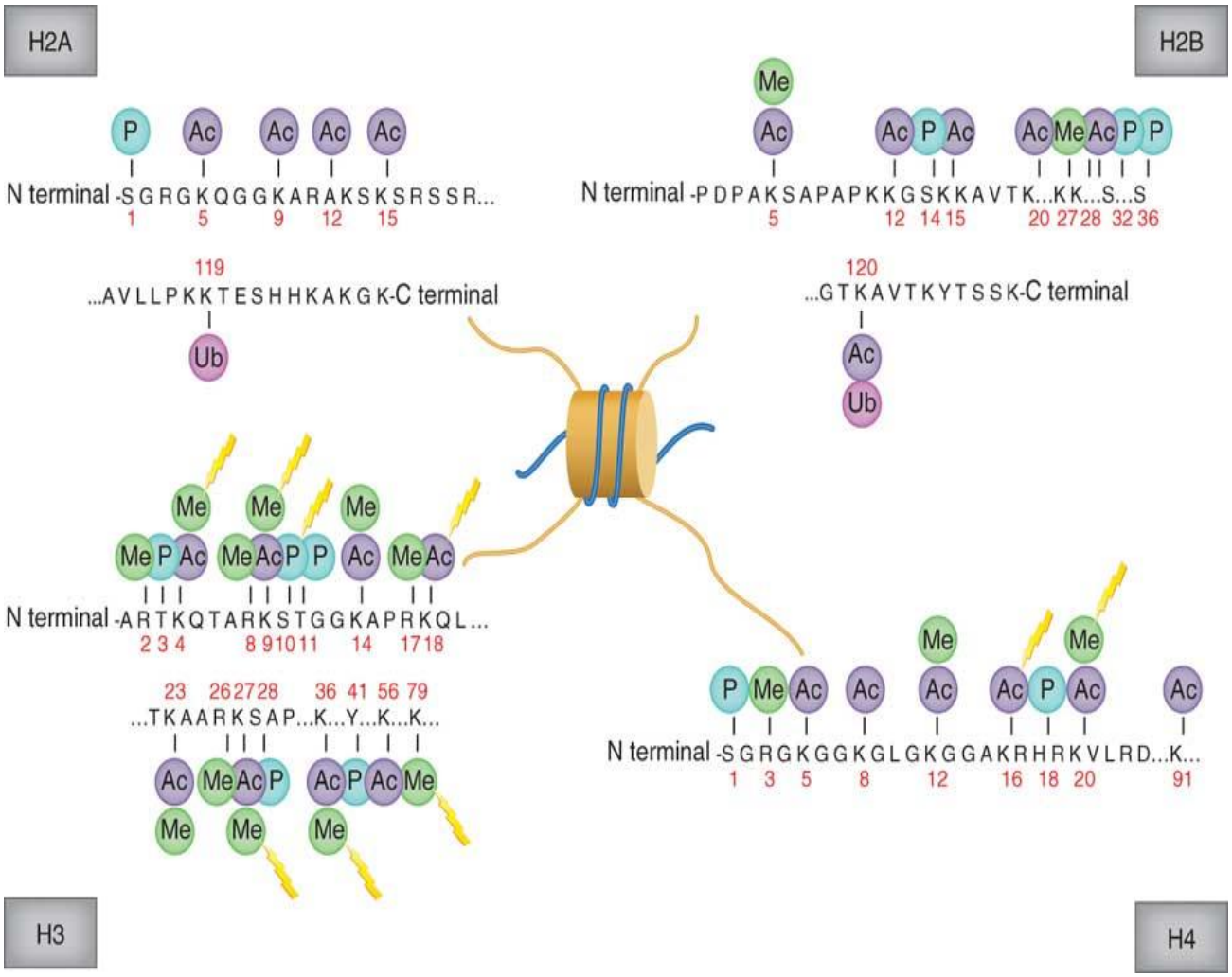


Figure 1.5: Histone modifications. Adapted from (Rodriguez-Paredes and Esteller, 2011). *The core histone proteins (H2A, H2B, H3 and H4) and their tails (N-terminal and C-terminal) are subjected to a variety of post-translational modifications such as acetylation (Ac) of lysine residues, methylation (Me) of lysine and arginine residues, phosphorylation (P) of serine, threonine and tyrosine residues, and mono-ubiquitination (Ub) of lysine residues. SUMOylation of lysine residues, and ADP-ribosylation of lysine residues (not show) are additional modifications.*

1.3.3 Epigenetic modification

Epigenetic modification is a heritable change in gene expression that results from molecular mechanisms that are not mediated through changes in the DNA sequence. In some cases, epigenetic modifications are inherited across generations (Cheung and Lau, 2009). Two major components of epigenetic modification are histone/chromatin modifications and DNA methylation (Kimura *et al.*, 2005, Bartova *et al.*, 2009). Histone modifications and DNA methylation play a key role in controlling differentiation during embryonic development, inactivation of the X chromosome in the female, genomic imprinting and in DNA repair and DNA double strand break (DSB) repair systems (Richardson, 2002, Celeste *et al.*, 2003).

Histone acetylation, which is catalyzed by histone acetyl transferases, results in a more open euchromatin configuration, which leads to activation of associated loci. This activation of euchromatin can be suppressed by histone deacetylases enzymes, which catalyse the histone deacetylation reaction. In contrast, histone methylation inactivates heterochromatin by providing a binding site for the chromodomain-containing heterochromatin protein 1, which leads to transcriptional repression (Eberharter and Becker, 2002; Bartova *et al.*, 2009).

A second major mechanism associated with epigenetic modification is DNA methylation. DNA methylation is a biological process that involves the addition of a methyl group at the fifth carbon position at cytosine residues in DNA by DNA methyltransferase enzymes (Figure 1.6) (Richardson, 2002).

There is a cyclical relationship between DNA methylation and histone modifications. DNA methylation affects histone modification through the recruitment of proteins including histone deacetylases. Histone acetylation and methylation can, in turn, influence DNA methylation (Cheung and Lau, 2009). DNA methylation levels can be altered, therefore, by changes in expression or acting of DNA methyltransferase enzymes or as a result of changes in histone acetylation status.

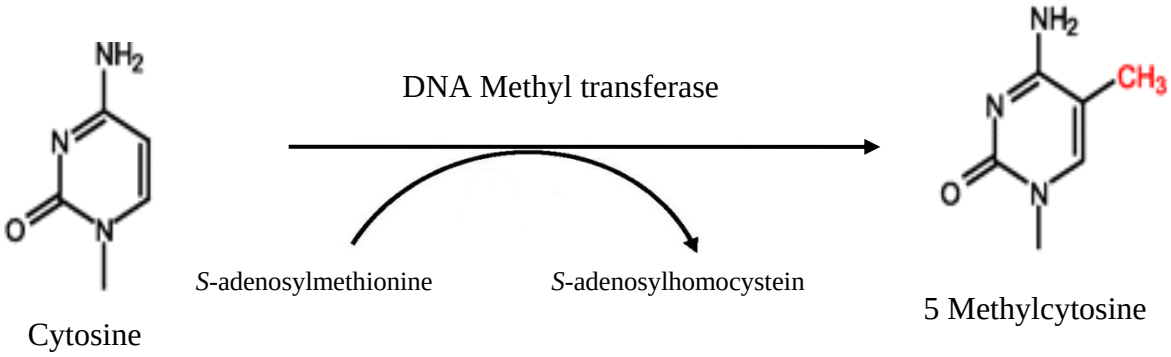


Figure 1.6: DNA methylation mechanism. Adapted from (Chen and Riggs, 2011)

1.4 Sirtuins, focusing on Sirt1

Silent information regulator (SIR) enzymes comprise a large family of proteins referred as sirtuins, which are expressed widely in various species from yeast to mammals (Baur *et al.*, 2012). The first sirtuin gene, Sirt2, was identified in *Saccharomyces cerevisiae* by Klar and colleagues (1979). Later on, Sirt2 homologues were identified in fruit flies, worms, bacteria, plants and mammals (Table 1.2). Sirtuins are NAD^+- dependent histone deacetylases and categorized as class III histone deacetylase (Figure 1.7) (Salminen *et al*, 2009).

In mammals, seven sirtuins (SIRT1-7) have been identified and roles in the regulation of metabolism, growth (McBurney *et al.*, 2003) and inflammation (Pfluger, 2008), as well as a role in lifespan extension, have been described. Among the sirtuins, Sirt1 has been studied extensively and appears to deacetylate a large number of cellular substrates (Baur et al., 2012).

In lower organisms, Sirt2 has been reported to regulate lifespan. For instance, overexpression of Sirt2 was observed to increase lifespan in yeast (Kaeberlein *et al*., 1999), worms (Tissenbaum and Guarente, 2001), and fruit flies (Rogina and Helfand, 2004). In contrast, deletion of Sirt2 in yeast resulted in reduction in lifespan (Kaeberlein *et al.*, 1999).

In mammals, Sirt1 plays an essential role in regulation wide range of cellular process such as transcriptional regulation, apoptosis, stress responses, and longevity by deacetylation of multiple proteins such as p53, p73, Ku70, and forkhead transcription factors (FOXOs). For example, Sirt1 has been reported to suppress the apoptosis

pathway by deacetylation the tumour suppressor p53 at multiple lysine residues. Moreover, Sirt1 has been reported to deacetylate and repress FOXO protein which leads to reduction of cellular stress (Brunet *et al.,* 2004; Motta *et al.,* 2004). Sirt1 has also been reported to induce apoptosis, increase expression of DNA repair and cell cycle checkpoint genes, and to protect pancreatic β-cells against cytotoxicity induced by glucose (Kitamura *et al.*, 2005, Banks *et al.*, 2008; review in Kelly, 2010).

It is believed that Sirt1 may promote longevity and protect from age- related diseases by regulating metabolism and endocrine pathways (Brooks *et al.,* 2009). Mice with Sirt1 overexpressed at low levels were protected from diabetes and hepaticlipid damage resulting from a high fat diet (Bordone *et al*, 2007; Banks *et al.*, 2008). Mice lacking Sirt1 (Sirt1-null mice) have been shown to have a metabolic dysfunction compared with wild-type mice (Boily *et al.,* 2008). Furthermore, heart-specific overexpression of Sirt1 in mice enhanced resistance to oxidative stress and slowed the heart-ageing process (Alcendor *et al*, 2007).

1.4.1 Sirt1 and insulin/ IGF signalling

The insulin/insulin-like growth factor 1 signalling (IIS) pathway has been reported to modulate lifespan in different species (Rinco *et al.,* 2004; Rinco *et al.,* 2005). A single gene mutation in the insulin like growth factor (IGF) signalling pathway has been reported to extend lifespan in various species including worms (Kenyon *et al.,* 1993), flies (Clancy *et al.,* 2001), and mice (Al-Regaiey *et al.,* 2003). In worms, mutants in this gene Daf2 (homologous to insulin receptor in mammals) had increased lifespan. In flies, lack of insulin receptor substrate CHICO (Clancy *et al.,* 2001) or insulin receptor IR (Tatar *et al.,* 2001) were shown to increase lifespan. Flies

homozygous for CHICO mutations had lifespan increased by 48% compared with an increase of 36% in heterozygotes.

In a mouse model, Holzenberger *et al* (2003) reported that heterozygous IGF-1 knockout resulted in extended lifespan in compared with wild type mice. The same finding has been reported for adipose-specific homozygous IGF-1 knockout mice (Blüher *et al.*, 2003).

1.4.2 Sirt1 and dietary restriction (DR)

Dietary restriction (DR), reduction of food intake below *ad libitum* without malnutrition, has been reported to promote longevity in diverse species from yeast to mammals (McCay *et al.*, 1935; Walker *et al.*, 2005) through a mechanism proposed by some researchers to be dependent on Sirt1 in mammals and on its homologue Sir2 in yeast (Cantó and Auwerx, 2009). In mammals, a growing body of evidence supports the view that Sirt1 is involved in mediating the effect of DR on lifespan extension. Firstly, levels of Sirt1 have been documented to increase in mammal tissue in response to DR (Brunet et al., 2004; Nisoli *et al.*, 2005; Barger *et al.*, 2008; Kanfi *et al.*, 2008). Secondly, mice lacking Sirt1 (Sirt1-null mice) showed a metabolism dysfunction and did not respond to DR compared with wild-type mice (Boily *et al.*, 2008). Additionally, the physical activity of these mice was decreased in response to DR (Chen *et al.*, 2005a). In contrast, whole-body overexpression of Sirt1 induced effects similar to the DR in mice, including reduced body weight and enhanced glucose homeostasis. In addition, there was a reduction in the level of insulin, glucose and cholesterol in pattern similar to those were subjected to DR (Bordone *et al.*, 2007).

Table1.2: List of sirtuin genes expressed in several species, their intracellular localisation and their enzyme activities. Adapted from (Dong and Zou, 2010). The identified sirtuins genes for each organism are listed and, where known, sub-cellular location and activity is stated. ART= ADP-ribosyltransferase.

Organism	Gene	Sub-cellular localization	Enzymatic activity
S. cerevisiae	Sir2	Nucleus	Deacetylase/ART
	Hst1	Nucleus	Deacetylase
	Hst2	Cytoplasm	Deacetylase
	Hst3	Nucleus	Unknown
	Hst4	Nucleus	Unknown
C. elegans	Sir-2.1	Nucleus	Deacetylase
	Sir-2.2	Unknown	Unknown
	Sir-2.3	Unknown	Unknown
	Sir-2.4	Unknown	Unknown
D. melanogaster	dSir2	Nucleus	Deacetylase
	dSirt2	Unknown	Deacetylase
	dSirt4	Unknown	Unknown
	dSirt6	Unknown	Unknown
	dSirt7	Unknown	Unknown
Mammals	SIRT1	Nucleus	Deacetylase
	SIRT2	Cytoplasm	Deacetylase/ART
	SIRT3	Mitochondria	Deacetylase/ART
	SIRT4	Mitochondria	ART
	SIRT5	Mitochondria	Unknown
	SIRT6	Nucleus	Deacetylase/ART
	SIRT7	Nucleus	Unknown

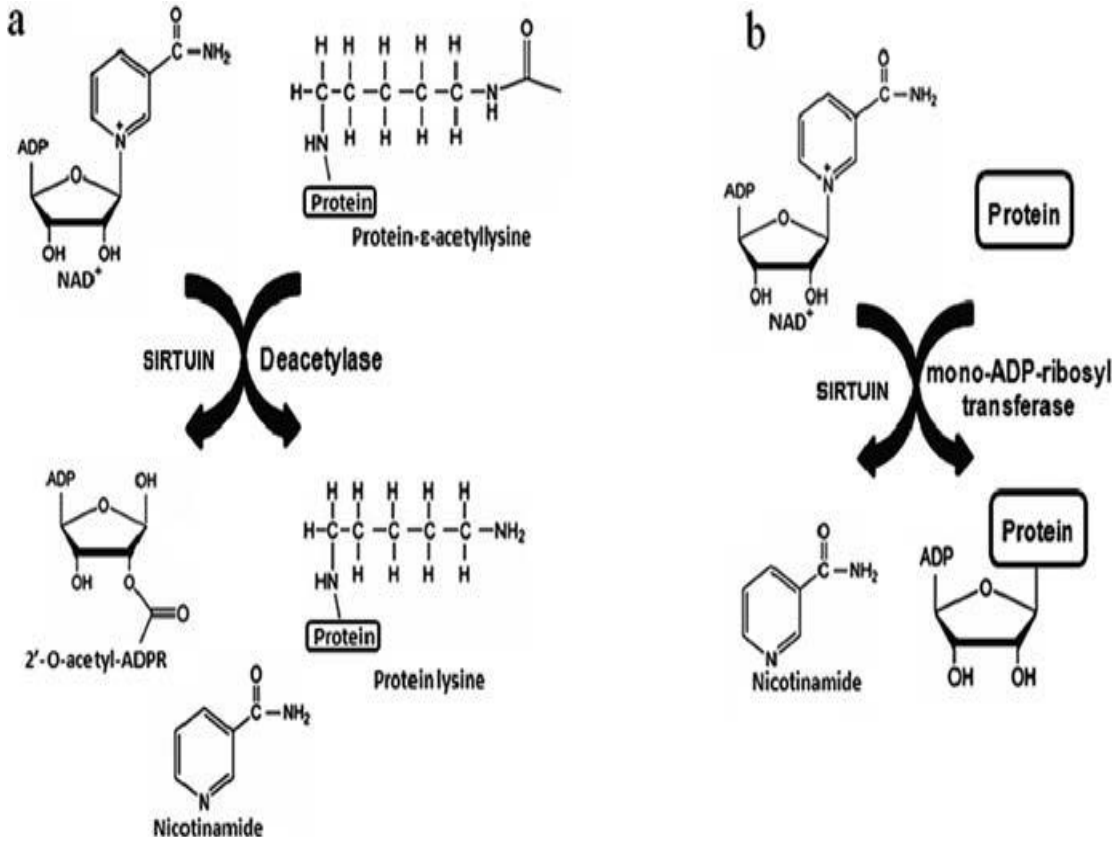


Figure 1.7: The enzymatic activities of sirturins. Sirtuin act as deacetylase (a) and or ADP ribosyltransferase (b) enzymes and regulate the activities of target proteins. Adapted from (Dong and Zou, 2010).

1.5 Hypothesis

The hypothesis underlying the study was that some of the beneficial effects of resveratrol on lifespan/aging are mediated through effects on histone expression that oppose changes observed in ageing, and that these actions of resveratrol influence the expression of genes that affect the ageing process. A second hypothesis was that these effects of resveratrol are mediated through ERα and/or ERβ.

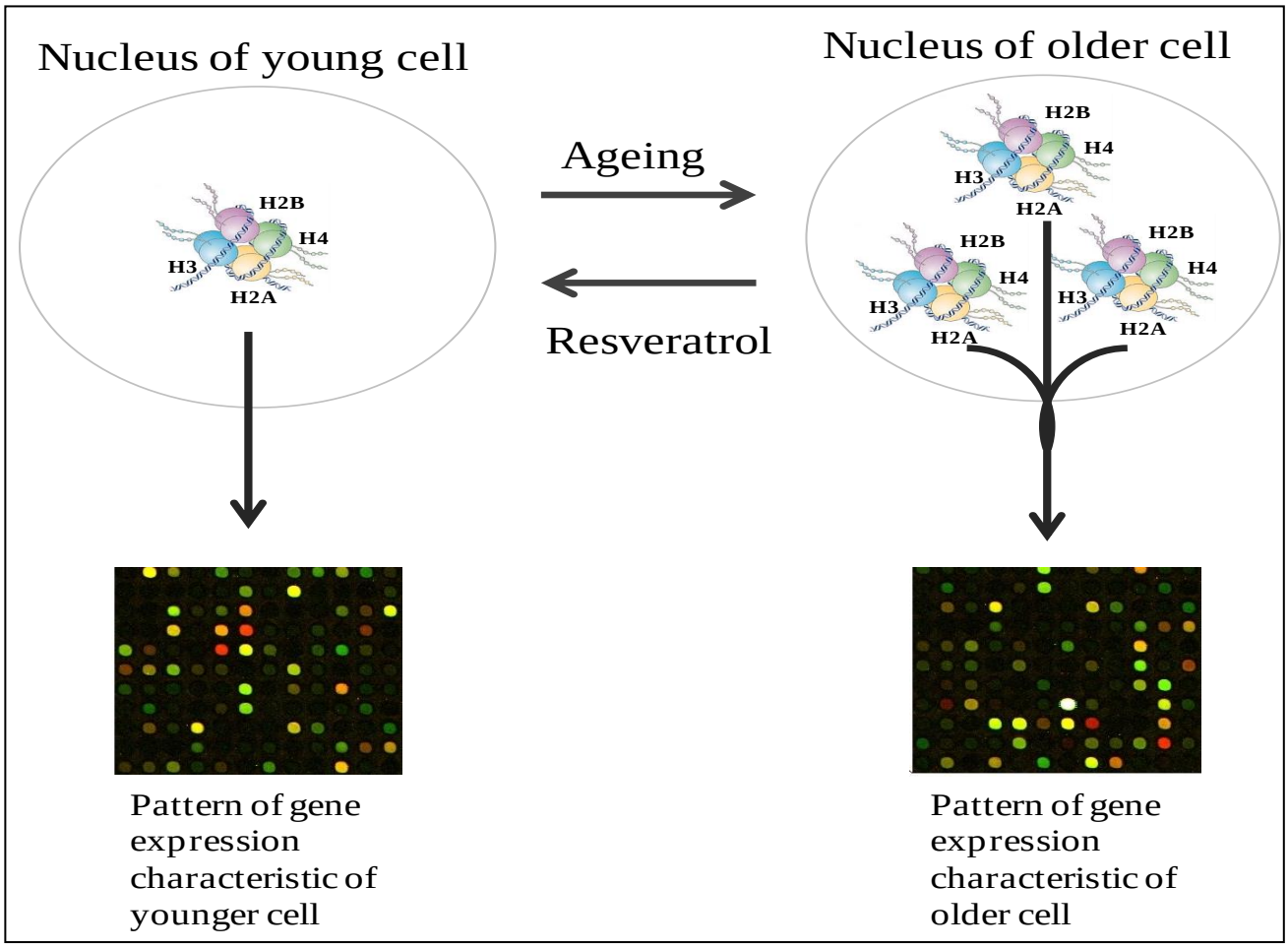


Figure 1.8: Diagrammatic representation of the hypothesis. It is hypothesised that histone (H2A, H2B, H3 and H4) expression may increase with age and the beneficial effects of resveratrol on lifespan/longevity are mediated through this effect on histone protein expression.

1.6 Objectives of the study

The specific objectives of the study were:

1. To examine the effect of resveratrol on histone expression.
2. To investigate the effect of age on histone expression.
3. To identify specific genes and pathways affected by resveratrol treatment.
4. To study whether the estrogen receptors are essential for resveratrol to affect histone expression.

2 Materials and Methods

Reagents and chemicals were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated.

All solutions and plastic consumables were sterilised by autoclaving at high pressure and temperature. Sterile MilliQ water was used and was generated by deionisation using a Millipore filtration unit (Millipore, Massachusetts, USA)

2.1 Cell culture

Tissue culture was carried out in a class II laminar flow hood using aseptic techniques. All culture medium and supplements were obtained sterile (Gibco BRL, UK) unless otherwise stated. Cells were grown as monolayer cultures in sterile 75 cm^2 flasks (Greiner Bio-one, Gloucestershire, UK) and were incubated at 37 ºC in a 5% CO_2 atmosphere.

2.1.1 Growth and maintenance of cells

Human breast cancer MCF-7 cells (passage number 72-89) were grown in improved minimal essential medium (IMEM) containing Glutamax plus 4.5 g/L glucose, supplemented with 10% (v/v) foetal calf serum (FCS), 10000 IU/ml penicillin, 10000 µg/ml streptomycin, 1% (v/v) nonessential amino acids (NEAA), and 1% (v/v) sodium pyruvate. Caco-2 human colonic adenocarcinoma cells (passage number 21-28) were grown in Dulbecco`s modified Eagle`s medium (DMEM) containing Glutamax plus 4.5 g/L glucose, supplemented with 10% (v/v) FCS, 1% (v/v) NEAA, and 0.06 % (v/v) gentamycin.

Cells were routinely subcultured when 90% confluent by removing the medium, washing with 15 ml sterile phosphate buffered saline (PBS), followed by adding 2 ml trypsin, and incubation for 5 minutes at 37°C. After incubation, cells were resuspended in 10 ml of medium and transferred to 25 ml universal tubes and centrifuged for 5 minutes at 1500 rpm. The supernatant fluid was removed and cell pellets were resuspended in 10 ml of normal growth medium. One milliter of the resuspended cells was transferred to a new 75 cm^2 flask and 15 ml of the medium was added and cells were incubated at 37 °C in 5% CO_2 in air.

2.1.2 Cell counting

Fifteen microlitres of cell suspension were mixed with equal volume of trypan blue dye solution. The trypan blue cell suspension was pipetted onto a haemocytometer and the viable cell numbers per ml calculated using the following formula:

Number of cells per ml = 2 (average number of viable cells in 16 squares) X 10000

2.1.3 Cell treatment

Resveratrol (trans-isomer), supplied in a powder form, was dissolved in DMSO (100 mM stock solution) and added to phenol red- free culture medium to reach a final concentration of 10 µM resveratrol. Caco-2 cells were seeded in 6 well culture plates at a density of $3x10^5$ cells/well. After 24 hours incubation in growth medium, cells were treated with either 0.01% DMSO (vehicle control) or 10 µM resveratrol for 48 hours in the presence or absence of the ER antagonist Fulvestrant (0.1 µM). Treated or untreated cells were then processed to extract total cell protein. MCF-7 cells were treated with resveratrol following the same procedure.

2.1.4 Preparation of cell lysate

Cells (Caco-2, MCF-7) were washed with phosphate buffered saline (PBS). PBS containing 1x protease inhibitor cocktail (Roche) was added just before lysis. Cells were scraped from the plastic, transferred into 1.5 ml microcentrifuge tubes and centrifuged at 13000 rpm for 15 minutes at 4 °C. The pellets were resuspended in 50µl of resuspension buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA and 1x protease inhibitor cocktail).

2.1.5 Determination of protein concentration

Protein concentrations were determined using the Nanodrop spectophotometer (Thermo, USA). Typically, 1µl of each sample was applied directly onto the nanodrop lower optical surface. Protein concentration was calculated by nanodrop software. MilliQ water was used as a blank.

2.2 Extraction of histone proteins from mouse intestine

Histone extraction was carried out according to a published procedure (Jeong, 2004, Druesne, 2004). Briefly, small pieces of small intestine from young and old female mice (12 months and 38 months) were homogenized in cold-ice 1x PBS, and centrifuged at 12,000 g for 10 minutes. Subsequently, pellets were resuspened in hypotonic buffer (1.5 mM $MgCl_2$, 10 mM HEPES, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 mM KCl) and kept on ice for 10 minutes. The suspension was then sonicated for 15 minutes. After sonication, suspensions were centrifuged at 3300 g for 15 minutes and the cytoplasmic fractions were discarded. The pellets were resuspended in 0.2 M H_2SO_4 and kept overnight at 4 ºC. The suspensions were then centrifuged at 10000 g for 15 minutes. Histone proteins were

precipitated using 1 ml acetone and incubating overnight at -20 ºC. Acetone was removed after centrifuging at 10000 g for 15 minutes at 4 ºC and the pellets were neutralized in 10 µl NaOH 2M and 40 µl MilliQ water. Protein concentrations were measured using the Nanodrop as described in section 2.1.5.

2.3 Human colonic tissue samples

Human samples used were kind gift from Professor John Mathers (Institute for Aging and Health, Newcastle University, UK). Total protein samples were extracted from human colon biopsies of eleven males (at 21, 23, 33, 43, 64, 66, 73, 74, 77 or 82 years old). Human samples were homogenised in a total volume of 40 µl buffer prepared by mixing 20 µl of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA and 1x protease inhibitor cocktail) with 20 µl of gel loading buffer (1 M Tris-HC pH6.8, 50% glycerol, 12.5 % SDS, 0.1 % bromophenol blue, 1 M dithiottheitol (DTT) and 5 % β-mercaptoethanol). Cell lysis was achieved through the SDS content (6.25%) in the combined buffer. Samples were then denatured at 95 ºC for 5 minutes, and then resolved by SDS-PAGE as described in section 2.4.4.

2.4 Western blot analysis

2.4.1 Antibodies

Primary antibodies, obtained from rabbit, were diluted in 1x PBS, 5% (w/v) non fat milk powder and 0.05 % (v/v) Tween-20 as follows: anti-H2A (1:250), anti-H2B (1:250), anti-H3 (1:1000), anti-H4 (1:250) and anti-alpha tubulin (1:250). Secondary antibody, anti rabbit IgG peroxidase conjugate, was diluted (1:3000) in 1x PBS, 5% (w/v) non fat milk powder and 0.05% (v/v) Tween-20 (Table 2.1).

2.4.2 Protein sample preparation

Protein samples were mixed with 5x protein loading buffer [1 M Tris-HC pH6.8, 50% glycerol, 12.5% SDS, 0.1% bromophenol blue, 1M dithiothreitol (DTT) and 5% β-mercaptoethanol]. Proteins were then denatured at 95 ºC for 5 minutes. The samples were briefly centrifuged at room temperature to collect the mixture at the bottom of the microfuge tube.

2.4.3 Gel preparation

Separating gel was prepared using 2.19 ml of 40 % bis acrylamide (37.5:1), 2.8 ml of 2.5x separating gel buffer [1.875 M Tris-HCl pH 8.9, 0.25 % SDS], 6 µl of TEMED, 65 µl of 10 % ammonium persulfate (APS) in a total volume of 5.45 ml. A 10 % stacking gel was prepared using the following reagents: 0.25 ml of 40 % bis acrylamide (37.5:1), 0.4 ml of 5x stacking buffer [0.3 M Tris-HCl pH 6.7, 0.5% SDS], 2.5 µl TEMED, 18 µl of 10 % APS in a total volume of 2.175 ml.

2.4.4 SDS polyacrylamide gel electrophoresis and transfer of protein to a solid support membrane

Proteins were subject to SDS polyacrylamide gel electrophoresis (SDS-PAGE). Five micrograms of protein was loaded in each lane of a 12.5 % polyacrylamide gel (section 2.4.3). Gels were run at 80V for 2 hours in 1x protein electrophoresis running buffer [made up as 5x/L: 60.6 g Tris Base, 144.1 g glycine, 5 g SDS]. ColorBrust electrophoresis marker (M.W 8000-220,000, Sigma) was used as a molecular weight standard. Following electrophoresis, proteins were transferred to activated PVDF membranes using a semi dry blotter at 15V for 50 minutes. The PVDF membranes were activated by incubating in methanol for 30 seconds, washing in milliQ water for

2 minutes and soaking in membrane transfer buffer (80 % 1x protein electrophoresis buffer, 20% methanol) for 10 minutes at room temperature. After protein transfer, the PVDF membranes were incubated in blocking solution [1x PBS, 5 % (w/v) non fat milk powder, 0.05 % (v/v) Tween-20] overnight on a rocking platform at 4 ºC.

2.4.5 Probing membrane with antibodies and signal detection

Following overnight blocking, PVDF membranes were stained with primary antibodies (anti-H2A, anti-H2B, anti-H3, or anti-H4) for 1 hour at room temperature on a rocking platform. Membranes were then washed 4 times, for 10 minutes each, in membrane wash solution [1x PBS, 0.05 % (v/v) Tween-20]. Subsequently, the membranes were incubated with secondary antibody (section 2.4.1) for 1 hour at room temperature on a rocking platform. Membranes were washed 5 times, for 10 minutes each, in membrane wash solution. Protein-antibody complexes were detected using the Enhanced Chemiluminescence System (ECL, Amersham, UK). Briefly, membranes were incubated with a 40:1 mixture of solutions A and B respectively. Then the excess liquid was removed using thick blotting paper (Whatman), and the membranes were wrapped in acetate membrane, placed in an X-ray film cassette and overlaid with high performance chemiluminescence film. Exposed film was developed using an automated developer/ fixer.

2.4.6 Probing membranes with antibody to a reference protein

To assess protein loading and transfer efficiency, membrane blots were probed for α-tubulin as a reference. Typically, membranes were incubated with a primary antibody immunoreactive against α-tubulin for an hour at a room temperature on a rocking platform. Membranes were then washed 4 times, for 10 minutes each, in membrane

wash solution (section 2.4.5). Membranes were then incubated with secondary antibody (section 2.4.1) for an hour at room temperature on a rocking platform, followed by washing 5 times, for 10 minutes each, in membrane wash solution. Protein-antibody complexes were detected using ECL as described in section 2.4.5.

2.4.7 Colloidal blue staining of protein gels

Colloidal blue stain was used to assess protein loading when a house keeping antibody could not be used. The colloidal blue stain was prepared following the manufacturer's instructions (Invitrogen, UK). Briefly, stain was made up of 55 % deionised water, 20 % methanol, 5 % stain B and 20 % stain A. Protein samples were resolved by SDS-PAGE, run at 80V for 2 hours in 1x protein electrophoresis running buffer. Gels were then incubated in colloidal blue stain overnight on a rocking platform at room temperature. Subsequently, gels were destained in distilled water for 2 hours on a rocking platform at room temperature. Gels were examined on a light transilluminator and images were recorded using Uvitec gel documentation with UVIband software (UVtech, Cambridge, UK).

2.4.8 Band quantification by densitometry

The intensities of protein bands were measured using densitometry software [UVItec (UVIband software), UK)] on a gel documentation system [UVItec (Model (BTS-26M), UK)].

2.4.9 Data analysis

All data were statistically analysed using a commercially available software package (InStat, Graphpad Software, USA). Differences between groups were detected by one

way ANOVA followed by Dunnett's post test, unless otherwise indicated in the text or figure legends. Results are expressed as mean ±standard error of the mean (SEM) for all experiments. Data were taken to differ significantly only at a *p* value of 0.05 or less.

2.5 RNA microarray and bioinformatics analysis

2.5.1 RNA extraction and purification

Human intestinal Caco-2 cells or human MCF-7 breast cancer cells were treated with resveratrol (10 µM) for 48 hours (section 2.1.3). Total RNA was extracted using TRIzol Reagent according to the manufacturer's instructions (PureLink®RNA Mini Kit, Invitrogen). One millilitre of Trizol was added to each well and the cell lysate was passed several times through a pipette tip before transferring into a microfuge tube. After incubation at room temperature for 5 minutes, 200µl chloroform was added. The tube was shakenvigorously by hand for 15 seconds and incubated at room temperature for 2-3 minutes and centrifuged at 12,000xg for 15 minutes at 4 °C. Four hundred microlitres of the colourless, upper phase containing the RNA was transferred to a fresh RNase-free tube. Equal volume of 70% ethanol was added and mixed well by vortexing. Up to 700 µl of samples were transferred to a spin cartridge with a collection tube and centrifuged at 12,000xg for 15 seconds at room temperature. The flow-through was discarded and the spin cartridge was reinserted into the same collection tube. 700 µl wash buffer 1 was added to the spin cartridge and centrifuged at 12,000xg for 15 seconds at room temperature. The flow-through and the collection tube were discarded and the spin cartridge was inserted into a new collection tube. Five hundred microlitres wash buffer II with ethanol was added to the spin cartridge and centrifuged at 12,000xg for 15 seconds at room temperature.

The flow-through was discarded and the spin cartridge was reinserted into the same collection tube and centrifuged at 12,000xg for 1 minute at room temperature to dry the membrane with bound RNA. The collection tube was discarded and the spin cartridge was inserted into a recovery tube. Thirty microlitres of RNase-free water was added to the centre of the spin cartridge, incubated at room temperature for 1 minute followed by centrifuging at 12,000xg for 1 minute at room temperature. The flow-through containing RNA was stored in -80 °C until used.

2.5.2 Determination of RNA concentration and purity

RNA concentration was measured using the Nanodrop spectophotometer (Thermo, USA). Briefly, 1µl of each sample was applied directly onto the nanodrop lower optical surface. RNA concentration was automatically measured by nanodrop software. RNA free water was used as a blank.

To assess RNA purity, the ratios of A260/A280 and A260/A230 were analysed by Nanodrop. Basically, RNA with an A260/A280 $\geq$ 2.0 and an A260/A230 $\geq$1.8 was considered as a highly intact RNA.

2.5.3 Determination of RNA stability using the Agilent 2100 bioanalyser

RNA integrity was measured using the Agilent 2100 Bioanalyzer (RIN=10). Agilent RNA 6000 Nanochips were loaded with RNA using the RNA 6000 Nano assay kit (Agilent Technologies, workingham, UK) following the manufacturer's instructions.

A RIN (RNA integrity number) is generated by an algorithm as a measure of the degradation of RNA molecules. The value of a RIN ranges from 1 (totally degraded RNA) to 10 (totally intact RNA). For downstream applications RINs above a threshold of 7 are considered acceptable.

2.5.4 Gene expression profiling

Global gene expression profiles were determined by hybridization to the whole genome Illumina HumanHT-12v3 single colour beadchip microarray. Two biological replicates for each cell line (Caco-2 and MCF-7) and each condition (control or resveratrol treated) were hybridised separately to arrays. Sample processing and hybridisation was carried out by Arrayexpression (Leiden, the Netherlands). Ingenuity Pathway Analysis (IPA) was used to identify the pathways affected by resveratrol treatment.

2.5.5 Analysis of microarray data

Microarray data were imported to GeneSpring GX 11 (Agilent) for visualisation. Hierarchical clustering analysis was used to measure the relative similarity among biological replicates. Probes with an lllumina detection *p* value >0.6 in all samples in replicate groups were considered as expressed. Rank products (Rank Prod) analysis was used to identify differential expression between groups as probes with a percentage of false positives (PFP) of <0.05 over 100 permutations of the class labels with a resulting fold change of >1.5.

2.5.6 Gene identifier conversion

To compare data generated by microarray analysis of RNA extracted from Caco-2 and MCF-7 cell lines and data compiled from published studies in mice, all gene identifiers were converted to the equivalent Ensembl identifier, using the Ensemble Biomart Gene Conversion Tool at www.ensembl.org/biomart.

2.5.7 Gene list intersections

Gene lists converted into Ensembl identifiers (section 2.5.8) were compared and duplicates identified using the Advanced Filter> Unique Records option in Excel (Microsoft).

2.5.8 Statistical analysis of gene list intersections

To determine whether the number of genes shaved between different lists was greater or fewer than expected of independent groups by chance, the representation factor was calculated by applying the following equation:

$$X/[(nD)/N]$$

where X was the number of genes common between groups, n was the number of genes in one list, D the number of genes in the other list and N the total number of genes. A representation factor of >1 indicated a larger intersections than would be expected from two unrelated independent groups.

The cumulative hypergeometric probability was calculated by applying the following equation:

$$h (\geq X; N, n, D) = [{}_{D}C_{X}] [{}_{N-D}C_{n-X}] / [{}_{N}C_{n}]$$

where N is the total number of genes, n is the number of genes in one list, D is the number of genes in the other list, X is the number of genes common between groups, and ${}_{D}C_{X}$ is the number of combinations of D event, taken X at a time. The StatTrek hypergeometric calculator tool at http://stattrek.com was used to calculate cumulative hypergeometric probability.

2.6 Gene expression analysis

2.6.1 Design of PCR primers

Gene or cDNA sequences were identified using tools available through NCBI http://www.ncbi.nlm.nih.gov/. Oligonucleotide primers were designed using PrimerQuest^{SM} (http://eu.idtdna.com/Scitools/Applications/Primerquest/Default.aspx). Primers were between 18 and 24 bases in length with a melting temperature greater than 55 °C and GC content 50-60%. To improve efficiency of binding to the DNA template, oligonuclotides contained a G or C residue at the 3` end. Primers were synthesised by MWG Biotech Ltd., UK.

2.6.2 RNA extraction

Total RNA was extracted from Caco-2 and MCF-7 cells using TRIzol reagent, according to the manufacturer's instructions (Invitrogen).

2.6.3 Determination of RNA concentration and purity

RNA concentration and purity were measured using the Nanodrop spectophotometer (Thermo, USA).

2.6.4 DNase treatment of RNA

RNA samples were routinely subjected to DNase treatment to remove any DNA contamination. Each reaction mixture contained 4.5µg of RNA, 4.5 U DNase (Roche) and 2µl of 10x DNase buffer (Roche). RNase free water was added to reach a final volume 20µl. The reaction was incubated at 37 ºC for 30 minutes, then 4 µl of stop solution (EDTA, 20 mM, PH 8.0) was added. The samples were incubated at 65 °C

for 10 minutes then the RNA was placed on ice if required for use immediately or stored at -80 °C.

2.6.5 Reverse transcription

Five microlitres (approximately 2µg) of RNA were reverse transcribed to complementary DNA (cDNA). The reverse transcription reaction consisted of two steps. In the first step, 5 µl of RNA (2 µg), 1 µl random primers, 0.4 µl dNTP (100 mM) and sterile MilliQ water were mixed to total volume of 13 µl. This mixture was incubated at 65 ºC for 5 minutes and then transferred to ice. The second step consisted of the addition 4 µl of 5x First Strand Buffer (250 mM Tris-HCl), 1µl 0.1 M DTT, 1µl RNase inhibitor (40 u/µl) and 1 µl Superscript reverse transcriptase (SSIII, Invitrogen) (200 u/µl). To act as a negative control a second reverse transcriptive reaction was carried out omitting the SSIII enzyme. These mixtures were incubated at 25 ºC for 5 minutes, 50 ºC for 45 minutes and the reaction was held at 4 ºC.

2.6.6 Polymerase chain reaction (PCR)

PCR reactions were performed using the following: 0.15 µl of 5 u/µl Taq DNA polymerase (Bioline), 1x Thermostart H-buffer ($MgCl_2$ free), 1.2µl of 25 mM $MgCl_2$, 0.3µl of 25 mM dNTPS, 2 µl of 5 µM sense primer and 2 µl of 5 µM antisense primer made up to final volume of 20 µl with MilliQ water and including 1 µl of reverse transcription-generated cDNA.

Thermal cycling for reactions to ERα and ERβ was as follows:

95 ºC for 15 minutes (hot start)

40 cycles
- 95 ºC for 30 seconds denaturation
- 60 ºC for 30 seconds annealing
- 72 ºC for 90 elongation

72 ºC for 2 minutes final elongation

4 ºC hold

Thermal cycling for amplification of GAPDH, a reference gene, was as follows:

95 ºC for 15 minutes (hot start)

29 cycles
- 95 ºC for 30 seconds denaturation
- 55 ºC for 30 seconds annealing
- 72 ºC for 90 elongation

72 ºC for 2 minutes final elongation

4 ºC hold

All primers are listed in Table 2.2 A. After PCR, the products were analysed by agarose gel electrophoresis.

2.6.7 Agarose gel electrophoresis

For DNA analysis, agarose gels were prepared by boiling 1% agarose in 1x TBE buffer (made up as 10x: 108g/l Tris base, 55 g/l boric acid, 40 ml 0.5 M EDTA; pH 8.0). Two microlitres of ethidium bromide (10 mg/ml) was added to the cooled gel solution before pouring into the gel apparatus. Five microlitres of PCR products were mixed with 2 µl 5x gel loading buffer (50 mM Tris-HCl pH 8, 5 mM EDTA, 20 % glycerol and 0.1 % Bromophenol Blue) and loaded onto agarose gels. DNA ladder-I

(Bioline) was used as a molecular weight standard. The gel was run for 30 minutes at 70V. DNA was visualised on a UV transilluminator and images were recorded using Uvitec gel documentation with UVIband software (UVtech, Cambridge, UK).

2.7 Generation and manipulation of DNA plasmid constructs

2.7.1 Determination of gene promoter sequence

The sequence of the promoter region of the histone H3 gene was retrieved in FASTA format using the Gene2promoter software (Genomatrix, Germany). To ensure the promoter lay upstream of the transcription start site (TSS), the promoter sequence was aligned against the transcript.

2.7.2 Polymerase chain reaction (PCR)

PCR reactions were performed as described in section 2.6.6. Thermal cycling for amplification of the histone H3 promoter was as follows:

95 ºC for 15 minutes (hot start)

35 cycles {
- 95 ºC for 30 seconds denaturation
- 60 ºC for 30 seconds annealing
- 72 ºC for 90 elongation
}

72 ºC for 2 minutes final elongation

4 ºC hold

After PCR, the products were analysed by agarose gel electrophoresis as described in section 2.6.7. All primers are listed in Table 2.2 B

2.7.3 Cloning of PCR products into pBlue-TOPO

PCR products were cloned into the pBlueTOPO vector (Invitrogen, UK) according to the manufacturer's protocol. Briefly, 4µl of PCR product were mixed with 1 µl salt solution and 1µl vector. The ligation reaction was incubated for 5 minutes at room temperature, and then 2 µl of this reaction was added to chemically competent OneShot TOP *E.coli* cells, which were then incubated on ice for 30 minutes, then heat-shocked at 42 °C for 30s, before adding 250 µl SOC media. Cells were incubated with shaking for 1 hour at 37 °C. The transformed cells were grown overnight at 37 °C on Luria Bertani (LB)-agar plates (1% peptone, 1% NaCl, 0.5% yeast extract, 15 g/L agar) containing 50 µg/ml ampicillin.

2.7.4 Plasmid DNA preparation

Single bacterial colonies were cultured in 5 ml LB-broth (1% peptone, 1% NaCl, 0.5% yeast extract) containing 50 µg/ml ampicillin at 37 °C with shaking overnight. Plasmid DNA was extracted using the Eppendrof Miniprep Kit (Qigaen) according to the manufacturer's protocol. To extract plasmid DNA in large quantities, an endotoxin-free maxiprep kit (Qiagen) was used according to the manufacturer's instructions.

2.7.5 Digestion of plasmid DNA with restriction endonucleases

To determine if plasmid DNA contained the correct insert, digestion with *Hind* III was used. The reaction was prepared by adding 5 U *Hind* III to 1x reaction buffer in a final volume of 20 µl. The reaction was then incubated at 37 °C for 1-2 hours. The digestion products were detected by agarose gel electrophorsis as described in section 2.6.7.

2.7.6 DNA sequencing

Plasmids were sequenced by MWG Biotech.

2.8 Mutagenesis of promoter-reporter plasmid constructs

Estrogen response elements (ERE) identified within the histone H3 promoter sequence included in the promoter reporter construct were mutated using a PCR-based method. Primers were designed to introduce mutations into three sites at which consensus ERE sequences were present within the histone H3 promoter region in the construct using PCR with the wild-type promoter-reporter construct as the template. PCR product including mutated regions was then subcloned into the pBlue TOPO vector (Invitrogen) as described in section 2.7.4.

The procedure involved introducing each mutation sequentially in three PCR reactions, the products of which were joined also by PCR. To avoid reamplifying the wild-type promoter sequence from contaminating wild-type plasmid in the final step, random primer sequences were introduced at the 5` and 3` ends of the required product, and primers matching these sequences were used for the final amplification. In the first PCR reaction the 5` region of the promoter including one of the EREs was generated and the ERE was replaced with random sequence. In the second reaction, the middle region of the promoter including the second ERE was generated and the ERE was replaced with random sequence. These two products were then joined in a third PCR reaction. In a fourth PCR reaction the 3` region of the promoter including the third ERE was generated and the ERE was replaced with random sequence. The products of the third and fourth PCR reaction were then joined using the unique outer

primers (to the random sequence). All PCR reactions were carried out using the following thermal cycling parameters:

95 ºC for 15 minutes (hot start)

40 cycles {
- 95 ºC for 30 seconds denaturation
- 65, 64, 63…,50 ºC for 30 seconds annealing
- 72 ºC for 90 elongation

72 ºC for 2 minutes final elongation

4 ºC hold

The successful mutation of the three EREs in the histone H3 promoter sequence was confirmed by sequencing (MWG Biotech). All primers are listed in Table 2.2 C

2.9 Transient transfection of mammalian cells

For transfection, Caco-2 cells were seeded into 6-well plates at a density $3.5x10^5$ cells/well and incubated for 24 hours at 37 °C. After 24 hours incubation, cells were transfected using GeneJammer tansfection reagent (Stratagene Europe, Netherlands). For each well, a 100 µl volume of transfection mixture was prepared by adding 4.5 µl GenJammer to 1.75 µg plasmid DNA in serum and antibiotic-free medium. Cells were supplemented with 900 µl growth medium and transfection mixture was added in dropwise manner. Then, cells were incubated for 4 hours at 37 ºC prior to the addition of 1ml of complete medium. Cells were incubated for a further 24 hours. Transfected cells were subjected to treatment with resveratrol as described in section 2.1.3.

2.10 Reporter gene assays

2.10.1 Preparation of whole cell lysate

Cells in 6-well plates were washed with 1 ml ice-cold PBS per well, followed by addition 100 µl of lysis buffer (0.25 M Tris (pH7.4), 0.25 % (v/v) Nonident P40, 2.5 mM EDTA). Cells were then frozen at -20 °C for 30 minutes. After 30 minutes, cells were thawed at room temperature. Cells in each well were harvested using a sterile cell scraper, transferred to 1.5 ml microcentrifuge tubes and centrifuged at 13,000 g for 5 minutes at 4 °C. The supernatant fluids were transferred to new tubes.

2.10.2 Determination of protein concentration

The bradford assay was used to measured protein concentration of cell lysates using a 96 well plate. A standard curve was generated by preparation of 0, 20, 40, 60, 80, 100 µM solution of bovine serum albumin (BSA) in a final volume 50 µl. Bradford reagent (Biorad, UK) was diluted (1:5), and 200 µl of the reagent was added to 50 µl of diluted cell lysates (1:50) or BSA standard. Standards were prepared and measured in triplicate and samples in duplicate. Absorbance was measured at 595 nm using a plate-reader (Thermo Labsystems Multiskan Ascent). Protein concentrations of samples were measured from the standard curve.

2.10.3 β-galactosidase reporter assays

Reporter assays were carried out by adding 20 µl of cell lysate to 130 µl of 1.2 mg/ml chlorophenol red-β-D-galactopyranoside (CPRG) in buffer containing 25 mM MOPS, 100 mM NaCl, 10 mM MgCl2, at pH 7.5 and incubating at 37 °C. When a red colour was observed, the reaction was stopped by the addition of 80 µl 0.5 M Na_2CO_3.

Absorbance was measured at 650 nm on a plate-reader (Thermo Labsystems Multiskan Ascent).

2.10.4 Data analysis

The activity of β-galactosidase was measured as nanomoles of chlorophenol red formed per minute per mg of total protein. All data were statistically analysed using a commercially available software package (InStat, Graphpad Software, USA). Differences between groups were detected by one way ANOVA followed by Dunnett's post test. Results are expressed as mean ±standard error of the mean (SEM) for all experiments. Significance was taken as a p value of 0.05 or less.

2.11 Antibodies and Oligonucleotide

Table 2.1: Antibodies

A

Primary antibodies	Symbol	Source	Dilution
Anti- histone 2A	H2A	Abcam	1:250
Anti- histone 2B	H2B	Abcam	1:250
Anti- histone 3	H3	Sigma	1:1000
Anti- histone 4	H4	Abcam	1:250
Anti-alpha tubulin		Sigma	1:250

B

Secondary antibody	Source	Dilution
Anti-rabbit IgG peroxidase conjugate	Sigma	1:3000

Table 2.2: Oligonucleotide

A

Target and Genbank accession no.	Primer sequences (5`→3`)	Product size (bp)
ERα	GGATACGAAAAGACCGAAGAG	
NM 000125	GTCTGGTAGGATCATACTCGG	236 bp
ERβ	TAGTGGTCCATCGCCAGTTATCAC	
NM 001437	GCACTTCTCTGTCTCCGCACAA	438 bp
GAPDH	TGAAGGTCGGAGTCAACGGATTTG	
NM 002046	CATGTAAACCATGTAGTTGAGGTC	170 bp

B

Target and Genbank accession no.	Product	Primer sequences	Product size (bp)
Histone 3 (H3m)	H3For	GTGGGAGAAGTGCCATGCAGCAC	862 bp
M26150	H3Rev	CTTGCCTGCAGAGACGTCTGTG	

C: The primers were used to generate the histone H3 promoter-reporter construct contained with mutations introduced at three identified sites with ERE consensus sequence. The unique primer sequences are in lower case, the mutated sequence is highlighted in red.

Product	Primer sequences (5`→3`)
H3unq1	tattaccgacgcccggcggc
H3unq2	aaagtgaatggcgtgtgggc
H3Fwdunq1	tattaccgacgcccggcggcGTGGGAGAAGTGCCATGCAGCAC
H3Revunq2	aaagtgaatggcgtgtgggcCTTCCTGCAGAGACGTCTGTG
H3MutFor1	GGAAGTGTTAAAACCCGCA**TCAAA**CACACAAGTTTGAATATG
H3MutRev1	CATATTCAAACTTGTGTGTTTGATGCGGGTTTTAACACTTCC
H3MutFor2	CTAAGAGCATTTTTCTAAT**ATAGA**ACACTTCTTATGCGACACCC
H3MutRev2	GGGTGTCGCATAAGAAGTGTTCTATATTAGAAAAATGCTCTTAG
H3MutFor3	CGTCAGAGTAGCTACGGTAAT**ACCAC**GGAGCCTCTCTTAATCTGC
H3MutRev3	GCAGATTAAGAGAGGCTCCGTGGTATTACCGTAGCTACTCTGACG

3 The effect of resveratrol on histone expression

3.1 Introduction

Resveratrol, a phytoalexin natural product, is produced in several plants to protect against infection by bacteria or fungi (Fremont, 2000). The presence of resveratrol in red wine attracted the attention of scientists as a possible explanation for the phenomenon known as the "French paradox", which is a term used to describe the relatively low rates of cardiovascular disease and obesity among the French population despite their high fat diet and regular consumption of red wine (Renaud and de Lorgeril, 1992). Several studies have further reported potential biological benefits of resveratrol as a factor in the aetiologies of diseases including cardiovascular disease (Wang *et al.,* 2012), cancer (Sun *et al.*, 2008), and diabetes (Palsamy and Subramanian, 2008). Additionally, resveratrol has been reported to promote longevity/lifespan in various species from yeast to mouse (Howitz *et al.*, 2003; Cohen *et al.,* 2004; Borra *et al.*, 2005), potentially through stimulating the NAD^+-dependent deacetylases enzymes, Sirt2 and Sir1, respectively (Howitz *et al.,* 2003; Cohen *et al.*, 2004). These enzymes deacetylate a large number of proteins and regulate many critical cellular processes including transcription, metabolism, DNA repair, and stress resistance (review in Kelly, 2010).

The mechanism by which resveratrol induces these biological effects is not completely understood. This study aimed to investigate the mechanism by which resveratrol influences longevity/lifespan. The ability of resveratrol to stimulate the histone deacetylase Sirt1 raises the question as to whether resveratrol promotes longevity through its effects on histone acetylation. To answer this question previous work in the laboratory investigated the effect of resveratrol on histone acetylation. Caco-2 cells were treated with 10 µM resveratrol for 48 hours, then semi-purified histone proteins were analysed by western blotting using an

antibody specific for histone 4 (H4) acetylated at Lys16 or using an antibody immunoreactive against H4 irrespective of acetylation status. As shown in figure 3.1 both anti-H4 antibodies revealed a marked reduction in the H4 signal after treatment of Caco-2 cells with resveratrol indicating that resveratrol treatment reduced H4 protein expression, rather than affecting acetylation status (L Wakeling and D Ford, personal communication).

Based on this finding, it was proposed that the ability of resveratrol in promoting longevity may be mediated through effects on histone expression. Therefore, the effect of resveratrol (at a physiological concentration at 10 µM) on histone expression was investigated using two different cell lines: Caco-2 (human intestinal) and MCF-7 (human breast cancer).

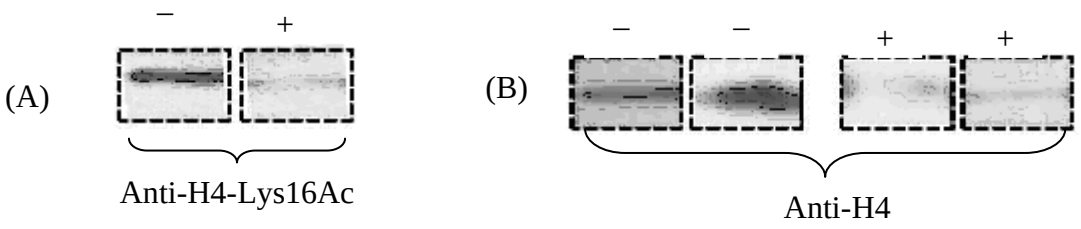


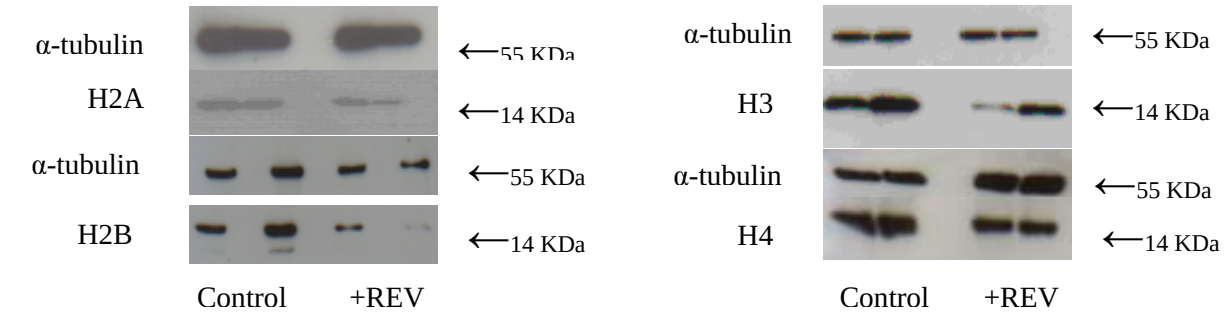
Figure 3.1: The effect of resveratrol (+) on histone 4 (H4) acetylation and H4 expression in the Caco-2 cell line. *H4 acetylation and H4 expression were detected by western blot analysis using an antibody specific for H4 acetylated at Lys16 or using antibody immunoreactive against H4 irrespective of acetylation status. (A) Western blot using an antibody with reacting against H4 acetylated at Lys16. A notable decreased in histone H4 acetylation in the cells treated with resveratrol is evident. (B) Western blot using antibody immunoreactive against H4 irrespective of acetylation status. There is a marked reduction in total H4 expression in treated cells with resveratrol (L Wakeling and D Ford, personal communication).*

3.2 Effect of resveratrol on histone expression in Caco-2 and MCF-7 cell lines

The current study investigated the hypothesis that some of the beneficial effects of resveratrol on lifespan/aging are mediated through effects on histone expression that oppose changes observed in ageing. A further suggestion, although not addressed through the work presented in this chapter, is that these epigenetic actions of resveratrol influence the expression of genes that affect the ageing process.

For this purpose, two different cell lines were used to determine whether or not the response to resveratrol observed previously was cell line specific and to indicate if estrogen receptor (ER) status was a determinant of responsiveness. The MCF-7 (ERα-positive, ERβ-positive) and Caco-2 (ERβ-positive) cell lines were treated with either 0.01% DMSO (vehicle control) or 10 μM resveratrol (in 0.01% DMSO) for 48 hours. Total cell lysate was then analysed by western blotting using antihistone antibodies immunoreactive against H2A, H2B, H3, and H4. To ensure equal amounts of protein were loaded, the same membrane was probed with an antibody immunoreactive against α-tubulin. Quantitative data were derived by densitometric quantification of band intensities. Densitometric analysis of the protein bands in relation to α-tubulin indicated that expression of histone H2A, H2B, H3, and H4 was decreased significantly in cells treated with resveratrol (Figure 3.2-3.3).

(A)



(B)

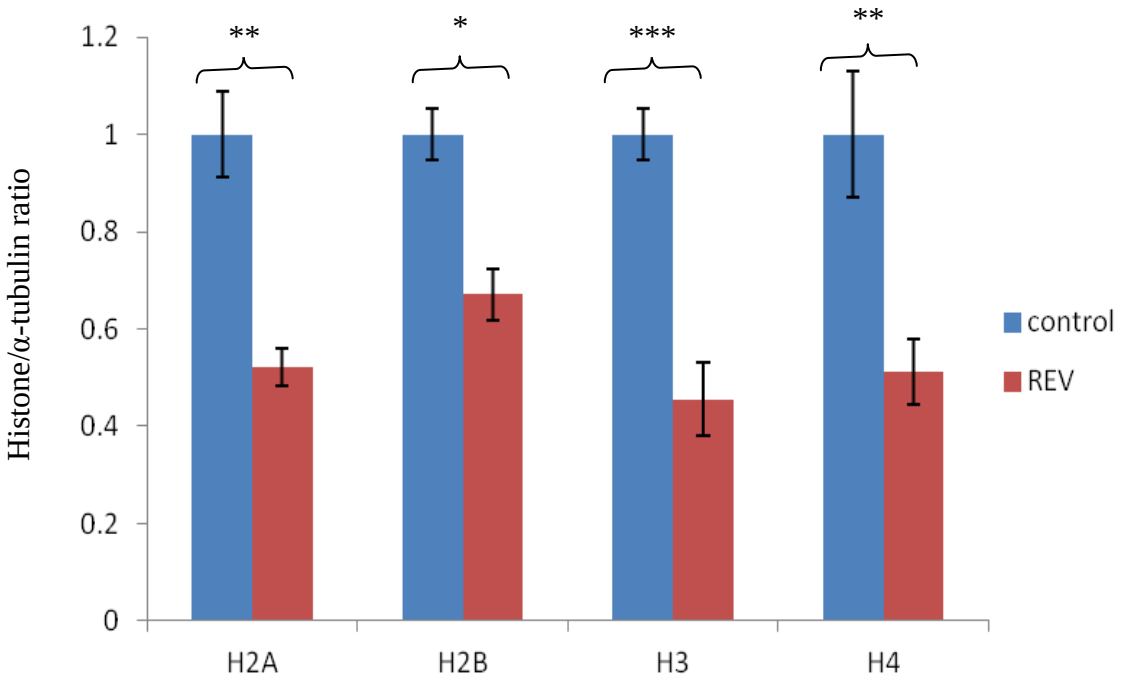


Figure 3.2: The effect of resveratrol (REV) on the level of histone expression in the MCF-7 cell line. ***(A)*** *Western blot analysis using anti-histone antibodies for histone H2A, H2B, H3, and H4. Typical data are shown.* ***(B)*** *Results of densitometric analysis of western blots detecting expression of histone proteins. Data are shown as mean ± standard errors (SEM) (n=9, based on four experiments), *P <0.05, **P<0.01, ***P<0.001. The expression of histone H2A, H2B, H3, and H4 were significantly decreased in cells treated with resveratrol.*

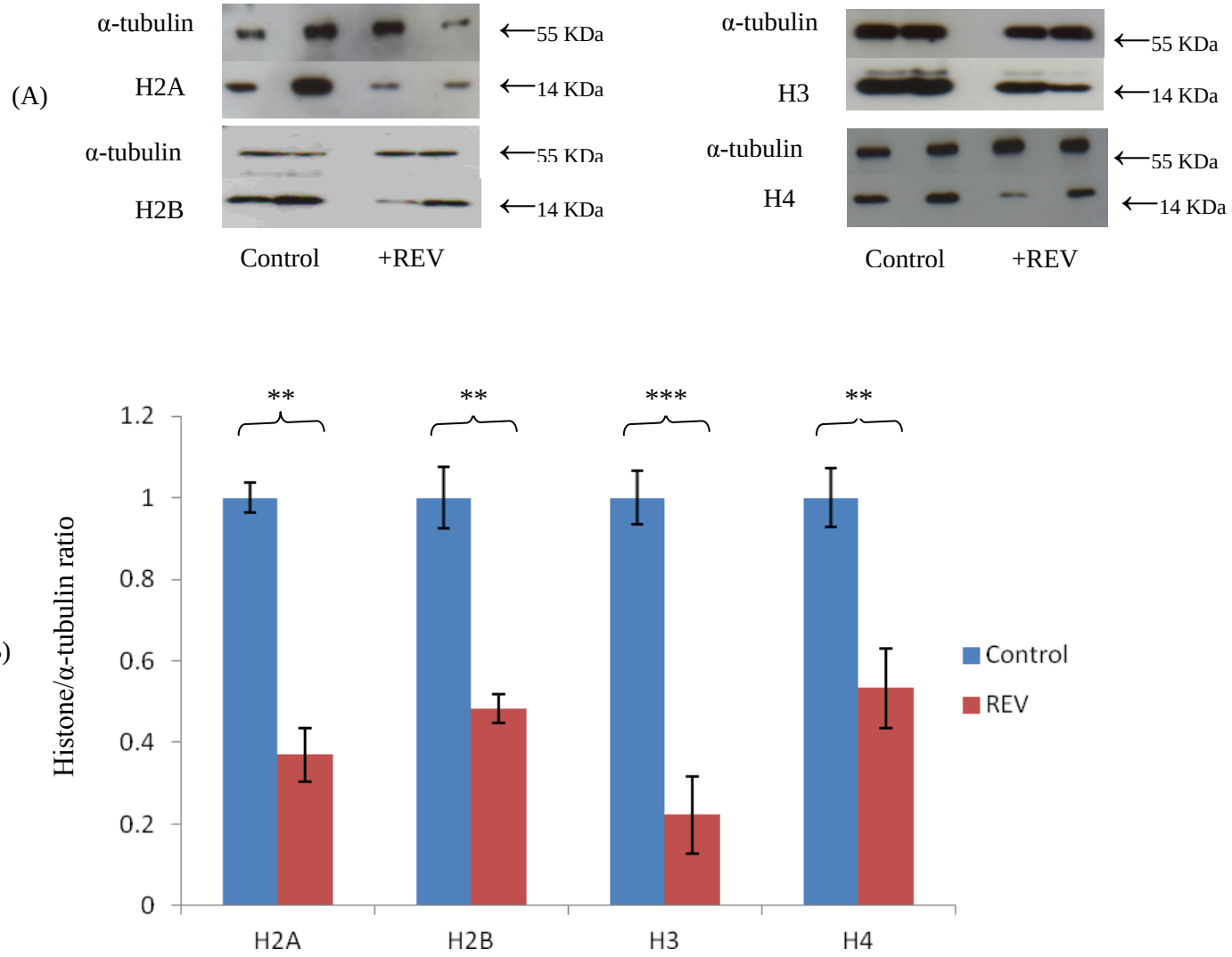


Figure 3.3: The effect of resveratrol (REV) on the level of histone expression in the Caco-2 cell line. (A) *Western blot analysis using anti-histone antibodies for histone H2A, H2B, H3, and H4. Typical data are shown.* ***(B)*** *Results of densitometric analysis of western blots detecting expression of histone protein. Data are shown as mean ± standard errors (SEM) (n=9, based on four experiments), **P <0.01, ***P<0.001. The expression of histone H2A, H2B, H3, and H4 were significantly decreased in treated cells with resveratrol.*

3.3 The effect of age on histone expression

Based on the observations that resveratrol reduced histone protein expression (H2A, H2B, H3, and H4) in cultured human (Caco-2 and MCF-7) cells, this study investigated if this reduction in histone expression could play a role in the regulation of lifespan/longevity by determining if histone expression changed with age. It was hypothesised that the ageing process may be associated with an increase in histone expression and that a reversal of this process may underline some of the apparent anti-ageing effects of resveratrol.

3.3.1 The effect of age on histone expression in mouse intestine

To investigate the effect of age on the level of histone expression, small intestinal tissues from young and old female mice (at 12 months or 38 months) were studied. Histone extraction was conducted using the low concentration acid extraction procedure, followed by western blot analysis using anti-histone antibodies for H2A, H2B, H3, and H4. Colloidal blue staining of protein preparations resolved by SDS PAGE on gels run in parallel to those used for western blotting was used to ensure equal loading of protein samples and to normalise protein concentrations.

Figure 3.4 shows histone expression in these young and old mice. The results of densitometric analysis of the protein bands show that histone H2A, H2B, and histone H4 were significantly increased in old mice (38 months) compared with younger mice (12 months). In contrast, histone H3 did not show a significant change in level of expression.

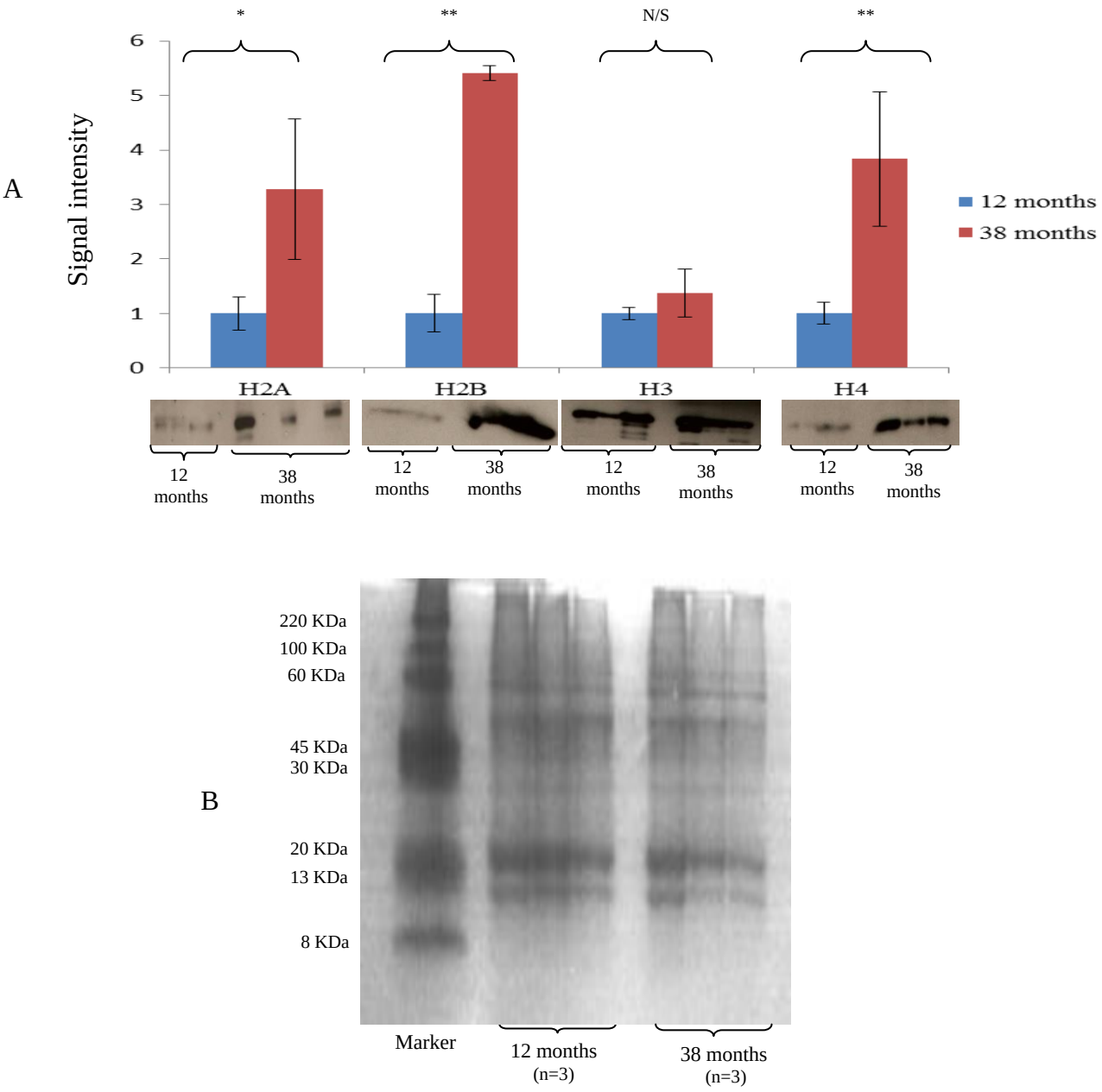


Figure 3.4: The effect of age on histone expression in mouse intestine. *Histones were detected by western blot analysis using anti-histone antibodies for H2A, H2B, H3, and H4.* ***(A)*** *Densitometric analysis of western blot data shown as mean ± standard errors (SEM) (n=3), *P <0.05, **P<0.01. Data are pooled from one analysis for each of H2A and H2B, three analyses for H3 and five analyses for H4 and are expressed normalised to the histone signal intensity for the young mice. Histone H2A, H2B, and H4 were significantly increased in old mice (38 months) compared with younger mice (12 months). Histone H3 did not show a significant change in level of expression.* ***(B)*** *Colloidal blue staining of a parallel gel was used to ensure equal loading of protein samples. Sizes of molecular weight markers running in parallel (left hand lane, as labelled) are indicated.*

3.3.2 The effect of age on histone expression in human intestine

To determine if the observation that histone expression increased with age in mouse intestine extended to humans, the effect of age on the level of histone expression in human subjects was investigated. Total protein was extracted from human colon biopsies of eleven males (at 21, 23, 33, 43, 64, 66, 73, 74, 77 or 82 years old). Western blot analysis was carried out using anti-histone antibodies for H2A, H2B, H3, and H4. Numerical data were derived by denstiometric quantification of band intensities. Blots were probed also with anti α-tubulin antibody and data expressed as a ratio of intensities of the two signals.

Figure 3.5 shows the level of histone expression measured in the colon biopsies of these young and old males. There was no apparent affect of age on the level of histone expression (H2A, H2B, H3, and H4) among these human subjects.

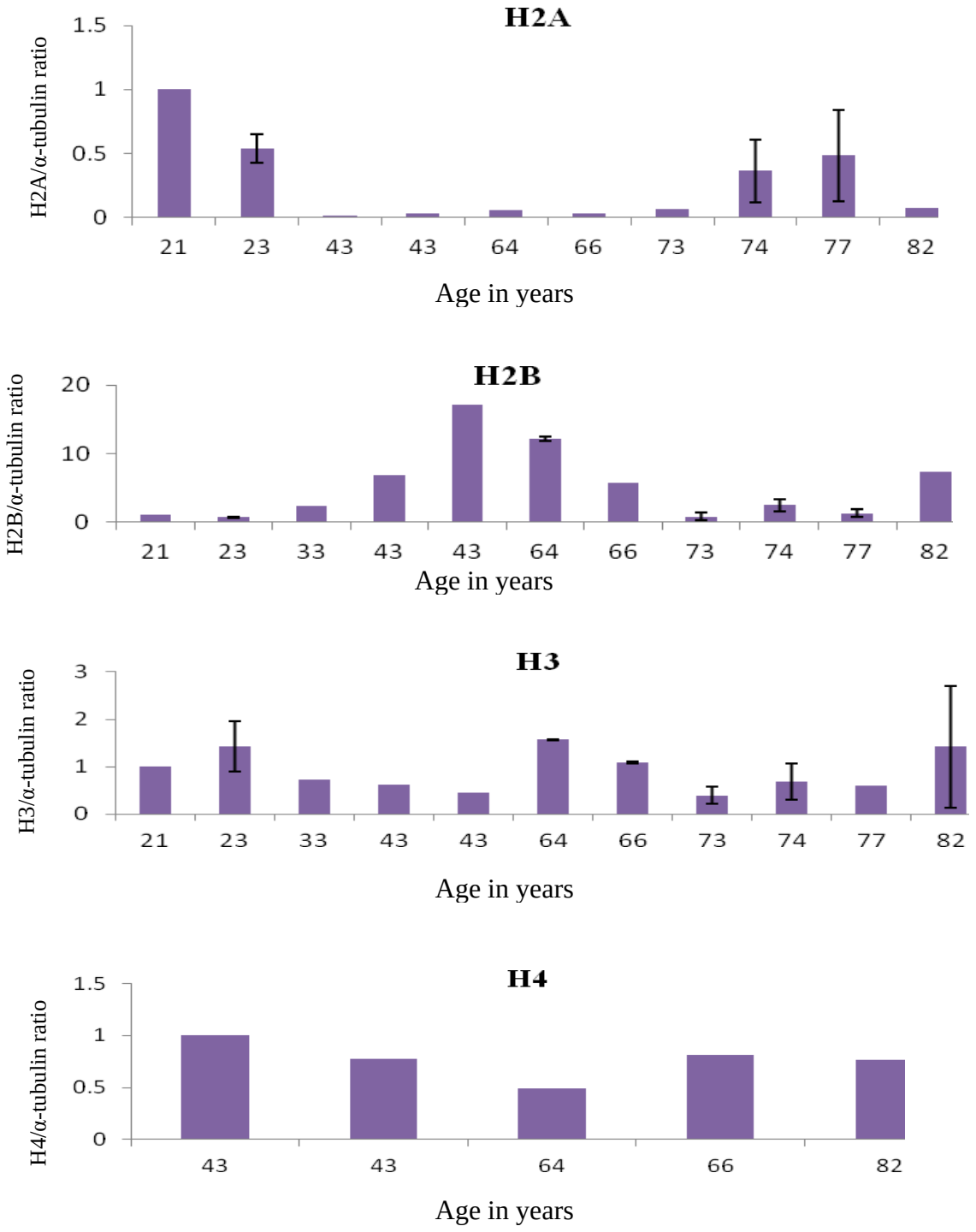


Figure 3.5: The effect of age on histone expression in human colon biopsies. *Densitometric analysis of western blot data shown as mean with standard errors. The age of each subject is indicated. Data are pooled from two analysis for each of H2A, H2B and H3 and one analysis for H4 and are expressed as mean ± standard errors (SEM) normalised to the histone signal intensity for the youngest subject. There was insufficient material to measure H4 expression in all subjects. There was no apparent effect of age on histone expression levels among these human subjects.*

3.4 Discussion

Histone proteins are essential proteins that are involved in DNA packing into nucleosomes. There are two types of histone proteins, core histone proteins including H2A, H2B, H3 and H4, and a linker histone known as H1. Each of the core histone proteins has a flexible N-terminal tail extending outward. In addition, histones H2A and H2B have a C-terminal tail that also extends outward from the nucleosome (Jenuwein *et al.*, 2001, cited in Bilsland, 2005).

The core histone proteins and their tails (N-terminal and C-terminal) undergo a variety of post-translational modifications, together comprising what is known as the "histone code". These modifications include acetylation, ubiquitination, methylation and phosphorylation (Jenuwein *et al.*, 2001, citied in Bilsland, 2005). These modifications of histone proteins play a fundamental role in the regulation of gene expression.

Recently, histone methylation/ acetylation has attracted increased attention as a result of findings that indicate role in regulating lifespan in different species. For example, mutations in members of the histone H3 methyltransferases have been documented to extend life span in the worm (Greer *et al.*, 2010; Maures *et al.*, 2011). Furthermore, stimulation of Sirt1 and Sirt2 histone deacetelyeses, by resveratrol or dietary restriction, has been reported to promote longevity in different species (Howitz *et al.*, 2003).

Studies in the laboratory, designed with the intention of investigating if resveratrol affects histone acetylation, indicated, unexpectedly, an effect on the expression level (rather than acetylation status) of histone proteins in the human intestinal Caco-2 cell line (L Wakeling and D Ford, personal communication).

The current study investigated the hypothesis that some of the beneficial effects of resveratrol on lifespan/aging are mediated through effects on histone expression. Further work would then investigate if these epigenetic actions of resveratrol influence the expression of genes that affect the ageing process. Towards this goal, the effect of resveratrol on the level of expression of histone proteins was investigated in two human cell lines. Caco-2 and MCF-7 cell lines were treated with resveratrol for 48 hours. Then western blot analysis using antihistone antibodies was conducted. The results showed that histone H2A, H2B, H3 and H4 expression was decreased in response to resveratrol treatment in both cell lines ($P<0.05$). Whereas both cell lines are reported to express ERβ, the Caco-2 cell line is reported to lack expression of ERα. The observations therefore indicate that if the observed effect of resveratrol is mediated through the ER then ERβ is functional in this regard. Further studies, presented in Chapter 5, investigated specifically the role of the ER in mediating effects of resveratrol on histone expression.

The concentration of resveratrol used in the current study (10 μM) is in line with concentrations considered achievable physiologically, for example through the consumption of resveratrol-rich foods (Mukherjee *et al.*, 2010). The observation may thus indicate an effect of resveratrol that could be achieved *in vivo*; however confirmation using either an animal model or, preferably, an intervention study in human participants is necessary to confirm such an effect. Studies *in vivo* would take into account the fact that resveratrol undergoes extensive metabolism, with glucuronide and sulphate conjugate being the prominent metabolite observed following ingestion (Yu *et al.*, 2002). Resveratrol glucuronide and sulphate conjugates are also major circulating metabolites observed in human subjects after oral ingestion of resveratrol (Goldberg *et al.*, 2003). Metabolism of resveratrol to dihdroresveratrol-glucuronides is also observed as reported in (Rotches-Ribalta *et al.*, 2012).

Other confirmatory research could include treatment of cells *in vitro* with metabolites of resveratrol to determine if these compounds have similar effects as the parent molecule on histone expression.

The impact, if any, of a reduction in histone expression on lifespan is still unclear but conceivable mechanisms through which effects may be mediated included effects on gene expression. To seek supporting data that resveratrol may promote lifespan through its effect on histone protein expression, further investigation focused on how histone expression changed with age.

There is little information about the effect of age on histone expression. To date, a small number of studies have reported a change in histone expression and/or chromatin configuration with age. For example, Chaturvedi *et al* (1985) reported that chromatin becomes more compact with age in the rat brain, and it has been observed that ageing in yeast is associated with a reduction in histone expression (Feser *et al.*, 2010).

The data presented here indicate that changes in histone expression are associated with age in mice. The expression levels of histone H2A, H2B and H4 were increased significantly in the intestine of old mice (at 38 months old) compared with young mice (at 12 months old). In contrast, no significant difference in the level of histone H3 expression between the two groups was detected, but a trend towards the same response as observed for the other histone proteins may indicate a need to analyse additional samples to detect an effect that reaches statistical significance. The mechanism underlying the influence of ageing on histone expression is still unclear. Similarly, the consequences of the observed reduction in histone expression are unknown but given that tight association of DNA with histones is a repressive

configuration, an expected effect may be reduced expression of genes associated with affected histones. Speculatively, such gene repression may lead to progressive ageing, age-related diseases such as cancer and, ultimately, death (Burzynski, 2003).

In contrast to the observations made in mouse intestine, Feser and colleagues (2010) reported a reduction in H3 and H4 expression with age in yeast. The possible explanation for the discordant observations may relate to the difference between unicellular yeast and mouse tissues.

In contrast to the observations made in mice, no age-associated difference in histone expression in the intestine of human subjects was detected.

The lack of consistency between the two sets of data-based on samples from mice and humans may be due to a number of different factors such as a difference between species, a difference between the small intestine (mice) and colon (humans) and a greater inter-individual variability in human samples, invoking the need to analyse a larger number of individuals to observe differences.

Since histone proteins were extracted from gut samples comprising the full thickness, cells in the sample would be from the mucosa, submucosa, muscularis, and serosa. The cell population would thus be heterogeneous and include absorptive cells, goblet cells, stem cells, enteroendocrine cells, and Paneth cells. (Treuting *et al.*, 2012). Levels of histone proteins detected would thus have reflected the levels of expression in these different cell types, and it is possible that the relative proportions of these cells differed with tissue age. An additional confounding factor may have been stage of cell cycle. During the cell cycle, histone mRNAs accumulate at the maximal levels during S phase. As cells exit S phase, the level of histone mRNAs decrease rapidly (Marzluff and Duronio, 2002). Thus, the histone protein quantity extracted would differ according to the proportion of cells at different stages in the cell cycle.

4 Global effects of resveratrol on gene expression

4.1 Introduction

Dietary restriction (DR), reduction of food intake below *ad libitum* without malnutrition, has been reported to promote longevity in diverse species from yeast to mammals (McCay *et al.*, 1935; Walker *et al.*, 2005). There is evidence that the mechanism is dependent on *Sirt*1 in mammals and on its homologue *Sir*2 in yeast, but this view has been to subject of vigorous challenge, based to a large degree on inconsistent observations made in yeast and flies (Burnett *et al.*, 2011). Evidence for the involvement of Sirt1 in mediating the effect of DR on lifespan extension in mammals includes that levels of Sirt1 have been documented to increase in some mammalian tissues in response to DR (Cohen *et al*, 2004; Nisoli *et al*., 2005), and that mice lacking *Sirt*1 (*Sirt*1-null mice) do not response to DR (Boily *et al*., 2008).

While there are potential benefits of DR, the challenges associated with this practice in humans has driven attempts to identify natural or artificial compounds with ability to mimic the effect of DR. Resveratrol has been reported to mimic to DR and extend lifespan in diverse species (Howitz *et al*., 2003; Wood *et al*, 2004; Baur *et al.*, 2006). Furthermore, resveratrol has been reported to promote longevity of mice under conditions of a high fat diet and to oppose the physiological effects of high caloric intake in manner similar to DR (Baur *et al.*, 2006). The mechanisms by which resveratrol mimics DR and influences lifespan are not completely understood. Opinions about the mechanism of action through which resveratrol has effects relevant to ageing/ or lifespan are at variance. Some investigators have a view that the mechanism is through activation of Sirt1 (Baur *et al.*, 2006), but others challenge this opinion (Pacholec *et al.*, 2010).

To begin to address the controversy around the possible role of Sirt1 in mediating effects of resveratrol that may mimic DR, the response of Caco-2 and MCF-7 cells to resveratrol was determined at the level of the transcriptiome with the aim of identifying if genes or pathways affected were also affected by manipulation of the expression level of *Sirt1*, for which a comparative data set was available from Caco-2 cells. These data were derived through previous work in the laboratory (LJ Ions and D Ford, personal communication). The extent to which genes affected by resveratrol intersected with a list of genes reported to be affected by dietary restriction (derived through previous *in silico* work in the laboratory; LJ Ions and D Ford, personal communication) was also determined.

4.2 The effect of resveratrol on global gene expression in Caco-2 and MCF-7 cell lines

Since reduced histone protein expression in response to resveratrol in Caco-2 and MCF-7 cells had been previously observed, it was important to conduct this transcriptiome analysis on cells where the same response was confirmed, reasoning that many effects on gene expression resulting from treatment with resveratrol could be a consequence of these changes in histone protein expression. After treatment of cells with 10 µM resveratrol for 48 hours, the usual response to resveratrol (reduction in histone protein expression) was confirmed. Total cell lysate from control and treated cells was analysed by western blotting using antihistone antibodies immunoreactive against H2A, H2B, H3 and H4. As shown in (Figure 4.1) histone H2A, H2B, H3 and H4 expression was decreased in response to resveratrol treatment in both cell lines.

RNA was extracted from Caco-2 and MCF-7 cells treated in parallel with 10 µM resveratrol for 48 hours. RNA concentration, purity and integrity were measured using the NanoDrop spectrophotometer and the Bioanalyzer (Figure 4.2). Global gene expression profiles were

determined by hybridization of RNA to the whole genome Illumina HumanHT-12v3 single colour beadchip microarray. Two biological replicates for each cell line (Caco-2 and MCF-7) and each condition (control or resveratrol treated) were hybridised separately to arrays. Sample processing and hybridisation was carried out by Arrayexpress (Leiden, the Netherlands).

Changes in gene expression were considered significant at $p < 0.05$ with a fold change ≥ 1.2. The 1.2 fold "cut off" was selected empirically based on the numbers of genes showing changes of particular magnitudes. Cut off values in microarray experiments are typically guided by characteristics of individual datasets in this way (McCarthy and Smyth, 2009; Bjornsdottir *et al.*, 2011). A relatively low threshold such as this is generally applied in studies looking at effects of dietary components on gene expression, where effects tend to be modest, and other studies have applied the same (Bjornsdottir *et al.*, 2011) or similar (1.3-fold) (Huggins *et al*., 2008) cut off values.

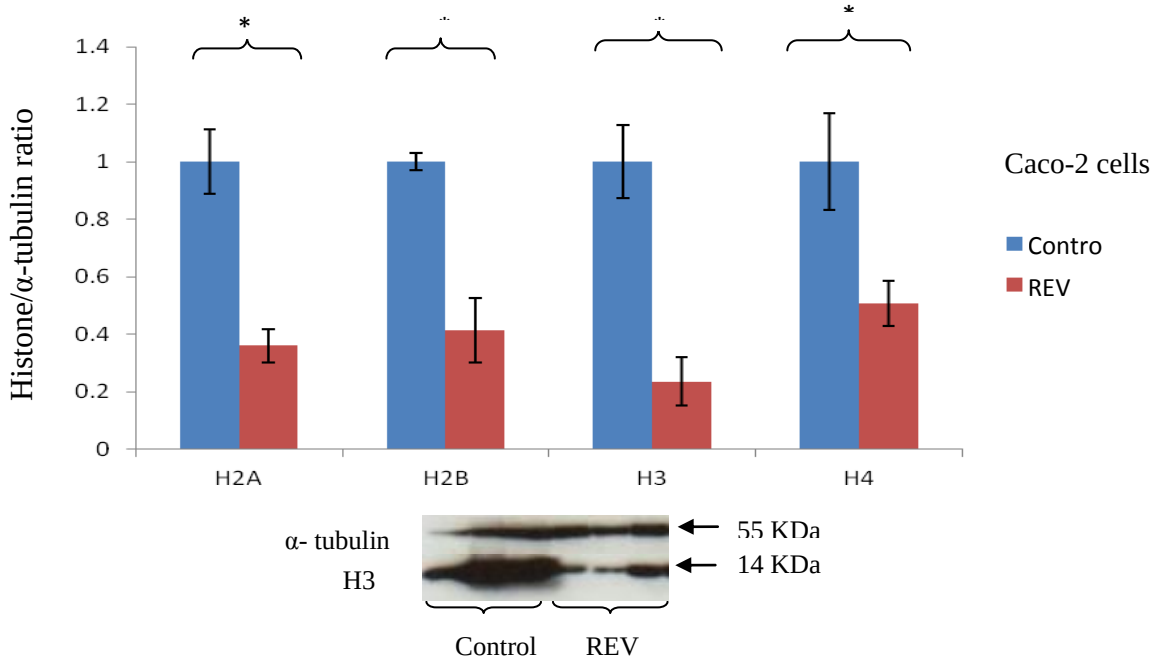
Tables 4.1 and 4.2 list the changes in gene expression in the Caco-2 cell line in response to resveratrol treatment. Tables 4.4 and 4.5 list the genes affected by resveratrol treatment in MCF-7 cells.

The pathways affected by resveratrol treatment were analysed using Ingenuity Pathway Analysis (IPA). The major pathways that were significantly affected by resveratrol treatment in Caco-2 and MCF-7 are presented in Table 4.3 and Table 4.6 respectively.

Resveratrol affected several metabolic pathways in the Caco-2 cell line. These pathways included propanoate metabolism, pyruvate metabolism and synthesis and degradation of ketone bodies. Estrogen receptor signalling and glucocorticoid receptor signalling pathways were also significantly affected by resveratrol treatment.

In the MCF-7 cell line, resveratrol affected several immune system pathways including: CTLA4 Signalling in Cytotoxic T Lymphocytes, PKCθ Signalling in T Lymphocytes, Calcium-induced T Lymphocyte Apoptosis, and Role of NFAT in Regulation of the Immune Response.

(A)



(B)

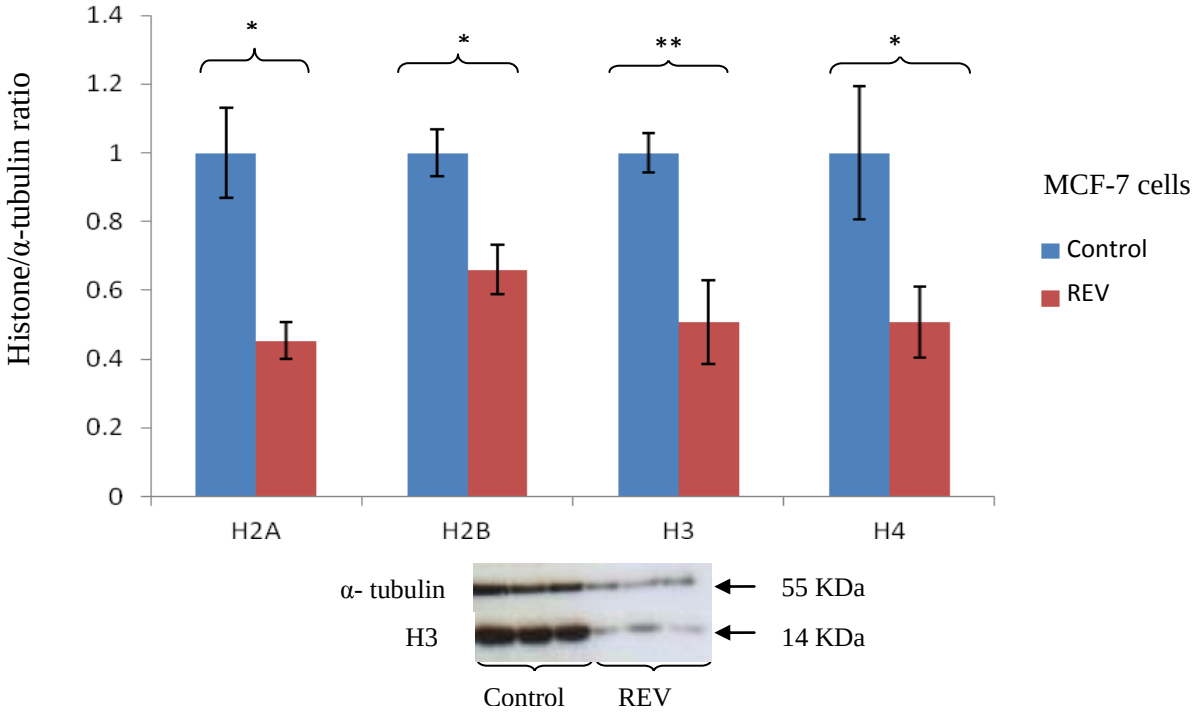


Figure 4.1: The effect of resveratrol (REV) on histone protein expression in Caco-2 and MCF-7 cells. (A) *Results of densitometric analysis of western blots detecting expression of histone proteins in Caco-2 cells are shown as mean with standard errors (n=3) *P <0.05, **P<0.01. The expression of histone H2A, H2B, H3 and H4 were significantly decreased in cells treated with REV.* ***(B)*** *Results of densitometric analysis of western blots detecting expression of histone proteins in MCF-7 cells are shown as mean with standard errors (n=3) *P <0.05. The expression of histone H2A, H2B, H3 and H4 were significantly decreased in cells treated with REV. Sample western blots are shown under each panel.*

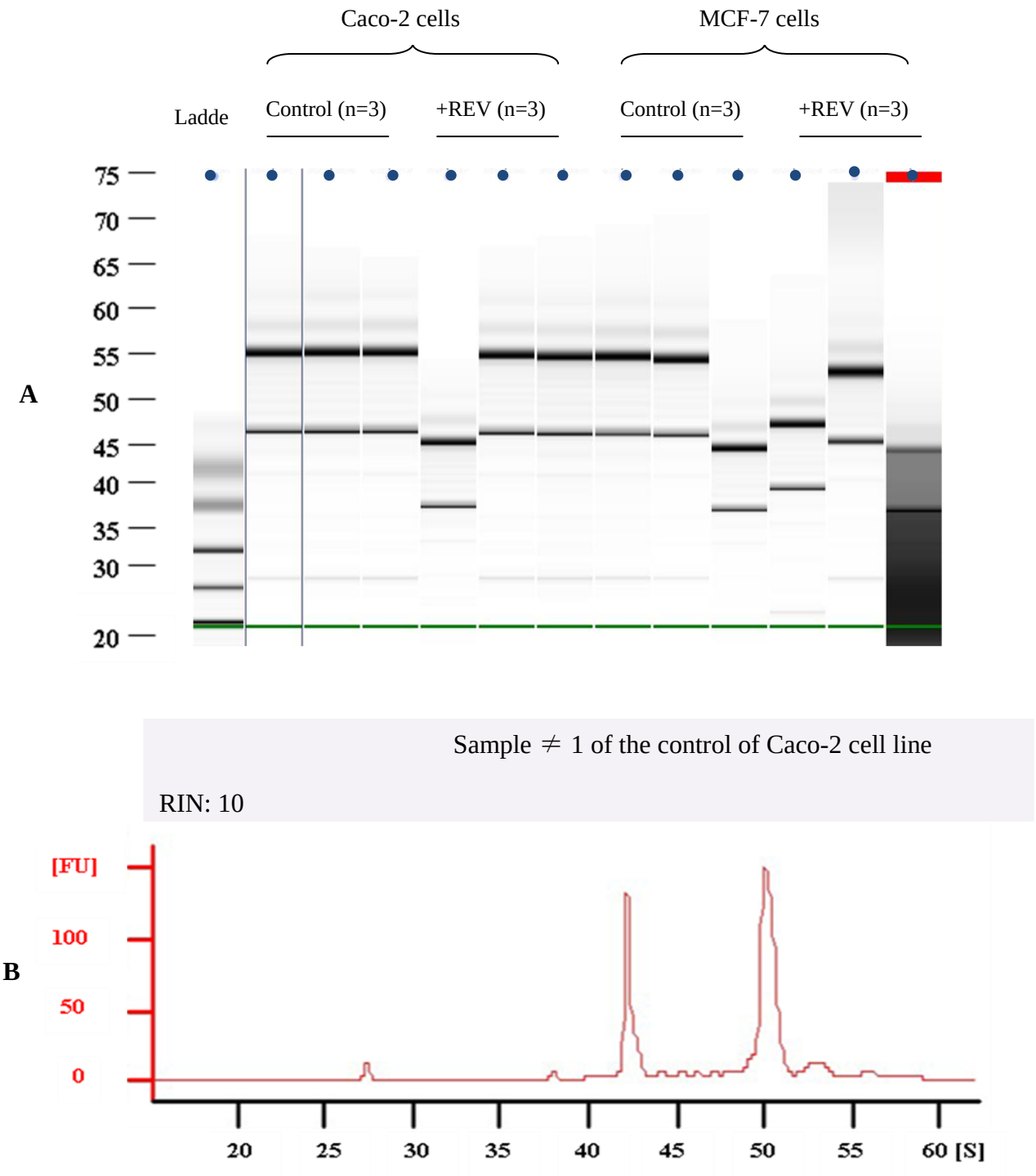


Figure 4.2: Analysis of RNA integrity using the Agilent 2100 bioanalyser. (A) *Gel-like image generated from bioanalyser electropherogram output of the analysis of RNA samples of the control (control) and treated with resveratrol (REV) extracted from Caco-2 and MCF-7cells.* ***(B)*** *Typical bioanalyser electropherogram output from the control (number1) of Caco-2 cell line. RNA integrity number (RIN) above the threshold of 7 was required for downstream application.*

***Table 4.1**: Genes upregulated in the Caco-2 cell line after treatment with resveratrol*

Gene symbol	Description	Fold change
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	1.26
APCDD1	Adenomatosis polyposis coli down-regulated 1	1.20
AQR	Aquarius homolog (mouse)	1.22
ATP10A	ATPase, class V, type 10A	1.25
BAT3	HLA-B-associated transcript 3	1.21
BOLA3	BolA homolog 3	1.22
CCNF	Cyclin F	1.32
DGCR11	DiGeorge syndrome critical region gene 11	1.20
FKBP5	FK506 binding protein 5	1.20
IGSF3	Immunoglobulin superfamily member 3	1.20
MIR586	MicroRNA 586	1.26
NAT5	n-terminal acetyltransferase b complex catalytic subunit nat5	1.20
NDUFA10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	1.20
PIGO	Phosphatidylinositol glycan anchor biosynthesis, class O	1.30
RPS27	Ribosomal protein S27 pseudogene	1.26
SCO2	SCO cytochrome oxidase deficient homolog 2 (yeast)	1.23
SNRPF	Small nuclear ribonucleoprotein polypeptide F	1.25
UTP14A	UTP14, U3 small nucleolar ribonucleoprotein, homolog A (yeast)	1.26

***Table 4.2**: Genes down regulated in the Caco-2 cell line after treatment with resveratrol*

Gene symbol	Description	Fold change
AADACL4	Arylacetamide deacetylase-like 4	1.28
AKR1D1	Aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase)	1.21
AMH	Anti-Mullerian hormone	1.20
AOF2	Amine oxidase (flavin containing) domain 2	1.25
ARID4B	AT rich interactive domain 4B (RBP1-like)	1.22
ARL3	ADP-ribosylation factor-like 3	1.21
ATF4	Activating transcription factor 4	1.21
ATP2B4	ATPase, Ca++ transporting, plasma membrane 4	1.22
BAZ1A	Bromodomain adjacent to zinc finger domain, 1A	1.20
C1GALT1C1	C1GALT1-specific chaperone 1	1.22
CBS	Cystathionine-beta-synthase	1.34
CCNC	Cyclin C	1.24
CCT3	Chaperonin containing TCP1, subunit 3 (gamma)	1.29
CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)	1.52
CGA	Glycoprotein hormones, alpha polypeptide	1.42
CNOT8	CCR4-NOT transcription complex, subunit 8	1.25
CREB1	cAMP responsive element binding protein 1	1.26
CSNK1D	Casein kinase 1, delta	1.24
DDX51	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	1.31
DENND1A	DENN/MADD domain containing 1A	1.24
DKFZP761E198	DKFZp761E198 protein	1.20
DKK3	Dickkopf homolog 3 (Xenopus laevis)	1.25
FLJ44124	Uncharacterized LOC641737	1.36
FOSB	Fosfomycin resistance protein FosB	1.20
FZD2	Frizzled homolog 2 (Drosophila)	1.25
GADD45G	Growth arrest and DNA-damage-inducible, gamma	1.30
HBP1	HMG-box transcription factor 1	1.22
HSPB1	Heat shock 27kDa protein 1	1.22
IFT172	Intraflagellar transport 172 homolog (Chlamydomonas)	1.21
KLF10	Kruppel-like factor 10	1.22
KLF4	Kruppel-like factor 4 (gut)	1.26
LGSN	Lengsin, lens protein with glutamine synthetase domain	1.20
MAK10	MAK10 homolog, amino-acid N-acetyltransferase subunit, (S. cerevisiae)	1.30
MCM8	Minichromosome maintenance complex component 8	1.42
MED1	Mediator complex subunit 1	1.20
MIR197	MicroRNA 197	1.24
NFAT5	Nuclear factor of activated T-cells 5, tonicity-responsive	1.30
NHLRC3	NHL repeat containing 3	1.25
NIPSNAP1	Nipsnap homolog 1 (C. elegans)	1.25

Continued

Gene symbol	Description	Fold change
OVOL1	OVO homolog-like 1 (Drosophila)	1.31
OXR1	Oxidation resistance 1	1.26
PAPOLA	Poly(A) polymerase alpha	1.26
PCSK5	Proprotein convertase subtilisin/kexin type 5	1.21
PCYOX1	Prenylcysteine oxidase 1	1.20
PINK1	PTEN induced putative kinase 1	1.23
RPS6KA3	Ribosomal protein S6 kinase, 90kDa, polypeptide 3	1.35
SAMD4B	Sterile alpha motif domain containing 4B	1.27
SCGN	Secretagogin, EF-hand calcium binding protein	1.28
SCML1	Sex comb on midleg-like 1 (Drosophila)	1.27
SCYL1	SCY1-like 1 (S. cerevisiae)	1.20
SKA2	Spindle and kinetochore associated complex subunit 2	1.40
SLC22A18AS	Solute carrier family 22 (organic cation transporter), member 18 antisenseBottom of Form Top of Form	1.26
SLC39A7	Solute carrier family 39 (zinc transporter), member 7	1.26
STXBP6	Syntaxin binding protein 6 (amisyn)	1.20
TINAGL1	Tubulointerstitial nephritis antigen-like 1	1.22
TP53INP1	Tumor protein p53 inducible nuclear protein 1	1.20
UBXN6	UBX domain protein 6	1.20
UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	1.21
ZNF607	Zinc finger protein 607	1.21
ZSWIM4	Zinc finger, SWIM-type containing 4	1.22

Table 4.3: *Pathways affected by resveratrol treatment in the Caco-2 cell line*

Ingenuity Canonical Pathways	-log(p-value)	Genes
Propanoate Metabolism	3.07E00	ALDH1A1, ACAT2, PCCA, ACAT1, ACACA, LDHA
p53 Signaling	3E00	SCO2, TP53INP1, JMY, GADD45G, MED1, CSNK1D, PML
Pyruvate Metabolism	2.66E00	ALDH1A1, ACAT2, PCK2, ACAT1, ACACA, LDHA
Melanocyte Development and Pigmentation Signaling	2.53E00	RPS6KB1, MITF, CREB1, ADCY1, RPS6KA3, ATF4
Hypoxia Signaling in the Cardiovascular System	2.26E00	CREB1, CSNK1D, ATF4, UBE2J1, LDHA
Glucocorticoid Receptor Signaling	1.86E00	TGFBR2, NFAT5, TAF5, PCK2, MED1, CREB1, NCOR2, NFATC4, FKBP5, SMARCA4
Circadian Rhythm Signaling	1.82E00	CREB1, CSNK1D, ATF4
Cleavage and Polyadenylation of Pre-mRNA	1.77E00	PAPOLA, CSTF3
Synthesis and Degradation of Ketone Bodies	1.77E00	ACAT2, ACAT1
ERK5 Signaling	1.68E00	RPS6KB1, CREB1, RPS6KA3, ATF4
Estrogen Receptor Signaling	1.68E00	TAF5, CCNC, PCK2, MED1, NCOR2, SMARCA4
Valine, Leucine and Isoleucine Degradation	1.53E00	ALDH1A1, ACAT2, PCCA, ACAT1
FLT3 Signaling in Hematopoietic Progenitor Cells	1.47E00	RPS6KB1, CREB1, RPS6KA3, ATF4
Role of IL-17F in Allergic Inflammatory Airway Diseases	1.44E00	CREB1, RPS6KA3, ATF4
VDR/RXR Activation	1.37E00	CCNC, MED1, NCOR2, KLF4

Table 4.4: *Genes upregulated in the MCF-7 cell line after treatment with resveratrol*

Gene symbol	Description	Fold change
CD79A	CD79a molecule, immunoglobulin-associated alpha	1.20
DBN1	Drebrin 1	1.23
GRINA	Glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)	1.22
GSTTP2	Glutathione S-transferase theta pseudogene 2	1.35
IMPDH2	IMP (inosine 5'-monophosphate) dehydrogenase 2	1.20
MIR1974	MicroRNA 1974	1.30
NUCB1	CG32190 gene product from transcript CG32190-RA	1.21
OAZ2	Ornithine decarboxylase antizyme 2	1.22
PGM5	Phosphoglucomutase-like protein 5	1.44
SS18L1	Synovial sarcoma translocation gene on chromosome 18-like 1	1.21
STXBP2	Syntaxin binding protein 2	1.20
ZNF334	Zinc finger protein 334	1.22
ZNF773	Zinc finger protein 773	1.22

Table 4.5: *Genes downregulated in the MCF-7 cell line after treatment with resveratrol*

Gene symbol	Description	Fold change
ANKRD32	Ankyrin repeat domain 32	1.20
ASB7	Ankyrin repeat and SOCS box containing 7	1.20
C6orf148	KH homology domain containing 1	1.24
DCDC2B	Doublecortin domain containing 2B	1.21
GRN	Granulin	1.39
HMG20A	High mobility group 20A	1.23
IFI6	Interferon, alpha-inducible protein 6	1.40
INSIG1	Insulin induced gene 1	1.21
KANK2	KN motif and ankyrin repeat domains 2	1.24
KIF23	Kinesin family member 23	1.21
LOC441131	Actin related protein 2/3 complex, subunit 3 pseudogene 5	1.24
LOC647349	AP-3 complex subunit sigma-1-like	1.22
MRPS16	Mitochondrial ribosomal protein S16	1.24
NUDT9	Nudix (nucleoside diphosphate linked moiety X)-type motif 9	1.23
PCGF3	Polycomb group ring finger 3	1.20
RABIF	RAB interacting factor	1.23
RAD51C	RAD51 homolog C (S. cerevisiae)	1.22
UBIAD1	UbiA prenyltransferase domain containing 1	1.20
ZNRD1	Zinc ribbon domain containing 1	1.23

Table 4.6: *Pathways affected by resveratrol treatment in the MCF-7 cell line*

Ingenuity Canonical Pathways	-log(p-value)	Genes
B Cell Development	2.2E00	HLA-DQB1, HLA-DRB5, CD79A
Role of NFAT in Regulation of the Immune Response	2.01E00	HLA-DQB1, GNB1, NRAS, LAT, HLA-DRB5, CD79A
Ephrin Receptor Signaling	2.01E00	PTK2, GNB1, NRAS, EPHA1, EFNA3, GRINA
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	1.89E00	HLA-DQB1, TNFSF11, HLA-DRB5, CD79A
Virus Entry via Endocytic Pathways	1.82E00	AP1G2, NRAS, PRKCD, TFRC
CTLA4 Signaling in Cytotoxic T Lymphocytes	1.79E00	HLA-DQB1, AP1G2, LAT, HLA-DRB5
One Carbon Pool by Folate	1.62E00	SHMT1, GART
Calcium-induced T Lymphocyte Apoptosis	1.56E00	HLA-DQB1, PRKCD, HLA-DRB5
PKCθ Signaling in T Lymphocytes	1.48E00	HLA-DQB1, NRAS, LAT, HLA-DRB5
IL-15 Production	1.46E00	PTK2, PTK7
IL-4 Signaling	1.46E00	HLA-DQB1, NRAS, HLA-DRB5
Macropinocytosis Signaling	1.46E00	NRAS, RAB5A, PRKCD
Glycine, Serine and Threonine Metabolism	1.37E00	TDH, SHMT1, SMOX

4.3 The intersections between lists of genes responding to dietary restriction (DR) and genes affected by resveratrol treatment

To investigate if resveratrol has effects similar to dietary restriction (DR), the intersections between the lists of genes affected by resveratrol treatment determined in the current investigation and a list of genes that respond to DR was identified. For this analysis the list of genes affected by dietary restriction was compiled from published studies in mice (LJ Ions and D Ford, personal communication). The representation factor (RF) and cumulative hypergeometric probability were calculated. A representation factor >1 indicates a greater overlap than expected between two independent groups, and hypergeometric probability indicates if any over-representation is statistically significant.

As shown in Figure 4.3, 51 genes were significantly changed in their expression in response to both DR and resveratrol treatment in the Caco-2 cell line (Table 4.7B). Out of these genes, ten genes were associated with pathways that significantly changed in response to resveratrol treatment. These genes are *ACAT*1, *ACAT*2, *ALDH1A*1, *ATF*4, *CSNK1D, FKBP*5, *GADD*45*G*, *KLF*4, *LDHA*, and *SMARCA*4.

In the MCF-7 cell line, 30 genes responded to both DR and resveratrol treatment (Table 4.7A). Seven of these genes were associated with pathways that significantly changed in response to resveratrol treatment. These genes are *AP1G2, CD*79*A, GRINA, NRAS, PRKCD, SHMT*1, and *TFRC*.

Importantly, both overlaps were greater than expected by chance (RF>1 and $P<0.05$).

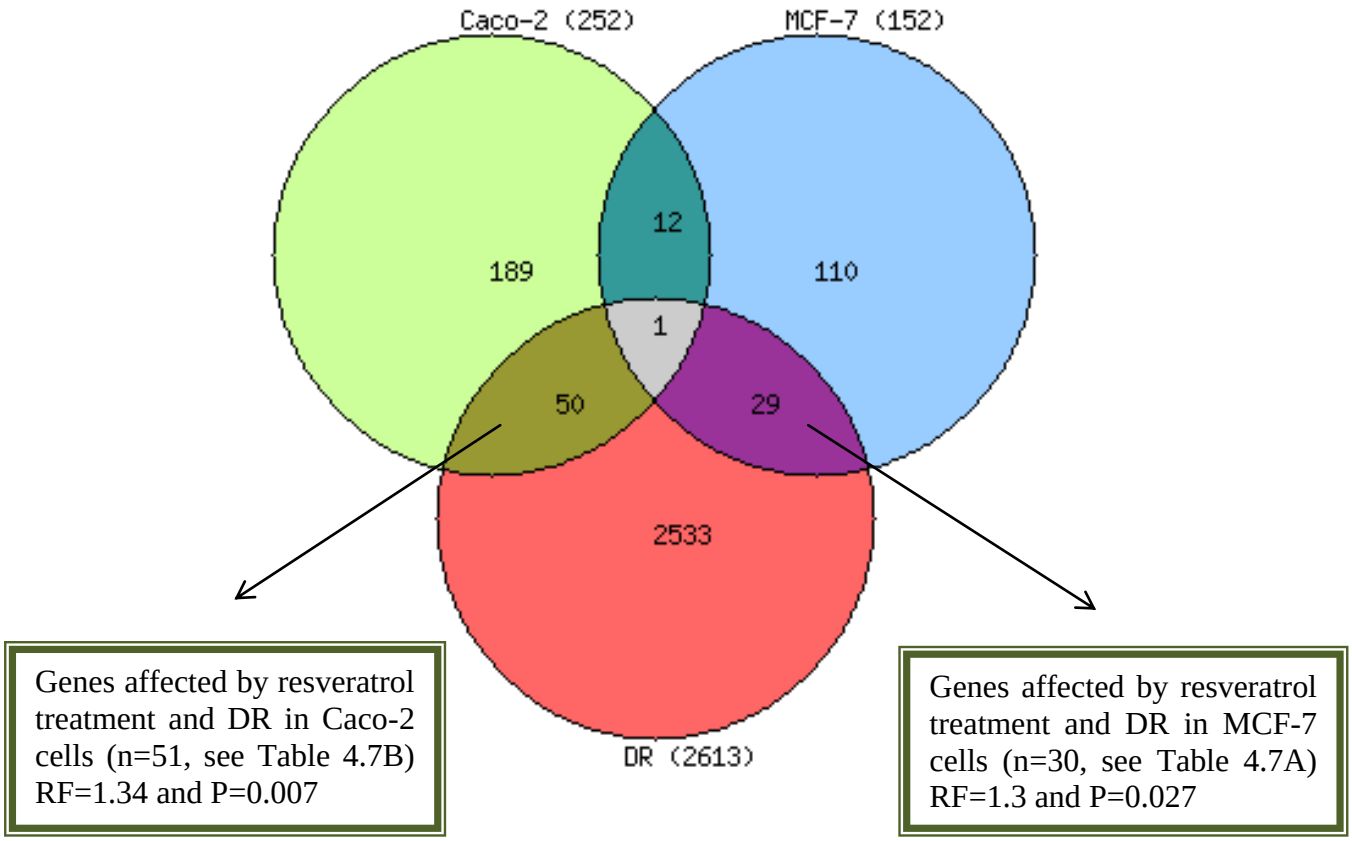


Figure 4.3: *Intersections between lists of genes affected by resveratrol treatment in Caco-2 and MCF-7 cell lines and genes responding to dietary restriction (DR), based on a list compiled from published studies in mice.*

Table 4.7: *List of genes affected resveratrol treatment and DR in Caco-2 and MCF-7 cell lines*

(A) Genes affected by resveratrol treatment and DR in MCF-7 cells (n=30)			
ENSMUSG00000003379	Cd79a	ENSMUSG00000029198	Grpel1
ENSMUSG00000018882	Mrpl45	ENSMUSG00000030824	Nucb1
ENSMUSG00000019889	Ptprk	ENSMUSG00000034308	Sdr42e1
ENSMUSG00000020241	Col6a2	ENSMUSG00000034708	Grn
ENSMUSG00000020534	Shmt1	ENSMUSG00000038145	Snrk
ENSMUSG00000021203	Otub2	ENSMUSG00000038582	Pptc7
ENSMUSG00000021286	Zfyve21	ENSMUSG00000039114	Nrn1
ENSMUSG00000021948	Prkcd	ENSMUSG00000040701	Ap1g2
ENSMUSG00000022464	Slc38a4	ENSMUSG00000049470	Aff4
ENSMUSG00000022564	Grina	ENSMUSG00000053916	Nanp
ENSMUSG00000022797	Tfrc	ENSMUSG00000055053	Nfic
ENSMUSG00000024145	Pigf	ENSMUSG00000056917	Sipa1
ENSMUSG00000026203	Dnajb2	ENSMUSG00000059208	Hnrnpm
ENSMUSG00000027852	Nras	ENSMUSG00000060126	Tpt1
ENSMUSG00000029064	Gnb1	ENSMUSG00000031438	Rnf128
(B) Genes affected by resveratrol treatment and DR in Caco-2 cells (n=51)			
ENSMUSG00000001416	Cct3	ENSMUSG00000028454	Pigo
ENSMUSG00000002910	Arrdc2	ENSMUSG00000028776	Tinagl1
ENSMUSG00000002996	Hbp1	ENSMUSG00000028967	Errfi1
ENSMUSG00000003032	Klf4	ENSMUSG00000029068	Ccnl2
ENSMUSG00000003545	Fosb	ENSMUSG00000030337	Vamp1
ENSMUSG00000018736	Ndel1	ENSMUSG00000030772	Dkk3
ENSMUSG00000018900	Slc22a5	ENSMUSG00000032041	Tirap
ENSMUSG00000020260	Pofut2	ENSMUSG00000032047	Acat1
ENSMUSG00000020262	Adarb1	ENSMUSG00000032187	Smarca4
ENSMUSG00000021453	Gadd45g	ENSMUSG00000034285	Nipsnap1
ENSMUSG00000022037	Clu	ENSMUSG00000034584	Exph5
ENSMUSG00000022415	Syngr1	ENSMUSG00000035248	Zcchc6
ENSMUSG00000022957	Itsn1	ENSMUSG00000037465	Klf10
ENSMUSG00000023075	Akirin1	ENSMUSG00000037601	Nme1
ENSMUSG00000023832	Acat2	ENSMUSG00000038641	Akr1d1
ENSMUSG00000024039	Cbs	ENSMUSG00000041058	Wwp1
ENSMUSG00000024222	Fkbp5	ENSMUSG00000042406	Atf4
ENSMUSG00000024487	Yipf5	ENSMUSG00000045160	Bola3
ENSMUSG00000024713	Pcsk5	ENSMUSG00000045954	Sdpr
ENSMUSG00000025035	Arl3	ENSMUSG00000050213	Snip1
ENSMUSG00000025129	Ppp1r27	ENSMUSG00000053279	Aldh1a1
ENSMUSG00000025162	Csnk1d	ENSMUSG00000055491	Pprc1
ENSMUSG00000025395	Prim1	ENSMUSG00000063229	Ldha
ENSMUSG00000026730	Pter	ENSMUSG00000066026	Dhrs3
ENSMUSG00000027803	Wwtr1	ENSMUSG00000072082	Ccnf
ENSMUSG00000031438	Rnf128		

4.4 The intersections between lists of genes affected by resveratrol treatment and genes affected by Sirt1 knockdown

Towards addressing the question of whether or not resveratrol acts through *Sirt*1, the intersections between lists of genes affected by resveratrol treatment as identified in the current analysis and genes affected by knockdown of *Sirt*1 expression was identified. For this analysis a list of genes affected by *Sirt*1 knockdown in Caco-2 cells derived through previous research in the laboratory was used (LJ Ions and D Ford, personal communication).

As shown in Figure 4.4, only one gene was changed in its expression in response to both knockdown of *Sirt*1 expression and resveratrol treatment in the Caco-2 cell line (Table 4.8B). In the MCF-7 cell line, two genes responded to resveratrol treatment that also appeared on the list of genes that were affected by *Sirt*1 knockdown in Caco-2 cells (Table 4.8A). There was a remarkable distinction between the genes that responded to resveratrol treatment and to *Sirt*1 knockdown in the Caco-2 cell line. As expected, since genes that responded to resveratrol treatment in the Caco-2 and MCF-7 cell lines tended also to be distinct, there was also little overlap between the list of genes that responded to resveratrol treatment in the MCF-7 cell line and to *Sirt*1 knockdown in Caco-2 cells.

4.5 Occurrence of the ERE in the promoter region of genes affected by resveratrol

Given that resveratrol has structural similarity to estrogen, promoter regions of the genes affected by resveratrol treatment in both cell lines were searched for occurrences of the estrogen-response element (ERE) consensus sequence using Genomatix software. Of the 448 genes affected by resveratrol treatment in Caco-2 cells, the ERE was identified in only 9 genes. These genes are: *NFATC*4, *NME*1, *NRG*1, *CCNC*, *MED*1, *NCOR*2, *PCK*2, *SMARCA*4 and *TAF*5. Thus, the analysis indicated either that there was no enrichment of genes likely to

respond to estrogen among those responsive to resveratrol or that genes that are estrogen responsive have binding sites for the ligand ER complex that differ from the sequence(s) used for this search.

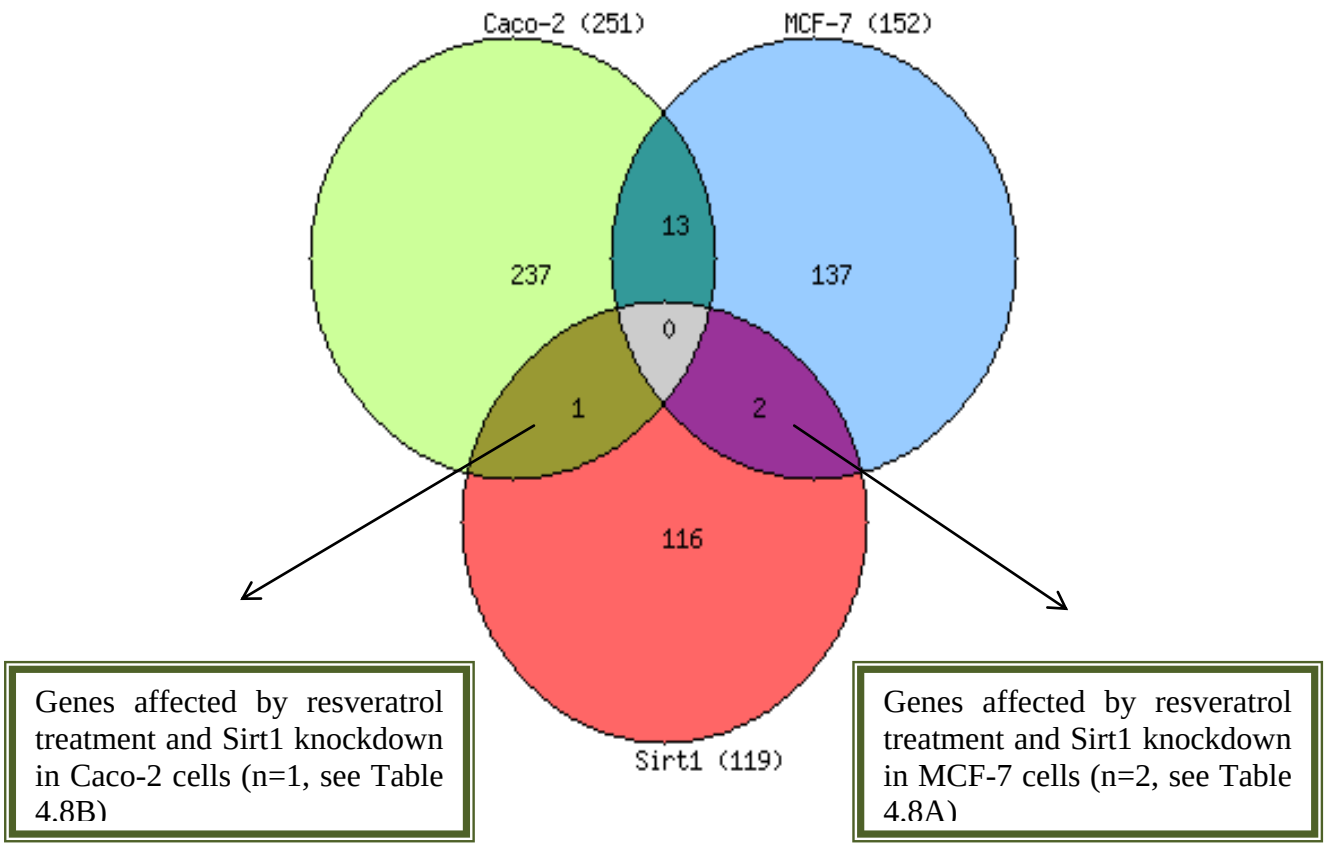


Figure 4.4: *Intersections between lists of genes affected by resveratrol treatment in Caco-2 and MCF-7 cell lines and genes affected by knockdown of Sirt1 expression in Caco-2 cells.*

Table 4.8: *Genes affected by knockdown of Sirt1 expression and resveratrol treatment in Caco-2 and MCF-7 cell lines*

(A) Genes affected by resveratrol treatment and *Sirt*1 knockdown in MCF-7 cells

Gene symbol	**Description**
RPS29	Ribosomal protein S29
TPT1	Tumor protein, translationally-controlled 1

(B) Genes affected by resveratrol treatment and *Sirt*1 knockdown in Caco-2 cells

Gene symbol	**Description**
IPO11	Importin 11

4.6 Discussion

Dietary restriction (DR) and resveratrol have been reported to promote longevity in diverse species from yeast to mammal through a mechanism that may be dependent on Sirt1 in mammals and on its homologue Sir2 in yeast.

In the work presented in this chapter, we investigated if resveratrol (at a concentration that is achievable physiologically) has a similar effect to that of DR or to that of manipulating Sirt1 expression at the level of gene expression in a human epithelial cell line model. Microarray analysis was carried out on RNA extracted from Caco-2 or MCF-7 cell lines treated with 10 μM resveratrol for 48 hours. The intersections between the derived list of genes affected by resveratrol treatment in both cell lines and an available list of genes responsive to DR were identified. The finding that both overlaps were greater than expected by chance lends support to the view that responses to resveratrol and to dietary restriction have some commonality and that resveratrol may mimic some effects of dietary restriction.

Towards investigating if resveratrol exerts its effects through Sirt1, the intersections between the lists of genes affected by resveratrol treatment and an available list achieved by knockdown of *Sirt1* expression in Caco-2 cells was identified. The finding that there was very little overlap between genes affected by resveratrol treatment and by knockdown of *Sirt1* expression adds to accumulating evidence that, contrary to previous opinion, resveratrol, at the concentration used in these experiments, does not act through effects on Sirt1. This view is supported by *in vivo* evidence based on a rodent model (Barger *et al.*, 2008a). In contrast, incubation of exogenous Sirt1 with human hyperacetylated histones caused a deacetylation at H4K16 and H3K9 (Vaquero *et al.*, 2004). Moreover, resveratrol treatment of a *Sirt*1$^{-/-}$ mouse myoblast cell line failed to induce the deacetylation of the Sirt1 substrate PGC-1α observed in wild-type myoblasts (Lagouge *et al.*, 2006), suggesting that the

resveratrol effect is mediated through Sirt1. Recently, Park and colleagues (2012) reported that the activation of Sirt1 by resveratrol appears to be indirect, through cAMP-mediated activation AMP-activated protein kinase (AMPK). Resveratrol-activated AMPK may increase the level of NAD^+ in the cell and thereby activate Sirt1 (Um *et al.*, 2010).

We observed a remarkable lack of commonality between lists of genes and between the pathways affected by resveratrol in Caco-2 (intestinal) and MCF-7 (breast cancer) cell lines. This observation is concordant with effects reported *in vivo*, indicating tissue-specific actions of resveratrol including actions on Sirt1. For example, Sirt1 expression was regulated differently by resveratrol in different tissues of resveratrol-treated mice. The expression of Sirt1 was downregulated in the heart but upregulated in the muscle, and did not change in the brain (Barger *et al.*, 2008b). Dose dependency is also a factor that should be considered. For instance, mice treated with high dose of resveratrol (22.4 mg/kg of body weight) showed enhanced longevity and also effects suggesting a role for Sirt1 (Baur *et al.*, 2006). In contrast, a low dose of resveratrol (4.9 mg/kg of body weight) showed no effect on lifespan in mice and no effect on *Sirt1* expression (Barger *et al.*, 2008a). However, direct comparison between these two studies is not possible because the background diet differed substantially; mice in the study by Baur and colleges received a high fat diet. Dietary levels of resveratrol that may be effective thus remain undefined. The concentration of resveratrol used in the current study (10 μM) is considered to be physiologically achievable through normal consumption of resveratrol-rich foods, but our cell culture models do not necessary reflect exposure of tissues *in vivo* to effects of dietary resveratrol, given that the compound undergoes substantial metabolism to glucuronide and sulphate conjugates (Goldberg *et al.*, 2003; Rotches-Ribalta *et al.*, 2012). The Caco-2 (intestinal) model may reflect more accurately effects of dietary resveratrol given that the metabolites are exposed to the parent compound. For both cell lines,

it would be of interest to measure the level of metabolism of resveratrol that occurs under our treatment conditions.

These data show that resveratrol affected several metabolic pathways in the Caco-2 cell line including propanoate metabolism, pyruvate metabolism and synthesis and degradation of ketone bodies. A possible explanation for these observations is that, as an intestinal cell line, Caco-2 represents the tissue that first encounters absorbed dietary metabolic substrates, so perhaps these pathways are particularly susceptible to perturbation. On the other hand, immune system pathways were the principal target of resveratrol in the breast cancer MCF-7 cell line. These pathways included calcium-induced T lymphocyte apoptosis, and role of NFAT in regulation of the immune response. The immune system has a contributory effect in early-stage breast cancer (Mouawad *et al.*, 2011), perhaps reflecting that these pathways are particularly prone to perturbation in breast cancer cells and thus providing a possible explanation for the observed effects of resveratrol. This apparent tissue-specific response to resveratrol is also apparent from the microarray data on an individual gene level. For example, amyolid precursor protein (*APP*) gene was upregulated in Caco-2 cells but downregulated in MCF-7 cells. This gene is of particular interest in the context of ageing. The *APP* gene is located on chromosome 21 ($21q^{21.2}$) and expressed in different tissues with the highest expression in neuronal cells in the central nervous system (CNS) (Zhang *et al.*, 2011). The functions of APP protein not fully understood, but a role in regulation of neuron growth and survival seems evident (Turner *et al.*, 2003; Priller *et al.*, 2006). The APP protein is the precursor molecule of beta-amyloid (Aβ), which is involved in the formation of amyloid plaques in the brain of Alzheimer disease patients. Mutation of the *APP* gene has been reported to increase the risk of early-onset of Alzheimer disease (Goate *et al.*, 1991). Furthermore, $APP^{-/-}$ mice showed features of ageing and neurodegenerative disease

including loss of body mass and weakness with reduced expression of synaptic markers associated with learning deficit and losses of memory (Prille *et al.*, 2006).

Some key genes - *FOXO1, PGC-1α* and *IGF-1*- that have been linked with ageing and particularly with a modulating effect of DR and/ or Sirt1- were absent from the lists of genes found to be affected by resveratrol in Caco2 and MCF-7 cells (Baur *et al.*, 2006). In contrast, these genes have been reported to be affected in different tissues including heart, liver, brain and skeletal muscle extracted from mice treated with resveratrol.

A possible explanation for these discordant observations is the apparent tissue/ cell specificity of resveratrol action at the level of gene expression, as indicated by the very different response we observed in Caco-2 compared with MCF-7 cells. Microarray data on gene responses to resveratrol in intestinal and breast tissues derived from mice treated with resveratrol would allow this idea to be tested, but appear unavailable at present in published resources.

Further analysis of the transcriptionic data involved investigating if the estrogen-response element (ERE) occurred at a higher frequency than expected by chance in genes regulated by resveratrol. The search for occurrences of the ERE consensus sequence in the promoter regions of the genes affected by resveratrol treatment in both cell lines was carried out using Genomatix software. The analysis indicated that genes including the ERE were not enriched within the population of genes that responded to resveratrol treatment in either cell line. This finding may indicate that resveratrol affects gene expression, generally, through a mechanism independent of direct binding of a resveratrol-ER complex to the ERE. Alternatively, an interpretation of the results could be that many of the genes do respond through binding of a resveratrol-ER complex to sequences in the promoter regions that act as EREs, but that the

criteria we used to define EREs were inappropriate for the detection of these sequences. A study that investigated the occurrence of the ERE in genes that responded to 17- β estradiol found that the ERE sequence was present in only a fraction (Bourdeau *et al.*, 2004). Indeed, there are numerous instances cited in the literature where genes without sequences that conform to the ERE consensus respond to ligands of the ER in the conventional manner, so it is difficult to define elements in gene promoter regions that function as ER-ligand binding sites. Should our analysis have shown enrichment for genes including the consensus ERE among those that responded to resveratrol then this would be evidence that supports the idea that some effects of resveratrol are ER-mediated. The negative results we obtained, however, are not sufficient to allow us to conclude that resveratrol actions on gene expression are generally independent of the ER. Further research on this topic is thus required. In chapter 5 of this thesis a direct approach to investigate if effects of resveratrol on histone H3 expression are ER-mediated is presented and further discussion around the general topic is included.

Further analysis of microarray data revealed that resveratrol action may be mediated through a non-genomic pathway of estrogen action. Two major signalling proteins have been reported to be involved in the activation of intracellular signaling cascades by estrogen through non-genomic pathway: physphatidylinosinol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPKs) (Suzuki *et al.*, 2008; Watson *et al.*, 2009). The microarray data revealed that resveratrol had an effect on extracellular-signal-regulated kinase 5 (ERK5) signalling in Caco-2 cells. ERK5 is a member of the MAPK family and plays an essential role in the regulation of cell proliferation, differentiation, and in cardiovascular development (Nishimoto and Nishida, 2006). The absence of this effect of resveratrol on the ERK5 pathway in MCF-7 cells provides further evidence of the tissue specific effect of resveratrol. Further research is

required to uncover the role of non-genomic signalling cascades in the mechanism of action of resveratrol.

Confirmation of the results of RNA microarray analysis using real time PCR is a standard step to validate these data. There was insufficient time to carry out this validation as a component of the research present in this thesis, but other researchers in the laboratory are currently confirming responses to resveratrol in MCF-7 and Caco-2 cells of genes identified by the microarray analyses presented. These data will be presented to validate the microarray studies in any published work that arises.

5 The mechanism of action of resveratrol

5.1 Introduction

Resveratrol has a structure similar that of known phytoestrogens so may mediate some of its action through its interaction with estrogen receptors ERα and/or ERβ. Some studies have reported that resveratrol may act as an ER agonist in the MCF-7 breast cancer cell line (Gehm *et al.,* 1997; Gehm *et al.,* 2004). In contrast, other studies documented apparent ER antagonist action in the same cell line (Kim *et al.,* 2004).

The work presented in this chapter aimed to investigate the mechanism through which resveratrol reduces histone protein expression and whether this effect is mediated through estrogen receptor. The hypothesis, based on the structural similarity between resveratrol and 17β-estradiol, was that effects of resveratrol on histone expression may be mediated through the estrogen receptor (ER).

5.2 Effect of resveratrol on histone expression in cells with different ER status in the presence and absence of the ER antagonist fulvestrant

As presented in chapter 3, resveratrol reduced histone protein expression in Caco-2 and MCF-7 cells. The work presented in this chapter was aimed to determine whether or not this response was related to the expression of estrogen receptors (ER), which may be a mechanism through which effects of resveratrol are mediated. For this purpose two different cell lines which differ in their estrogen receptor expression: MCF-7 (ERα-positive, ERβ-positive) and Caco-2 (ERβ-positive) were used.

5.2.1 Confirmation of the ER status of Caco-2 and MCF-7cells

To confirm the ER status of Caco-2 and MCF-7 cells, RNA was extracted from the cells then cDNA reversed transcribed from RNA was amplified using specific primers to ERα and ERβ (primer sequences are presented in table 2.2 A). Figure 5.1 presents the PCR products obtained. Products appeared to be of the predicted size for both ERα (236 bp) and ERβ (438 bp). The band derived using the ERα primers was confirmed as ERα by sequencing. Sequencing of the ERβ product was unsuccessful, thus its identity was not confirmed. ERα expression was detected only in the MCF-7 cell line. In the Caco-2 cell line no expression of ERα was detected. In contrast, ERβ expression was detected in both cell lines.

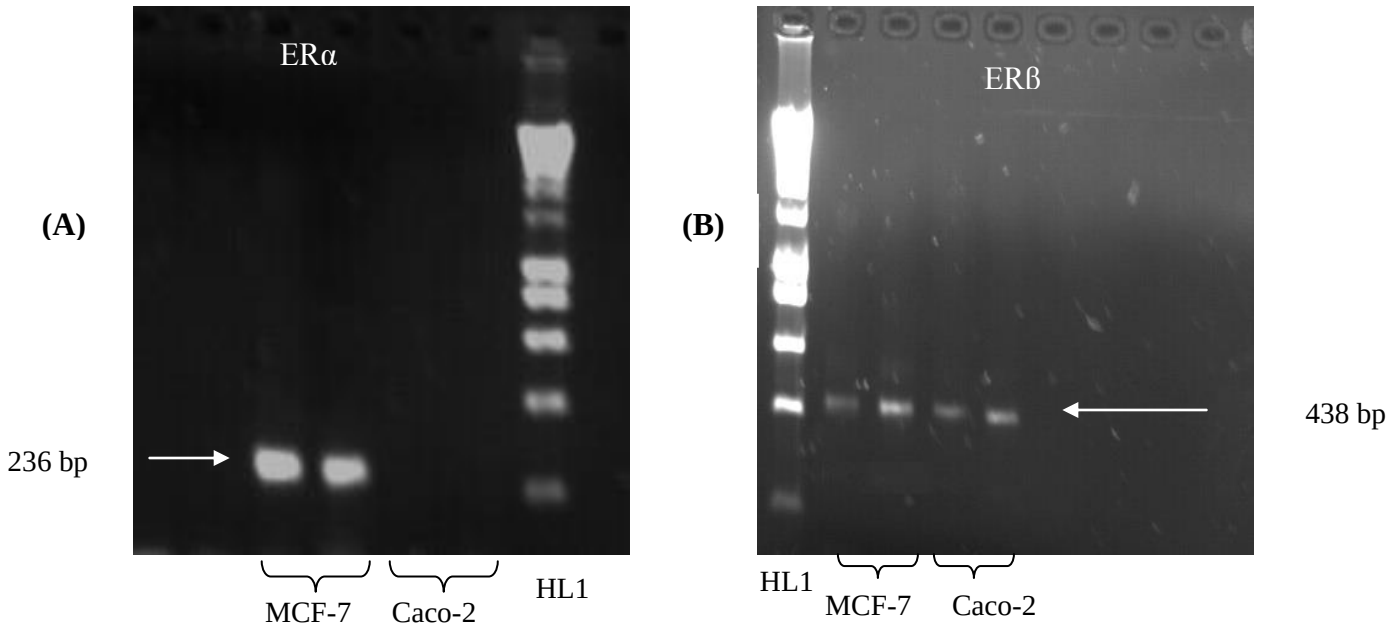


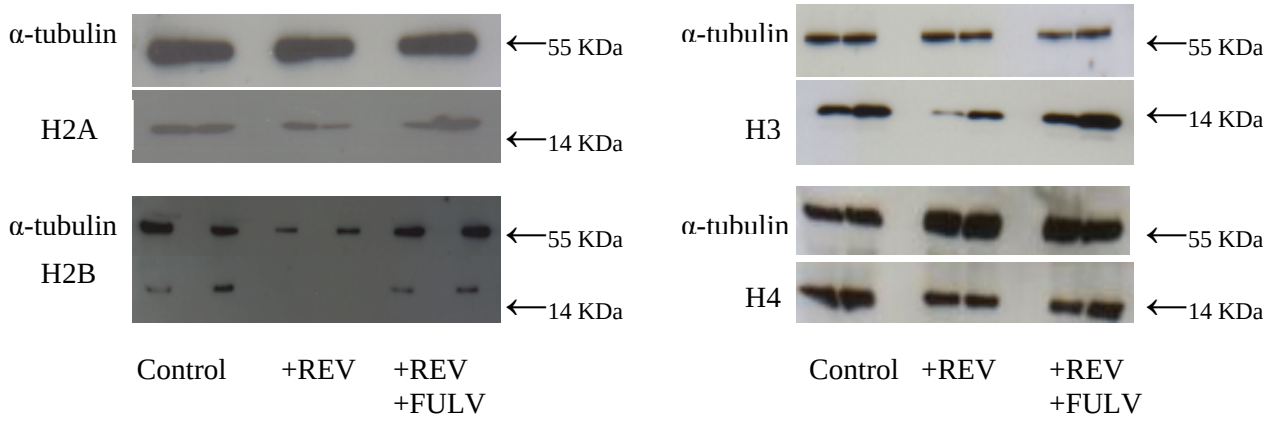
Figure 5.1: The expression of estrogen receptors ERα and ERβ in MCF-7 and Caco-2 cell lines. (A) *RT-PCR using primers for ERα. ERα expression was observed in the MCF-7 cell line. In contrast, no expression of ERα was detected in Caco-2 cell line.* ***(B)*** *RT-PCR using primers for ERβ. ERβ expression was observed in both cell lines. When reverse transcriptase was omitted from all reactions no products were observed (data not shown).*

5.2.2 Effects of resveratrol in the presence and absence of fulvestrant on histone protein expression on Caco-2 and MCF-7 cells

MCF-7 and Caco-2 cells were treated with either 0.01% DMSO (vehicle control) or 10 μM resveratrol (in 0.01% DMSO) for 48 hours in the presence or absence of the ER antagonist fulvestrant (0.1 μM). Total cell lysate was analysed by western blotting using antihistone antibodies immunoreactive against H2A, H2B, H3 and H4. To ensure equal amounts of protein were loaded, the same membrane blot was probed with an antibody immunoreactive against α-tubulin. Quantitative data were derived by denstiometric quantification of band intensities in relation to α-tubulin.

Visual inspection of the data shown in Figure 5.2A and Figure 5.3A indicates that treatment of MCF-7 and Caco-2 cells with resveratrol resulted in a decrease in histone H2A, H2B, H3 and H4 expression. Densitometric analysis of the protein bands in relation to α-tubulin indicated that expression of histone H2A, H2B, H3 and histone H4 was decreased significantly in cells treated with resveratrol. In both cell lines (Caco-2 and MCF-7), the effect of resveratrol on histone expressions was reversed in the presence of ER antagonist, fulvestrant, consistent with the action of resveratrol to reduce histone expression being an ER- mediated response (Figure 5.2B and 5.3B).

(A)



(B)

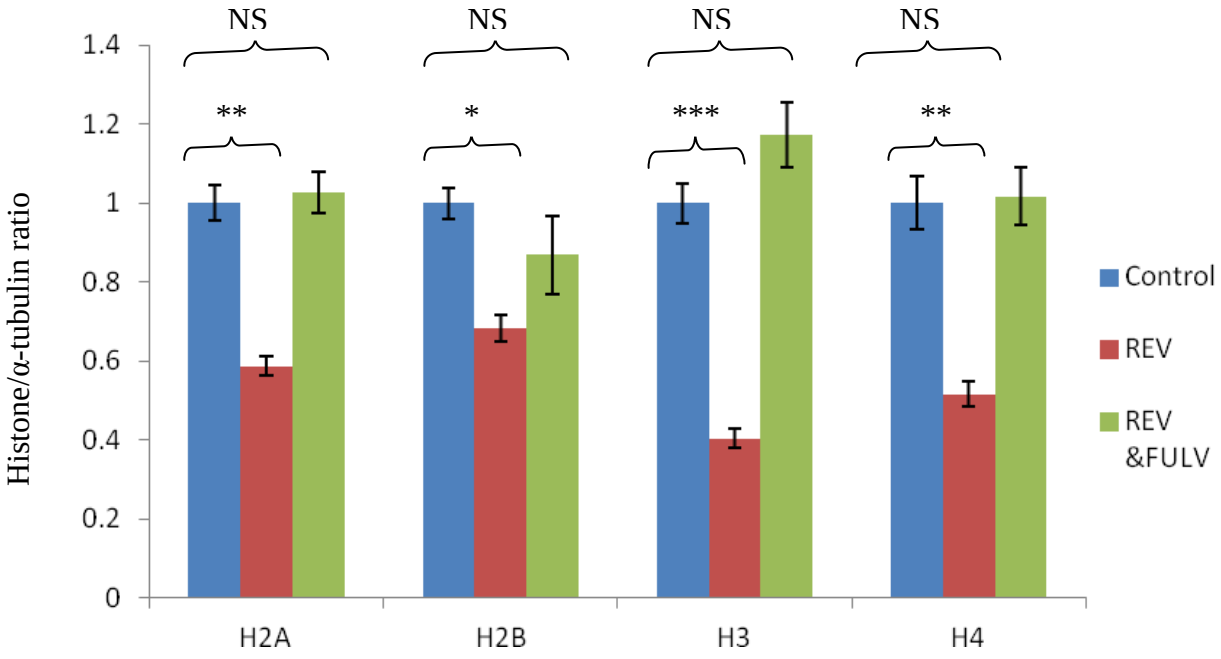
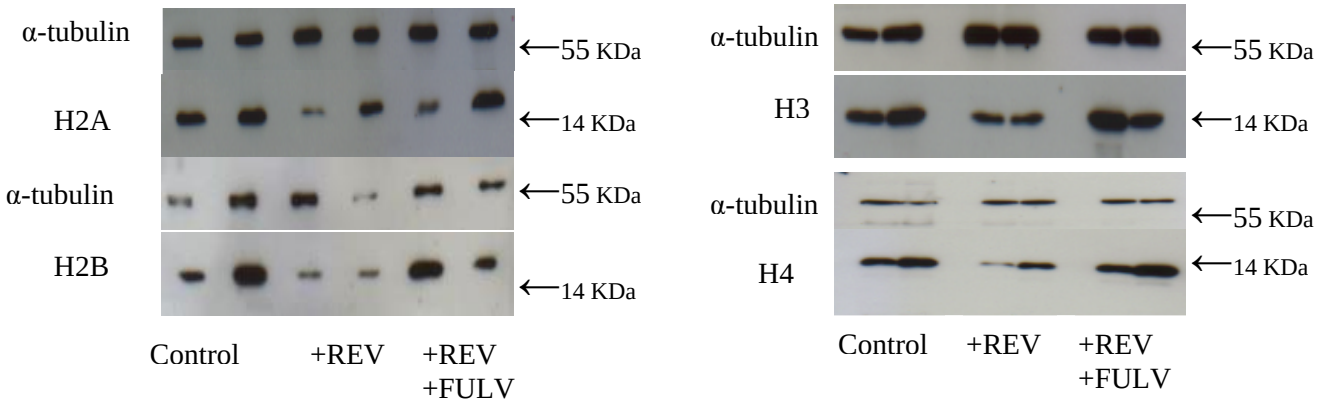


Figure 5.2: The effect of resveratrol on histone expression in MCF-7 cells in the presence and absence of an ER antagonist. (A) *Western blot analysis of histone H2A, H2B, H3 and H4. Typical data are shown.* ***(B)*** *Results of densitometric analysis of western blots detecting expression of histone proteins. Data are shown as mean ± standard error (SEM) (n=6, based on three experiments), *P <0.05, **P<0.01, ***P<0.001 by one way ANOVA followed by Dunnett's post test. The expression of histone H2A, H2B, H3 and H4 were significantly decreased in cells treated with resveratrol (REV). In contrast, there was no significant change in histone H2A, H2B, H3 and H4 expression in the treated cells with REV in the presence of the ER antagonist fulvestrant (FULV).*

(A)



(B)

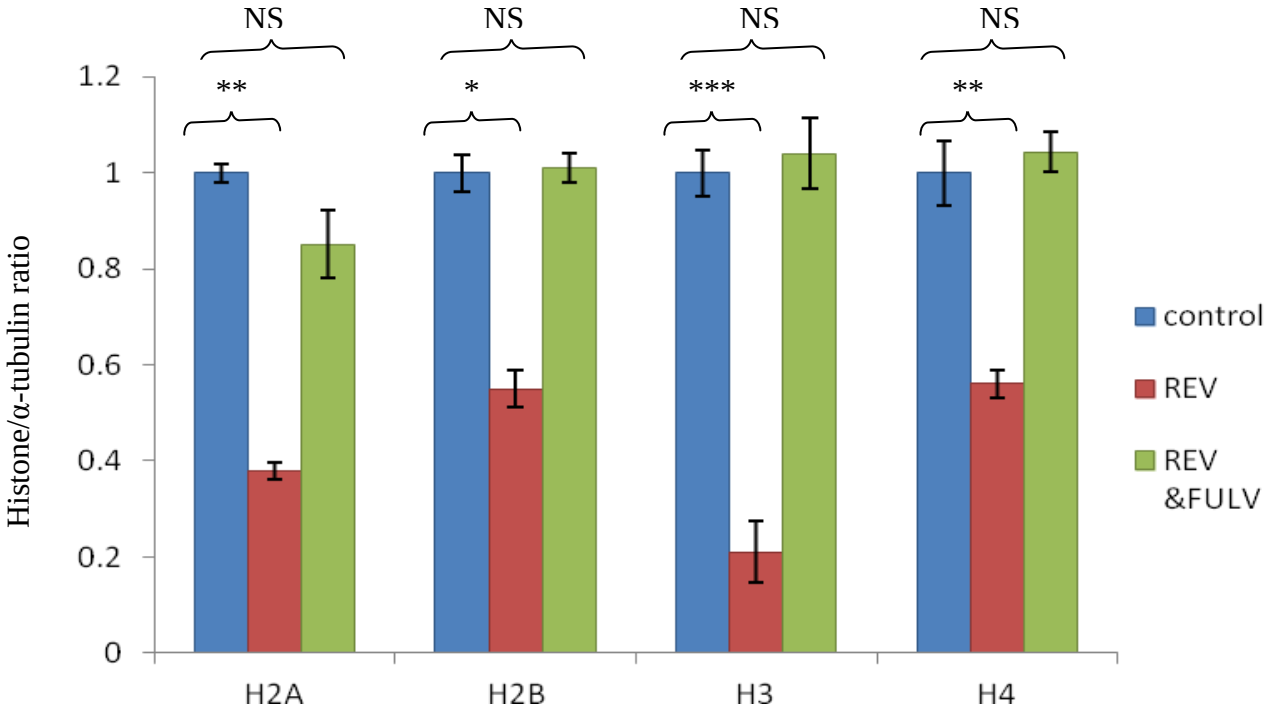


Figure 5.3: The effect of resveratrol on histone expression in Caco-2 cell line in the presence and absence of an ER antagonist. ***(A)*** *Western blot analysis of histone H2A, H2B, H3 and H4. Typical data are shown.* ***(B)*** *Results of densitometric analysis of western blots detecting expression of histone proteins. Data are shown as mean ± standard error (SEM) (n=6, based on three experiments), *P <0.05, **P<0.01, ***P<0.001 by one way ANOVA followed by Dunnett's post test. The expression of histone H2A, H2B, H3 and H4 were significantly decreased in cells treated with resveratrol (REV), whereas there was no significant change in histone H2A, H2B, H3 and H4 expression in the cells treated with REV in the presence of ER antagonist fulvestrant (FULV).*

5.3 The effect of resveratrol and ER antagonist fulvestrant on the activity of a histone H3 promoter-reporter construct in Caco-2 cells

For further investigation of the mechanism through which resveratrol acts to reduce histone expression, a promoter-reporter construct comprising the histone H3 promoter upstream of the β-galactosidase reporter gene in the vector pBlue TOPO (Invitrogen) was generated. Primers (see table 2.2 B) were designed to amplify 826 bp region of the histone H3 promoter using PCR (Figure 5.4). PCR products (Figure 5.5) were then subcloned directly into the pBlue TOPO vector (Invitrogen). Transformed *E.coli* colonies containing insert in the vector were detected by restriction digest using *Hind* III (Figure 5.5). The insert in the plasmid generated was sequenced (MWG Biotech) to confirm identity to the required sequence (Figure 5.6).

Caco-2 cells were transfected transiently with the H3 promoter-reporter construct. After transfection (24 hours later), cells were treated with 10 μM resveratrol in the presence or absence of the ER antagonist fulvestrant (0.1 μM) for 48 hours. The activity of the histone H3 promoter-reporter construct was determined by measuring the expression of β-galactosidase.

As shown in the Figure 5.7 resveratrol reduced reporter gene expression and this effect was attenuated by fulvestrant, demonstrating that resveratrol acts to reduce histone H3 expression at the level of transcription through an ER-mediated mechanism. In contrast, cells treated with fulvestrant were associated with an increase in histone H3 expression. A possible explanation of this finding is the ability of fulvestrant to promote the degradation and clearance of ERs, resulting in reduced availability of ERs to bind with phenolic compounds that may present in the medium and the serum used in cell treatments.

Accession number: M26150

TTTTGCCTAATATTCAGGCGGTGCTGCTGCCTAAGAAAACTGAGAGCCATCATAAGGCCAAGGGAAAGTG
AAGAGTTAACGCTTCATGCACTGCTGTTTTTCTGTCAGCAGACAAAATCAGCCTAACAGCAAAGGCTCTT
TTCAGAGCCACCTACGACTTCCATTAAATGAGCTGTTGTGCTTTGGATTATGCCGCCCATAAAGATGTTT
TTGAGGTGTTTTTAATGGCTTTGAGTGTGGCACTTTTAGTAATTTGTCCTGCAGAAATTAGATCCATAGA
AACCTCAGGAATTCTAGGTAT**GTGGGAGAAGTGCCATGCAGCAC**AAAACATGTTTACAGGGGTGATTCGC
GTTAAGTTTCACACACAGCAGTTACTACATTTTAGAGGAAGGAAATTATACCCATGAGTGCATTCCTAAC
TATCTTGAATGGAAGTGTTAAAACCCGCATGCCCCACACAAGTTTGAATATGTCATACCATTTGCTGTAG
CAATTAATGGCATACACAATTGAGAGCACACACATTACCACTGAACATTTGAGTATGTATTTCCCAAAAT
GAGCTTTTTTCCAGTTTGGGGATGTTTTGCTTTGTTTTGGGGTGGAGTCTCCCTCTCGCCCAAGCTGCAG
TGCAGCGGCGTGATAACAGCTCACTGTAACCTCGAACTCGGGCTCAAGCGATCCTCTTGACAGCCTTCTG
AGTAGCTGGGATTACAGGCGAGAGCCGCCACGCCCGGCTAAGAGCATTTTTCTAATTGCCCACACTTCTT
ATGCGACACCCAGAAAAATACAATTTTAAATAAAGCGCATATGCAAATTTCCCTAATCGTCTCCAATATT
CTCTGATTTCTTTTTTATATTTTAACTAGAAACAATTGGAGGTTTCCGCGTTGCTTTGTGTGGTTGTAAA
TTTTAAGACTTCAGGAAACTTTTCCAGTACAAGACTTGTCCACAGTGGATATAGCAGCTAAGGGGTTAAC
AAAATGACGTCAGAGTAGCTACGGTAATGGGCAGGAGCCTCTCTTAATCTGCAACCAGGCACAGAGATGG
ACCAATCCAAGAAGGGCGCGGGGATTTTTGAATTTTCTTGGGTCCAATAGTTGGTGGTCTGACTCTATAA
AAGAAGAGTAGCTCTTTCCTTTCCTC**CACAGACGTCTCTGCAGGCAAG**CTTTTCTGTGGTTTTGCCATGG
CTCGTACTAAACAGACAGCTCGGAAATCCACCGGCGGTAAAGCGCCACGCAAGCAGCTGGCTACCAAGGC
TGCTCGCAAGAGCGCGCCGGCTACCGGCGGCGTGAAAAAGCCTCACCGTTACCGCCCGGGCACTGTGGCT
CTGCGCGAGATCCGCCGCTACCAAAAGTCGACCGAGTTGCTGATTCGGAAGCTGCCGTTCCAGCGCCTGG
TGCGAGAAATCGCCCAAGACTTCAAGACCGATCTTCGCTTCCAGAGCTCTGCGGTGATGGCGCTGCAGGA
GGCTTGTGAGGCCTACTTGGTAGGGCTCTTTGAGGACACAAACCTTTGCGCCATCCATGCTAAGCGAGTG
ACTATTATGCCCAAAGACATCCAGCTCGCTCGCCGCATTCGCGGAGAAAGAGCGTAAATGTAAAGTCACT
TTTTCATCAGTCTTAAAACCCAAAGGCTCTTTTCAGAGCCACCCACTTATTCCAACGAAAGTAGCTGTGA
TAATTTTTTGTTGTCTTAACAGAACAAATTTCTAAGGACCCCCCCGGAAAGCATTAGACTATGGTCTTAA
AGTTGATTAACAGAAATAACGGTTTGGTCAGTCTTGCAGTGTAGGTTATTTCTGACCTTATTAAGGTGCT
ATTTGGAGAGAAGCTGTGTAAGTCCACTATCATTCAGGCCTCTAGCTTGCTATGATTAGCATTTGTTTAA
ACAACTTTGTAAGAGTAAGGGAAAAATCTGGTAAGTAGTTAACTGGCGCTTACTAGGCATTTTTGCAAAG
CTTTGAAAAGATTAGAAAATTGTGTCTTGCGAGTTCCAGTG

Figure 5.4: The histone H3 promoter region sequence and related primers. *The promoter sequence was taken from the human genome sequence. Primers (underlined) were designed for amplification by PCR of an 826 bp region of the histone H3 promoter sequence to generate a PCR product to sub-clone into the pBlue-TOPO reporter vector.*

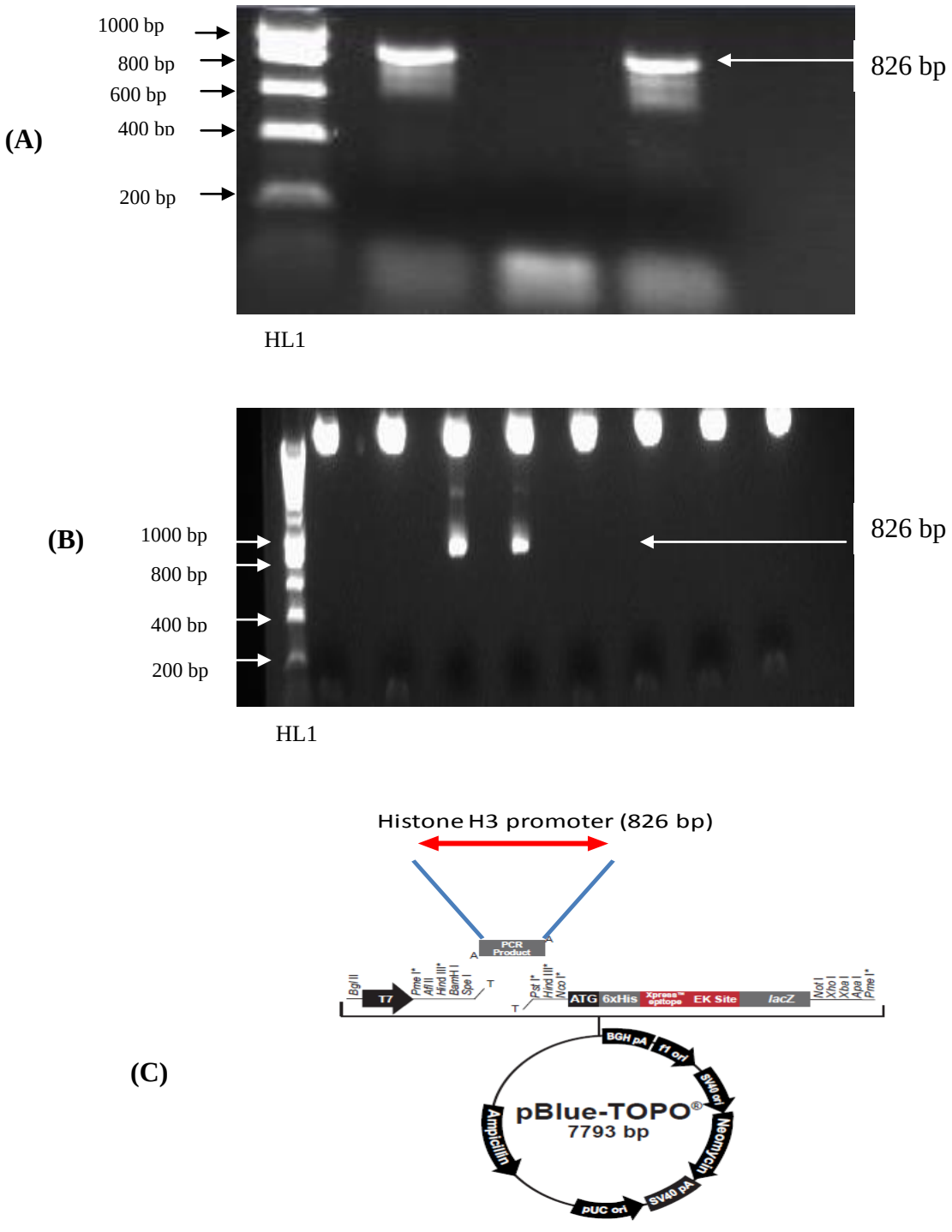


Figure 5.5: ***(A)*** *Agarose gel electrophoresis of human histone H3 promoter PCR products. cDNA reverse transcribed from Caco-2 RNA was amplified using specific primers to produce an 826 bp product comprising the H3 promoter sequnce.* ***(B)*** *Agarose gel electrophoresis of restriction digest by Hindi*III *of recombinant plasmids to screen for those containing the H3 promoter sequence. A DNA molecular weight marker (Hyperladder I) was run in the lane marked (HL1) sizes are indicated.* ***(C)*** *Diagrammatic representations of insertion of the histone H3 promoter sequence into the vector pBlue TOPO to generate the H3 promoter- reporter construct.*

```
301   TGTGGGAGAAGTGCCATGCAGCACAAAACATGTTTACAGGGGTGATTCGCGTTAAGTTTC  360
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
58    tgtgggagaagtgccatgcagcacaaaacatgtttacaggggtgattcgcgttaagtttc  117

361   ACACACAGCAGTTACTACATTTTAGAGGAAGGAAATTATACCCATGAGTGCATTCCTAAC  420
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
118   acacacagcagttactacattttagaggaaggaaattatacccatgagtgcattcctaac  177

421   TATCTTGAATGGAAGTGTTAAAACCCGCATGCCCCACACAAGTTTGAATATGTCATACCA  480
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
178   tatcttgaatggaagtgttaaaacccgcatgccccacacaagtttgaatatgtcatacca  237

481   TTTGCTGTAGCAATTAATGGCATACACAATTGAGAGCACACACATTACCACTGAACATTT  540
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
238   tttgctgtagcaattaatggcatacacaattgagagcacacacattaccactgaacattt  297

541   GAGTATGTATTTCCCAAAATGAGCTTTTTTCCAGTTTGGGGATGTTTTGCTTTGTTTTGG  600
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
298   gagtatgtatttcccaaaatgagctttttccagtttggggatgttttgctttgttttgg  357

601   GGTGGAGTCTCCCTCTCGCCCAAGCTGCAGTGCAGCGGCGTGATAACAGCTCACTGTAAC  660
      | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
358   gatggagtctccctctcgcccaagctgcagtgcagcggcgtgataacagctcactgtaac  417

661   CTCGAACTCGGGCTCAAGCGATCCTCTTGACAGCCTTCTGAGTAGCTGGGATTACAGGCG  720
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
418   ctcgaactcgggctcaagcgatcctcttgacagccttctgagtagctgggattacaggcg  477

721   AGAGCCGCCACGCCCGGCTAAGAGCATTTTTCTAATTGCCCACACTTCTTATGCGACACC  780
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
478   agagccgccacgcccggctaagagcatttttctaattgcccacacttcttatgcgacacc  537

781   CAGAAAAATACAATTTTAAATAAAGCGCATATGCAAATTTCCCTAATCGTCTCCAATATT  840
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
538   cagaaaaatacaattttaaataaagcgcatatgcaaatttccctaatcgtctccaatatt  597

841   CTCTGATTTCTTTTTTATATTTTAACTAGAAACAATTGGAGGTTTCCGCGTTGCTTTGTG  900
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
598   ctctgatttctttttatattttaactagaaacaattggaggtttccgcgttgctttgtg  657

901   TGGTTGTAAATTTTAAGACTTCAGGAAACTTTTCCAGTACAAGACTTGTCCACAGTGGAT  960
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
658   tggttgtaaattttaagacttcaggaaacttttccagtacaagacttgtccacagtggat  717

961   ATAGCAGCTAAGGGGTTAACAAAATGACGTCAGAGTAGCTACGGTAATGGGCAGGAGCCT  1020
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
718   atagcagctaaggggttaacaaaatgacgtcagagtagctacggtaatgggcaggagcct  777

1021  CTCTTAATCTGCAACCAGGCACAGAGATGGACCAATCCAAGAAGGGCGCGGGGATTTTTG  1080
      |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
778   ctcttaatctgcaaccaggcacagagttggaccaatccaagaagggcgcggggatttttg  837

1081  AATTTTCTTGGGTCCAATAGTTGGTGGTCTGACTCTATAAAAGAAGAGTAGCTCTTTCCT  1140
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
838   aattttcttgggtccaatagttggtggtctgactctataaaagaagagtagctctttcct  897

1141  TTCCTCCACAGACGTCTCTGCAGGCAAG  1168
      ||||||||||||||||||||||||||||
898   ttcctccacagacgtctctgcaggcaag  925
```

Figure 5.6: Alignment of sequence data generated from the histone H3 promoter-reporter construct (bottom line, lower case) with the required sequence.

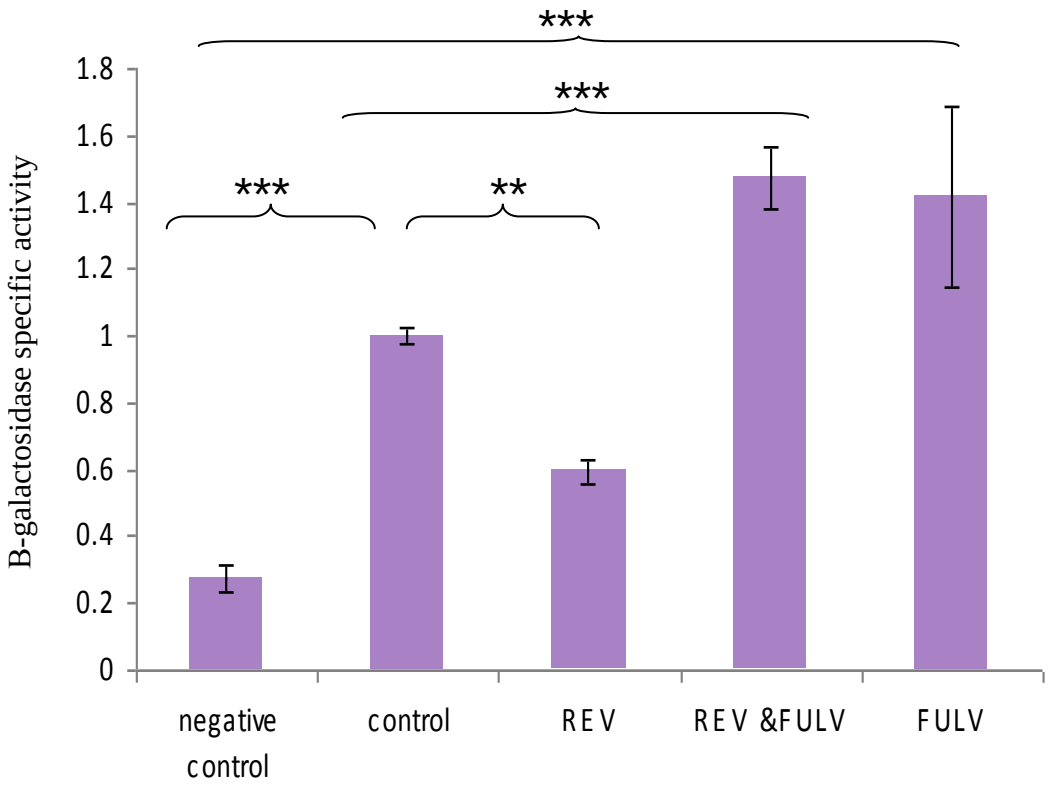


Figure 5.7: The effect of resveratrol and the ER antagonist fulvestrant on the activity of a histone H3 promoter-reporter construct in Caco-2 cells. *Caco-2 cells were transfected with a promoter-reporter construct comprising either the histone H3 promoter or with a corresponding negative control construct (negative control), without a promoter sequence insert. After transfection (24 hours later), cells were treated with resveratrol (RSV; 10 μM) in the presence and absence of fulvestrant (FULV; 0.1μM) for 48 hours. Control cells (Control) were treated with DMSO vehicle only. Data are shown as mean ± standard error (SEM) (n=9, based on four experiments), **P<0.01, ***P<0.001 by one way ANOVA followed by Dunnett's post test. The expression of the reporter gene was decreased significantly in response to the REV treatment and this effect was attenuated by fulvestrant.*

5.4 Investigation of the role of EREs in resveratrol-mediated effects on histone H3 gene transcription

Estrogen binds ER receptors then regulates the transcription of estrogen-responsive genes by either binding to specific sequences (estrogen response elements) within gene promoter regions, or by binding to other DNA-bound transcription factors, such as SP1, AP1 or NF-kappaB (Sun, 1998).

To address the potential role of EREs in mediating the effect of resveratrol on histone H3 gene expression, EREs identified within the histone H3 promoter sequence included in the promoter reporter construct were mutated using a PCR-based method. The primers were designed to introduce mutations into three sites at which consensus ERE sequences were present within the histone H3 promoter region in the construct using PCR (Figure 5.8). The PCR product including the mutated sites was subcloned into the pBlue TOPO vector (Invitrogen). Presence of insert in the vector from colonise of transformed *E.coli* was detected by digestion with *Hind* III then analysis by agarose gel electrophoresis (Figure 5.9 and 5.10). The successful mutation of the three EREs in the histone H3 promoter sequence was confirmed by sequencing (MWG Biotech) (Figure 5.11). All primers are listed in table (2.2 C).

Caco-2 cells were transfected transiently with either the histone H3 promoter reporter construct or with the equivalent construct containing the introduced mutations at the EREs. After transfection (24 hours later), cells were treated with 10 µM resveratrol in the presence or absence of the ER antagonist fulvestrant (0.1 µM) for 48 hours. The activity of the reporter gene (β-galactosidase) was then measured in cell lysates.

The activity of both the H3 promoter- reporter construct and of the equivalent construct containing mutations to the EREs was decreased significantly in response to the resveratrol treatment and this effect was attenuated by fulvestrant in both cases (Figure 5.12). The results therefore indicated that resveratrol reduced histone H3 expression at the level of transcription through the ER but through a mechanism independent of the EREs identified in the region of promoter sequence included in the promoter reporter construct.

Accession number: M26150

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AAGAGTTAACGCTTCATGCACTGCTGTTTTTCTGTCAGCAGACAAAATCAGCCTAACAGCAAAGGCTCTT
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GTTAAGTTTCACACACAGCAGTTACTACATTTTAGAGGAAGGAAATTATACCCATGAGTGCATTCCTAAC
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CAATTAATGGCATACACAATTGAGAGCACACACATTACCACTGAACATTTGAGTATGTATTTCCCAAAAT
GAGCTTTTTTCCAGTTTGGGGATGTTTTGCTTTGTTTTGGGGTGGAGTCTCCCTCTCGCCCAAGCTGCAG
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ATGCGACACCCAGAAAAATACAATTTTAAATAAAGCGCATATGCAAATTTCCCTAATCGTCTCCAATATT
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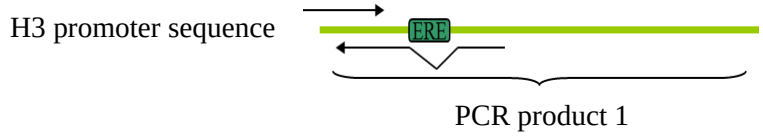
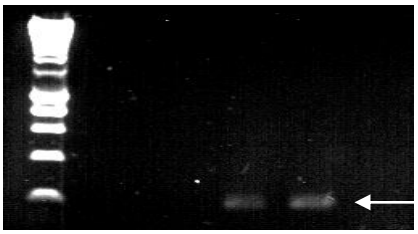
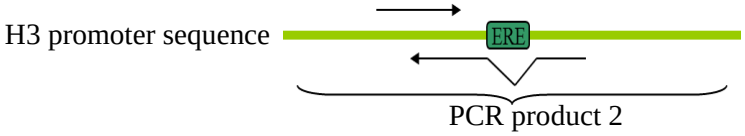
ATTTGGAGAGAAGCTGTGTAAGTCCACTATCATTCAGGCCTCTAGCTTGCTATGATTAGCATTTGTTTAA
ACAACTTTGTAAGAGTAAGGGAAAAATCTGGTAAGTAGTTAACTGGCGCTTACTAGGCATTTTTGCAAAG
CTTTGAAAAGATTAGAAAATTGTGTCTTGCGAGTTCCAGTG

Figure 5.8: The histone H3 promoter region sequence and related primers used to introduce mutations at sites with ERE consensus sequences. *The primers which introduce mutations at the ERE sites are underlined and in bold. The 5 bp sequences highlighted in blue are the ERE sequences to be mutated.*


H3 promoter sequence
ERE
PCR product 1

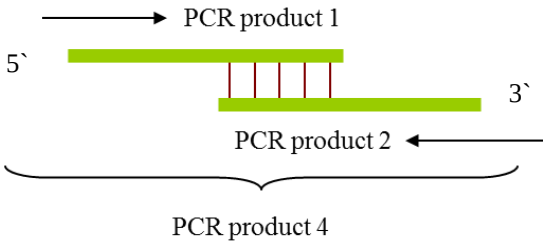

171 bp
HL1

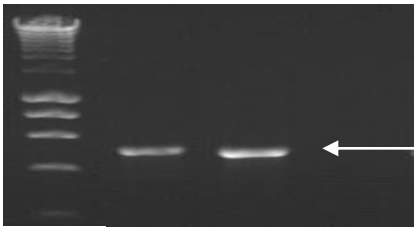

H3 promoter sequence
ERE
PCR product 2

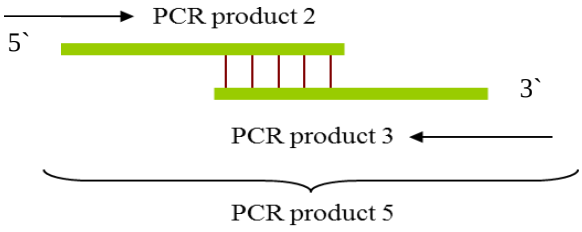

295 bp
HL1

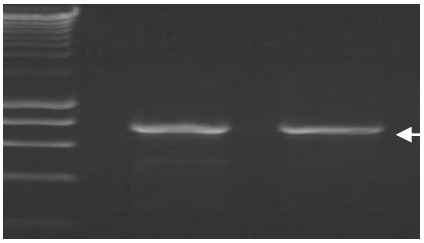

H3 promoter sequence
ERE
PCR product 3

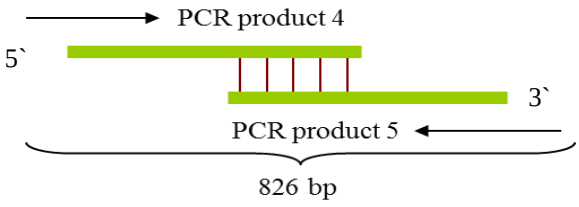

246 bp
HL1


PCR product 1
5`
3`
PCR product 2
PCR product 4


481 bp
HL1


PCR product 2
5`
3`
PCR product 3
PCR product 5


727 bp
HL1


PCR product 4
5`
3`
PCR product 5
826 bp

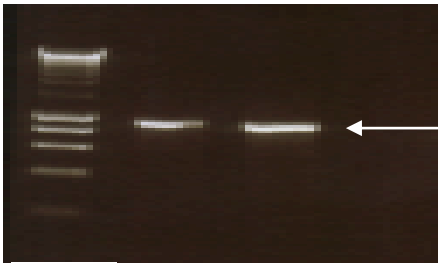

826 bp
HL1

Figure 5.9: *Agarose gel electrophoresis of PCR products at stages in the procedure used to obtain a histone H3 promoter-reporter incorporating with mutations at three identified sites with ERE consensus sequence. In the first PCR reaction the 5` region of the promoter including one of the EREs was generated and the ERE was replaced with random sequences. In the second reaction, the middle region of the promoter including the second ERE was generated and the ERE was replaced with random sequence. These two products were then joined in a third PCR reaction. In a fourth PCR reaction the 3` region of the promoter including the third ERE was generated and the ERE was replaced with random sequence. The products of the third and fourth PCR reaction were then joined using the unique outer primers (to the random sequence). A DNA molecular weight marker (Hyperladder I) was run in the lane marked (HL1*)**.**

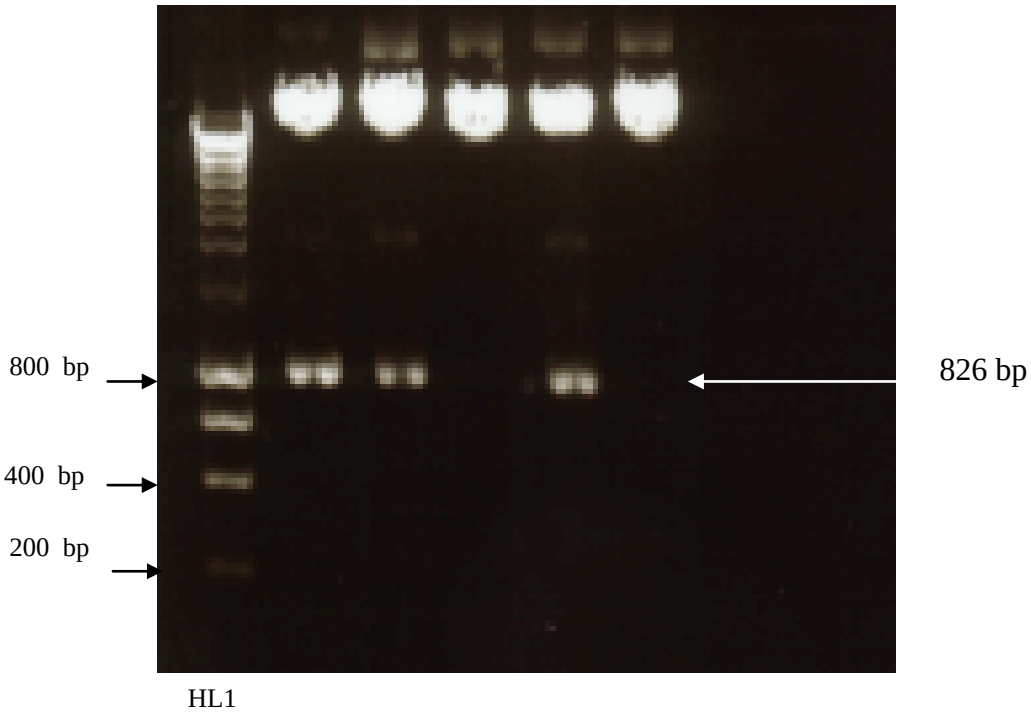


Figure 5.10: *Agarose gel electrophoresis of restriction digest by Hind III of recombinant plasmids to screen for those containing the H3 promoter sequence. A DNA molecular weight marker (Hyperladder I) was run in the lane marked (HL1*)**.**

```
37   GTGGGAGAAGTGCCATGCAGCACAAAACATGTTTACAGGGGTGATTCGCGTTAAGTTTCA  96
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
867  gtgggagaagtgccatgcagcacaaaacatgtttacaggggtgattcgcgttaagtttca  808

97   CACACAGCAGTTACTACATTTTAGAGGAAGGAAATTATACCCATGAGTGCATTCCTAACT  156
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
807  cacacagcagttactacattttagaggaaggaaattatacccatgagtgcattcctaact  748

157  ATCTTGAATGGAAGTGTTAAAACCCGCATCAAACACACAAGTTTGAATATGTCATACCAT  216
     ||||||||||||||||||||||||||||||    ||||||||||||||||||||||||||||
747  atcttgaatggaagtgttaaaacccgcatgcccacacaagtttgaatatgtcataccat  688

217  TTGCTGTAGCAATTAATGGCATACACAATTGAGAGCACACACATTACCACTGAACATTTG  276
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
687  ttgctgtagcaattaatggcatacacaattgagagcacacacattaccactgaacatttg  628

277  AGTATGTATTTCCCAAAATGAGCTTTTTTCCAGTTTGGGGATGTTTTGCTTTGTTTTGGG  336
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
627  agtatgtatttcccaaaatgagctttttccagtttggggatgttttgctttgttttggg  568

337  ATGGAGTCTCCCTCTCGCCCAAGCTGCAGTGCAGCGGCGTGATAACAGCTCACTGTAACC  396
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
567  gtggagtctccctctcgcccaagctgcagtgcagcggcgtgataacagctcactgtaacc  508

397  TCGAACTCGGGCTCAAGCGATCCTCTTGACAGCCTTCTGAGTAGCTGGGATTACAGGCGA  456
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
507  tcgaactcgggctcaagcgatcctcttgacagccttctgagtagctgggattacaggcga  448

457  GAGCCGCCACGCCCGGCTAAGAGCATTTTTCTAATATCAGAACACTTCTTATGCGACACC  515
     |||||||||||||||||||||||||||||||| |    ||||||||||||||||||||
447  gagccgccacgcccggctaagagcatttttctaat-tgcccacacttcttatgcgacacc  389

516  CAGAAAAATACAATTTTAAATAAAGCGCATATGCAAATTTCCCTAATCGTCTCCAATATT  575
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
388  cagaaaaatacaatttaaataaagcgcatatgcaaatttccctaatcgtctccaatatt  329

576  CTCTGATTTCTTTTTTATATTTTAACTAGAAACAATTGGAGGTTTCCGCGTTGCTTTGTG  635
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
328  ctctgatttctttttatattttaactagaaacaattggaggtttccgcgttgctttgtg  269

636  TGGTTGTAAATTTTAAGACTTCAGGAAACTTTTCCAGTACAAGACTTGTCCACAGTGGAT  695
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
268  tggttgtaaattttaagacttcaggaaacttttccagtacaagacttgtccacagtggat  209

696  ATAGCAGCTAAGGGGTTAACAAAATGACGTCAGAGTAGCTACGGTAATACCACGGAGCC  754
     ||||||||||||||||||||||||||||||||||||||||||||||||     ||||||
208  atagcagctaaggggttaacaaaatgacgtcagagtagctacggtaatgggcaggagcc  150

755  TCTCTTAATCTGCAACCAGGCACAGAGTTGGACCAATCCAAGAAGGGCGCGGGGATTTTT  814
     |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
149  tctcttaatctgcaaccaggcacagagatggaccaatccaagaagggcgcggggatttttt  90

815  GAATTTTCTTGGGTCCAATAGTTGGTGGTCTGACTCTATAAAAGAAGAGTAGCTCTTTCC  874
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
89   gaattttcttgggtccaatagttggtggtctgactctataaaagaagagtagctctttcc  30

875  TTTCCTCCACAGACGTCTCTGCAGG-AAG  902
     ||||||||||||||||||||||||| |||
29   tttcctccacagacgtctctgcaggcaag  1
```

Figure 5.11: Alignment of sequence data generated from the histone H3 promoter-reporter construct in which ERE sequences were mutated (bottom line, lower case) with the required sequence. *The 5 bp sequence highlighted in blue are the ERE sequences to be mutated.*

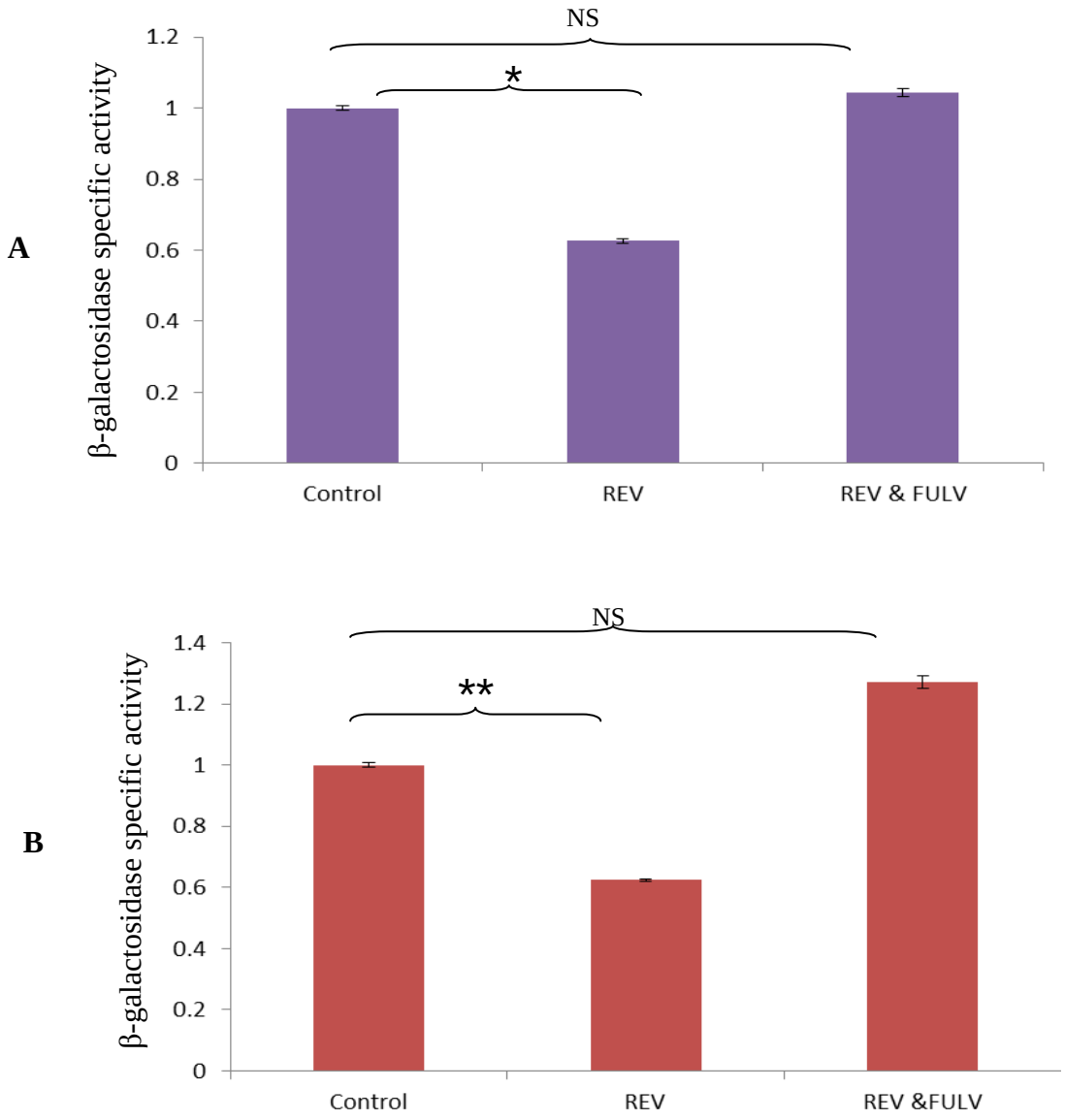


Figure 5.12: The effect of resveratrol and the ER antagonist fulvestrant on the activity of an H3 promoter-reporter construct in which three identified ERE sequences were mutated in Caco-2 cells. *Caco-2 cells were transfected with a promoter-reporter construct including either* ***(A)*** *the histone H3 promoter or* ***(B)*** *an equivalent construction in which three ERE sequences were replaced with random sequence. After transfection (24 hours later), cells were treated with resveratrol (RSV; 10 μM) in the presence and absence of fulvestrant (FULV; 0.1μM) for 48 hours. Control cells (Control) were treated with DMSO vehicle only. Data are shown as mean ± standard error (SEM) (n=9, based on four experiments), **P<0.01, ***P<0.001 by one way ANOVA followed by Dunnett's post test. There was a reduction in the expression of the reporter gene in response to the resveratrol (REV) treatment and this effect was attenuated by fulvestrant. There was no difference in the response of the two different constructs tested.*

5.5 The effect of 17-β estradiol and the ER antagonist fulvestrant on the activity of a histone H3 promoter-reporter construct in Caco-2 cells

The observed ability of resveratrol to downregulate transcription from the histone H3 promoter through the ER (attenuated by the ER antagonist fulvestrant) but through a mechanism independent of ERE sequences identified within the promoter raises the key question as to whether 17-β estradiol influences the histone H3 promoter in the same way. The effect of 17-β estradiol (at a physiological concentration of 10 nM) on the activity of both the histone H3 promoter-reporter construct and the equivalent construct in which ERE sequences were mutated in Caco-2 cells in the presence and absence of the ER antagonist fulvestrant was therefore measured.

Caco-2 cells were transfected transiently with one of the two promoter-reporter construct to be tested. After transfection (24 hours later), cells were treated with resveratrol (10 μM or 200 μM) or 17-β estradiol (10 nM) in the presence or absence of the ER antagonist fulvestrant (0.1 μM) for 48 hours. The activity of reporter gene (β-galactosidase) was measured.

As shown in Figures 5.13 and 5.14 both 17-β estradiol and resveratrol (at physiological concentration (10 μM) or pharmacological concentration (200 μM)) reduced the activity of the histone H3 promoter irrespective of whether the identified ERE sequences were intact or had been replaced with random sequence and this effect was attenuated by fulvestrant. These data demonstrated that 17-β estradiol modifies histone H3 expression through the ER but, like resveratrol, this action is not dependent on the ERE sequences that identified.

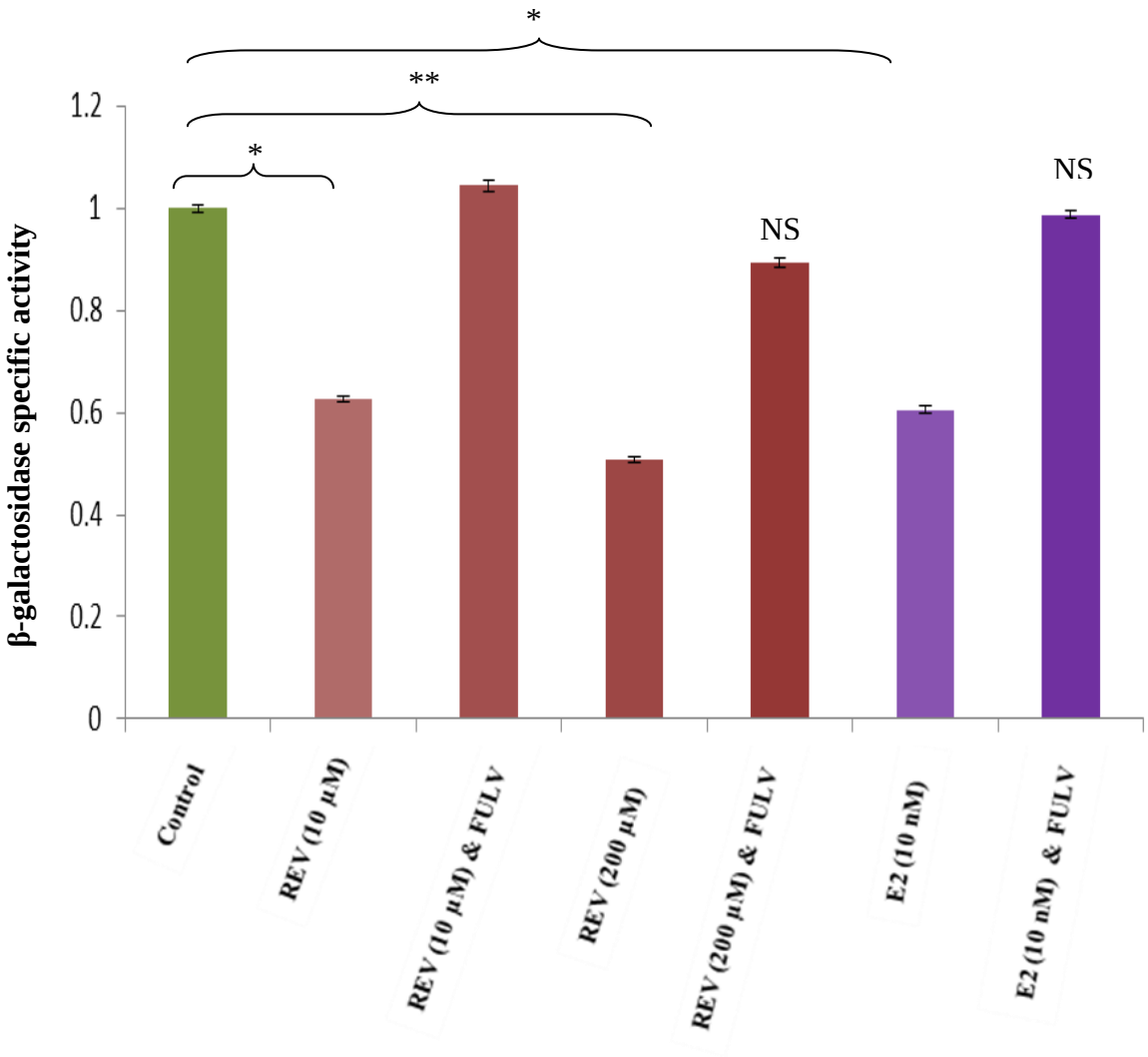


Figure 5.13: The effect of 17-β estradiol, resveratrol and the ER antagonist fulvestrant on the activity of a histone H3 promoter-reporter construct in Caco-2 cells. *Caco-2 cells were transfected with a promoter-reporter construct comprising a region of the histone H3 promoter. After transfection (24 hours later), the cells were treated with resveratrol (RSV; 10 μM, 200 μM) or 17-β estradiol (E2; 10 nM) in the presence or absence of fulvestrant (FULV; 0.1μM) for 48 hours. Control cells (Control) were treated with DMSO vehicle only. Data are shown as mean ± standard error (SEM) (n=9, based on three experiments), **P<0.01, ***P<0.001 by one way ANOVA followed by Dunnett's post test. Both 17-β estradiol and resveratol reduced the activity of the histone H3 promoter and this effect was attenuated by fulvestrant.*

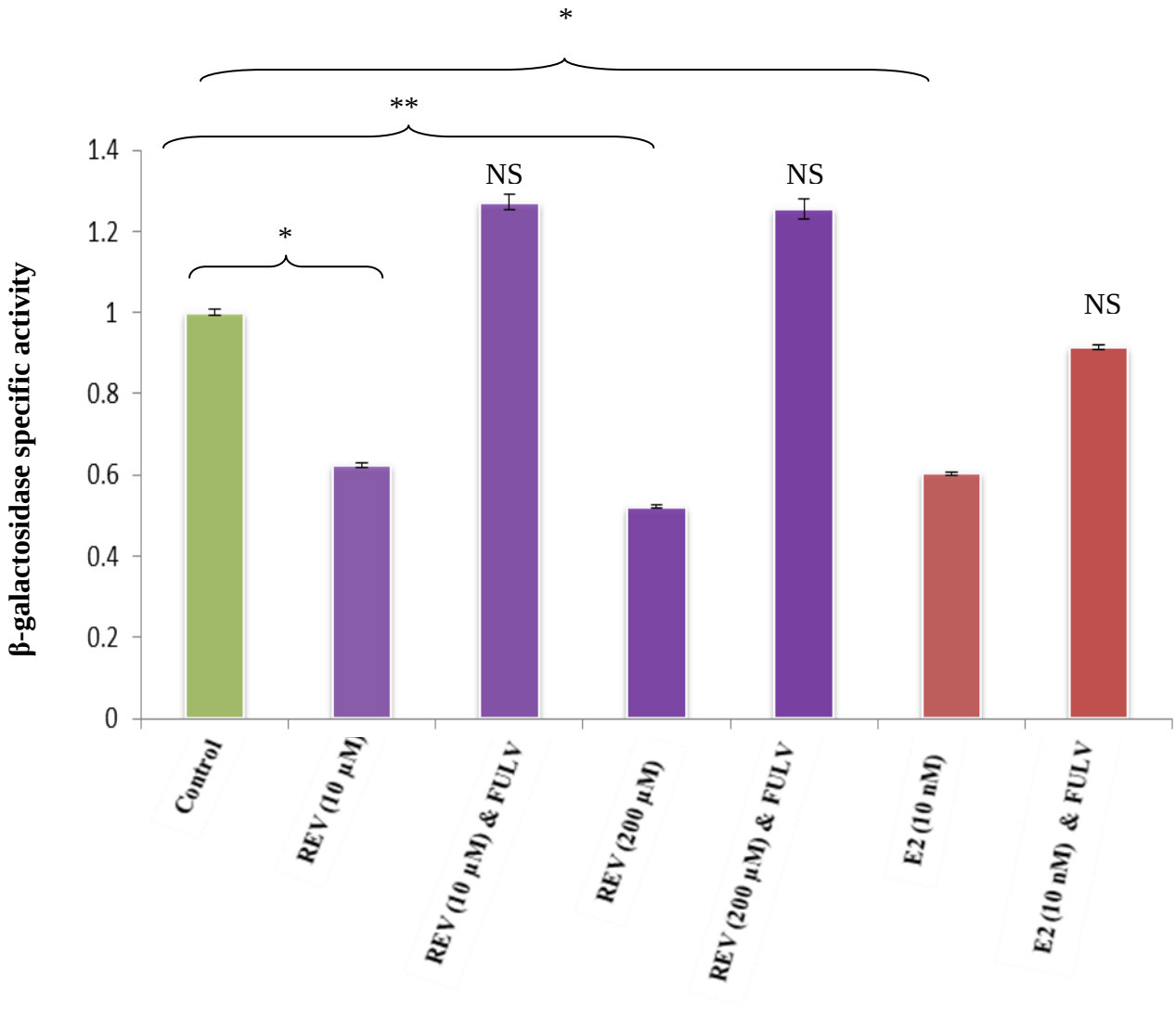


Figure 5.14: The effect of 17-β estradiol, resveratrol and ER antagonist fulvestrant on the activity of an H3 promoter-reporter construct in which three identified ERE sequences were mutated in Caco-2 cells. *Caco-2 cells were transfected with a promoter-reporter construct comprising a region of the histone H3 promoter in which ERE sequences was mutated. After transfection (24 hours later), the cells were treated with resveratrol (RSV; 10 µM, 200 µM) or 17-β estradiol (E2; 10 nM) in the presence or absence of fulvestrant (FULV; 0.1µM) for 48 hours. Control cells (Control) were treated with DMSO vehicle only. Data are shown as mean ± standard error (SEM) (n=9, based on three experiments), **P<0.01, ***P<0.001 by one way ANOVA followed by Dunnett's post test. Both 17-β estradiol and resveratol reduced the activity of the histone H3 promoter in which ERE sequences were mutated and this effect was attenuated by fulvestrant.*

5.6 Discussion

The estrogen receptor is a nuclear hormone receptor. There are two known estrogen receptor subtypes, ERα and ERβ, each encoded by different genes. These genes are located on chromosome 6q25 and chromosome 14q23-24.1 respectively *(*Li, 2003). These receptors mediate the biological functions of the estrogen hormone. Although ERα and ERβ bind with high affinity to the same ligand, they respond differently and induce opposite effects *(*Dahlman-Wright, 2006). Transcription of estrogen-responsive genes is affected by the estrogen-receptor complex through either binding to specific sequences, known as estrogen response elements, or by binding to other DNA-bound transcription factors, such as SP1, AP1 or NF-kappaB (Sun, 1998).

Resveratrol is a natural product that belongs to a large group compounds often referred to as phytoestrogens. These compounds have a chemical structure similar to 17β-estradiol (Kuiper *et al*, 1997), and have an ability to bind to estrogen receptors to produce effects that mimic biological actions of estrogen (Grynkiewicz and Opolski, 2005).

The work presented in this chapter investigated the hypothesis that effects of resveratrol - in particular the reduction that was observed in histone protein expression- are mediated through the estrogen receptor (ER). These effects of resveratrol to reduce histone expression could potentially be mediated through either ERα, ERβ or both. Thus, the ER status of the cell lines used in this study was confirmed by RT-PCR. In accordance with published information, the data confirmed that MCF-7 cells but not Caco-2 cells express ERα. Since both cell lines showed reduced histone expression in response to resveratrol, this finding indicates that the response to resveratrol is not ERα-dependent. On the other hand, expression of ERβ was detected in both cell lines, so it is possible that the response to resveratrol is mediated through ERβ. To

investigate if resveratrol reduces histone expression through ERs, Caco-2 (ERβ-positive) and MCF-7 (ERα and ERβ- positive) cell lines were treated with resveratrol in the presence and absence of the ER antagonist fulvestrant, and then western blot analysis using antihistone antibodies was conducted. The results show that histone H2A, H2B, H3 and H4 expression was decreased in response to resveratrol treatment in both cell lines (statistically significant). It was observed blockade of the effect of resveratrol to reduce histone expression in both cell lines by fulvestrant, consistent with resveratrol acting through the ER to bring about this downstream response. The fact that these responses were observed in the Caco-2 cell line, which lacks ERα, indicates that the responses are mediated through ERβ. This view is recently supported by *in vitro* evidence on mammal cell lines including MRC5 and SHSY5Y (Robb and Stuart, 2011).

To investigate the mechanism of action of resveratrol further it was investigated if action to reduce histone expression was at the level of histone gene transcription. To achieve this goal, a reporter gene assay was used. Caco-2 cells were transfected with a histone H3 promoter reporter construct and treated with resveratrol (10 μM, 24 hours) in the presence and absence of fulvestrant. The data indicated that the ER-mediated mechanism through which resveratrol caused a downregulation in histone H3 expression was to affect histone gene transcription. Since additional potential mechanisms – such as effects on histone mRNA stability and translation and on histone protein turnover were not explored- it cannot be concluded that transcriptional actions account entirely for the observed reduction in histone expression and further studies are warranted.

The experiments were extended to investigate if the response to resveratrol treatment was through interaction with estrogen response elements (EREs). Three potential EREs were identified within the histone H3 promoter region included in the promoter-reporter construct and replaced with random sequence. Caco-2 cells were transfected with this construct and treated with resveratrol. Also the effects of the endogeneous ER ligand 17-β estradiol on both the original and mutated promoter-reporter constructs were tested. Both compounds gave the same response profile: both reduced reporter gene expression from both promoter reporter constructs. These observations indicate that effects of neither compound, although mediated through the ER, are on the specific sequences region we identified and replaced. It is likely that a ligand-ER complex binds to other sequences in the promoter region that act as EREs and that we failed to identify. Additional modifications to the promoter-reporter construct may reveal these sequences. Although effects of the endogenous ligand 17-β estradiol could be mimicked in this experiment by resveratrol, the data are insufficient to allow us to determine if resveratrol acts primary as an ER-agonist (that activates transcription of EREs) or ER-antagonist (that inhibit agonist-induced transcriptional). Complex agonist/antagonist/partial agonist effects of dietary phytoestrogens, in particular isoflavones, have been reported. The concentration of resveratrol that was used (10 μM) is far in excess of that 17-β estradiol (10 nM) so further work is needed to examine in detail dose-response profiles and competition between resveratrol and 17-β estradiol for ER binding and downstream effects.

The ability of resveratrol to interact with the ER indicates the potential for resveratrol to be used in protection against a range of diseases associated with reduced secretion of estrogen hormone, including osteoporosis and alleviation of menopausal symptoms. Such actions have been attributed to the partial ER-agonistic actions of other dietary

phytoestrogens - notably the isoflavones – but the potential for resveratrol to act in a similar way remains relatively unexplored. Similarly, the implications of the finding that resveratrol acts through EREs should be explored with respect to potential and reported effects of resveratrol to protect against hormone-dependent cancer, including breast cancer (Garvin *et al.*, 2005; De Amicis *et al.*, 2011), prostate cancer (Fang *et al.*, 2011), and colon cancer (Tessitore *et al.*, 2000).

In ER-positive cancer such as breast cancer, which has an overexpression of ERα (Dotzlaw *et al.*, 1999; Speirs *et al.*, 1999), resveratrol has been seen to inhibit growth of cancer cells (Garvin *et al.*, 2005; Amicis *et al.*, 2011) through a mechanism that may be dependent on downregulation of ERα expression (Amicis *et al.*, 2011), suggesting that resveratrol may be considered as a natural selective estrogen receptor modulator (SERM). This view may explain the reduction incidence of breast cancer in Asian women where the consumption of phytoestrogens is high (Messina *et al.*, 1994). However, factors including ethnicity, inherited mutations in genes such as *BRCA1* and *BRCA2* also affect the incidence of breast cancer (Petrucelli *et al.*, 2011), and different distributions within populations could be a factor that confounds apparent links with diet.

Although the estrogenic/antiestrogenic effects of resveratrol may have many biological benefits, no data are available on its interaction with endogenous estrogen during embryonic development and early childhood. Resveratrol has a structure similar to diethylstilbestrol (DES), a potent synthetic estrogen used widely to protect pregnancies from abortion. This medicine has been reported to increase the incidence of undescended testes and abnormalities in urogenital tract in male newborns of mothers were administrated DES during early pregnancy (Gill *et al.*, 1976). This phenotype may be associated with reduced fertility and increased the risk of tumour in the

testicular germ cell in later life (Pettersson *et al.*, 2007). This view is supported by observation based on mice injected with DES between 9 to16 days of gestation, which induced features such as intra-abdominal testes (the testes are located inside the abdominal cavity), infertility, and urogenital tract abnormalities in male offspring (Greco *et al.*, 1993). Based on these observations and the similarity in the structure between resveratrol and DES, consideration should be given to resveratrol as a potential endocrine disruptor and clinical investigation of its side-effects during pregnancy and neonatal life may be warranted. For this purpose, further research using a rodent model is encouraged to examine effects of resveratrol including dosage and exposure time on the fetal development.

A further related area for research would be the role – if any- of reduced levels of histone expression (downstream of interaction with the ER) on health and disease risk. Other ER-mediated actions of resveratrol are likely to be components of the biological action of the compound.

The finding that the effects of resveratrol on histone protein expression are ER-mediated actions may be relevant to the well – studied and highly topical actions of resveratrol to protect against phenotypes associated with ageing and against other ageing-relating diseases, but the links require extensive further investigation. For example, differential effects in tissues of different ER expression profile, sex-specific effects and modifying actions of other ER agonists or antagonist *in vivo* could all be explored to begin to address this point.

Further studies *in vivo* are required to understand the impact of resveratrol on general health and on ageing and on ageing-related diseases and whether such effects mimic and/or antagonise estrogen actions.

6 Discussion and conclusion

Resveratrol has attracted particular interest as a result of reports that it can delay features of the ageing process and/or for extend lifespan in different species in a manner that mimics DR (Howitz *et al.,* 2003; Wood *et al,* 2004; Baur *et al.,* 2006). The mechanisms by which resveratrol brings about these effects are not fully understood, and are a subject of controversy.

This study aimed to investigate mechanisms through which resveratrol may have biological activity of benefit during the ageing process. The initial hypothesis was that some of the beneficial effects of resveratrol on lifespan/aging are mediated through effects on histone expression that oppose changes observed in ageing. To investigate this hypothesis human epithelial cell line models (Caco-2 and MCF-7 cells) were used.

Initially, it was investigated the effect of resveratrol, at a concentration achievable physiologically (10 µM), on the level of histone proteins in Caco-2 and MCF-7 cells. The results showed that histone protein (H2A, H2B, H3 and H4) expression was decreased in response to resveratrol treatment in both cell lines. To seek further evidence to support the hypothesis that resveratrol regulates lifespan/longevity by its effect on histone expression, it was essential to investigate how histone expression changes with age. Based on the findings concerning the ability of resveratrol to reduce histone expression, it was reasoned that the response could potentially counteract/ oppose feature of ageing if the ageing process were associated with an increase in histone expression. This idea was explored using small intestinal tissue from young and old female mice. A notable increase in histone expression (particularly H2A, H2B and H4) was observed in the tissue from the older mice, consistent with resveratrol having an effect that opposes changes observed during ageing. To extend the observations made in mice to humans, the effect of age on histone protein expression in the

intestine of human subjects was examined. No age-associated differences in histone expression in human subjects were observed. The fact that a difference was observed in mice but not in human subjects may be due to a number of different factors including: a difference between species, a difference between small intestine (mice) and colon (humans), and greater inter-individual variability in human samples, which were very limited in number. Samples from a larger number of individuals and extracted from specific layers of intestine/ colon, such as the mucosal layer, should be analysed to draw a more robust conclusion.

The mechanism underlying the influence of ageing on histone expression is still unclear. There is very little information in the literature about the effect of age on the level of histone expression in mammals or on functional consequences of reduced levels of histone expression. It is therefore difficult to predict the impact of this change in histone expression on the ageing process. The close relationship between histone association or modification and expression of genes encoded by the associated DNA makes it likely that there will be profound effects on gene expression

This work studied the effect of resveratrol at the level of transcriptiome, to address several objectives including: (1) investigating if resveratrol has an effect similar to that of DR at the level of gene expression; (2) identifying if genes or pathways affected by resveratrol were also affected by manipulation of the expression level of *Sirt1*, since Sirt1 has been implicated as factor that can modify the ageing process; (3) examining if resveratrol influenced the expression of genes/pathways related to the ageing process. These objectives were addressed using micro-array based technology, as described in Chapter 4. Genes within the intersection of those found to be affected by resveratrol treatment in Caco-2 and MCF-7 cell lines and a compiled list of genes responsive to DR were identified. The number of genes in the intersection for both cell types was greater than expected by chance, supporting to the view

that resveratrol may mimic some effects of DR. In contrast, the intersections between the lists of genes affected by resveratrol treatment in both cell lines and a list of genes affected by knockdown of *SIRT1* expression in Caco-2 cells were very small. This finding indicted that resveratrol does not act through effects on Sirt1. This view is supported by previous research in *vivo* based on a mouse model (Barger *et al.,* 2008a), but others challenge this opinion (Baur *et al*, 2006; Park *et al.*, 2012).

To address the third objective, we focused on *FOXO1, IGF-1* and *PGC-1α,* which all appear to be linked closely with determining lifespan and/ or modifying the ageing process, particularly in response to DR (Baur *et al.*, 2006). The microarray data revealed no apparent effect of resveratrol on these genes in either cell line. These findings are at variance with other microarray analyses based on mouse models (Baur *et al.*, 2006; Barger *et al.*, 2008a). Several factors may explain these differences, including the fact that the published *in vivo* analyses are based on organs such as heart, liver, brain and muscle, rather than intestine or breast epithelium as represented by the cell lines studied in this work. Tissue-specific actions of resveratrol are indicated by the current study, in the lack of commonalty between genes and the pathways affected by resveratrol in Caco-2 (intestinal) and MCF-7 (breast cancer) cell lines, and has also been highlighted previously on the basis of *in vivo* studies (Barger *et al.*, 2008b). A second factor that may account for differences is the dose-dependent action of resveratrol, as reported in *in vivo* studies (Barger *et al.*, 2008a, Barger *et al.*, 2008b, Pearson *et al.*, 2008). In these experiments, a low concentration of resveratrol that can be achievable physiologically through the diet (10 μM) was used. Higher doses of resveratrol may be necessary to elicit effects on these genes. A further consideration is that resveratrol is rapidly metabolised to metabolites including glucuronide and sulphate conjugates (Goldberg *et al.*, 2003; Rotches-Ribalta *et al.*, 2012). It is largely unknown to what extent the activities of

resveratrol and its major metabolites differ so it is possible that many of the gene responses observed *in vivo* are to metabolites of resveratrol rather than to the parent compound itself, which we assume to be the form to which our cell lines were exposed predominantly (unless substantial metabolism occurred under our tissue culture conditions). Many other differences/limitations associated with use of cell culture models may lead to results from *in vivo* studies differing from our own observations.

Since resveratrol has structural similarly to 17-β estradiol, we also analysed the microarray data to investigate if the estrogen-response element (ERE) occurred at a higher frequency than expected by chance in genes regulated by resveratrol. The analysis indicated that genes including the ERE were not enriched within the population of genes that responded to resveratrol treatment in either cell line. This finding may indicate that resveratrol affects gene expression, generally, through a mechanism independent of direct binding of a resveratrol-ER complex to the ERE. Alternatively, an interpretation of the results could be that many of the genes do respond through binding of a resveratrol-ER complex to sequences in the promoter regions that act as EREs, but that the criteria we used to define EREs were inappropriate for the detection of these sequences. A study that investigated the occurrence of the ERE in genes that responded to 17- β estradiol found that the ERE sequence was present in only a fraction (Bourdeau *et al.*, 2004). Thus, the negative results we obtained are not sufficient to allow us to conclude that resveratrol actions on gene expression are generally independent of the ER.

To investigate further if effects of resveratrol - in particular the reduction in histone protein expression - are mediated through the ER, two different cell lines - Caco-2 and MCF-7 - were used. Firstly, the ER status of the cell lines used in this study was confirmed by RT-PCR.

The data confirmed that MCF-7 cells but not Caco-2 cells express ERα whereas expression of ERβ was detected in both cell lines. Both cell lines were treated with resveratrol in the presence and absence of the ER antagonist fulvestrant. The results showed that the effect of resveratrol to reduce histone expression (H2A, H2B, H3 and H4) was reversed by fulvestrant, suggesting that resveratrol acts through the ER to elicit this downstream response. The fact that these responses were observed in the Caco-2 cell line, which lacks ERα, indicates that the responses are mediated through ERβ, consistent with other work in cell lines (Robb and Stuart, 2011). It was not possible to rule out that the response could also be mediated by ERα; to draw such a conclusion a cell line that expresses only ERα would be required.

Next, it was investigated if the reduction in histone protein expression in response to resveratrol treatment was at the level of histone gene transcription using reporter a gene assay (described in Chapter 5). Caco-2 cells were transfected with a histone H3 promoter reporter construct and treated with resveratrol (10 μM, 24 hours) in the presence and absence of fulvestrant. The data indicated that the ER-mediated mechanism through which resveratrol caused a downregulation in histone H3 expression was to affect histone gene transcription. The experiments were extended to investigate if the response to resveratrol treatment is through interaction with EREs. First, three potential EREs within the histone H3 promoter region included in the promoter-reporter construct were replaced with random sequence. Caco-2 cells were then transfected with either the original or mutated promoter-reporter construct and treated with resveratrol or the endogenous ER ligand 17-β estradiol. The results showed that both compounds reduced reporter gene expression from both promoter reporter constructs. These observations indicate that effects of neither compound, although mediated through the ER, are through the specific sequences we identified and replaced. It is likely that a ligand-ER complex binds to other sequences in the promoter region that act as

EREs and that we failed to identify. Although effects of the endogenous ligand 17-β estradiol could be mimicked in this experiment by resveratrol, these data are insufficient to allow us to determine if resveratrol acts primarily as an ER-agonist or ER-antagonist.

The finding that the effects of resveratrol on histone protein expression are ER-mediated actions has important implications for the use of resveratrol as a natural hormone-replacement therapy, for example to protect against osteoporosis (Rayalam *et al.*, 2011) and to alleviate of menopausal symptoms (Zern *et al.*, 2005). On the other hand, caution must be applied, as resveratrol may interact with endogenous estrogen during embryonic development and early childhood. Further study using a rodent model to examine the effect of resveratrol, including dosage and exposure time, on fetal development could be valuable in this regard.

In conclusion, the data indicate that resveratrol reduces histone expression in both Caco-2 and MCF-7 cells through an ER-mediated mechanism acting at the level of transcription and that this effect may oppose an accumulation of histone proteins (observed in mouse small intestine) that accompanies ageing. Microarray data indicated that resveratrol at a concentration achievable through diet has an effect similar to DR with respect to many of the genes regulated, but (in these experiments) excluding some key genes with well-established roles in the ageing process and/or in promoting longevity in response to DR. Effects on gene expression were cell line-specific and distinct from effects of Sirt1 manipulation.

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Appendix A:: Genes affected by resveratrol treatment in Caco-2 cell

ProbeID	p-value	Regulation	Symbol	Chromosome	GI	ILMN_Gene
7160239	3.24E-04	up	FOSB	chr19	5803016	FOSB
6200390	2.15E-04	down	PRIM1	chr12	41349493	PRIM1
5550242	0.00822	up		chr17	561211	HS.554608
2900674	6.27E-10	down	IGSF3	chr1	55953132	IGSF3
3840632	0.001924	up	ACSM3	chr16	47458816	ACSM3
3180168	7.75E-04	down	LOC646764	chr19	89052462	LOC646764
4390661	0.009971	up	DDX51	chr12	37059776	DDX51
5360491	0.00533	down		chr10	27878712	HS.61151
4250181	9.36E-04	down	EXOC5	chr14	82546833	EXOC5
6650176	1.08E-05	up	TRMT11	chr6	94420682	TRMT11
6130382	4.56E-06	up	PTER	chr10	47933342	PTER
4760079	1.73E-04	up	ADARB1	chr21	75709170	ADARB1
1010367	0.016954	up	CSTF3	chr11	75709188	CSTF3
10161	0.002524	down	MOBKL2A	chr19	40018625	MOBKL2A
4120504	0.008054	down	LOC643834	chr11	89033999	LOC643834
130673	5.45E-10	down	NHP2	chr5	53729323	NHP2
1300519	0.003014	up	ZNF597	chr16	22748966	ZNF597
1570414	5.45E-06	down	NAT5	chr20	89993684	NAT5
4760630	0.002505	up	RAB11FIP2	chr10	7662393	RAB11FIP2
4150709	7.13E-04	up	LOC651774		89062149	LOC651774
7320437	2.41E-07	up	DSCR3	chr21	5174424	DSCR3
5340315	0.001115	down	DEFB32	chr20	46409557	DEFB32
5340382	0.001197	down	BAT3	chr6	18375631	BAT3
2190470	0.012785	up	KLF10	chr8	5032176	KLF10
3400379	1.89E-04	up	STXBP6	chr14	46048194	STXBP6
20435	0.00931	down	LOC729279		169213739	LOC729279
20010	4.14E-06	up	CARS	chr11	62240993	CARS
2510689	6.72E-04	up	ROGDI	chr16	13375778	ROGDI
1450026	0.006174	down	LOC389101		113415004	LOC389101
1430487	5.14E-09	up	MGP	chr12	49574513	MGP
5130414	1.17E-06	down	C9orf23	chr9	22325369	C9ORF23
4250093	0.001689	up	LCA5	chr6	32171218	LCA5
2230743	0.015216	down	LOC649578		89026013	LOC649578
160639	1.86E-08	up	EBAG9	chr8	37694064	EBAG9
3800753	6.50E-06	down	CDCA7L	chr7	31542536	CDCA7L
2970605	0.009297	up	LOC100132098	chr19	169213500	LOC100132098
1570221	0.007926	up	C5orf34	chr5	38348407	C5ORF34
4150204	9.58E-04	up	PRRX2	chr9	38505203	PRRX2
3190195	1.50E-04	down	LOC646609	chr9	89029207	LOC646609
6290114	0.012692	down	SDPR	chr2	66346738	SDPR
5340324	0.006735	up	FUT7	chr9	56090657	FUT7
4850470	2.95E-07	down	ITSN1	chr21	47717122	ITSN1
3360553	0.011777	up	RP5-1022P6.2	chr20	153218549	RP5-1022P6.2

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2100519	0.006668	down	IPP	chr1	5174472	IPP
6250450	0.003379	down	LOC400558		169209573	LOC400558
1850041	3.62E-04	down	DPM2	chr9	24497593	DPM2
6560672	3.00E-04	down	HNRPR	chr1	14141188	HNRPR
6960241	0.011511	up	PSAPL1	chr4	145977197	PSAPL1
5420731	3.36E-04	down	CNKSR3	chr6	74316005	CNKSR3
4070632	0.012494	down		chr3	14292080	HS.563147
150180	0.013242	down	MMP10	chr11	4505204	MMP10
2070392	0.005635	down	SLFN12	chr17	31542644	SLFN12
1300402	0.008527	up	TRMT2A	chr22	51173877	TRMT2A
7200601	4.32E-11	up	MUC1	chr1	113206023	MUC1
5260296	0.012736	up	LOC100128905	chr2	169163414	LOC100128905
5050047	0.001716	down	KIAA1967	chr8	40548406	KIAA1967
6840673	0.012241	down	LOC100133288	chr8	169172702	LOC100133288
520678	0.008923	up	CD53	chr1	91106722	CD53
7570338	0.005277	down	LOC728138	chr16	169209809	LOC728138
4010187	4.58E-07	up	UGCGL2	chr13	11386200	UGCGL2
6520661	1.78E-11	down	C9orf46	chr9	142352128	C9ORF46
5390288	5.03E-04	up	C1GALT1C1	chrX	58532583	C1GALT1C1
3780148	1.03E-06	up	C7orf26	chr7	21362069	C7ORF26
1940050	0.00225	down	LOC388720		113412527	LOC388720
7650192	5.02E-09	up	LOC651102		89057454	LOC651102
940288	2.37E-07	up	BAZ1A	chr14	32967604	BAZ1A
5420687	2.92E-05	up	DACT2		141801653	DACT2
610082	0.011357	up	SKA2	chr17	154689645	SKA2
540193	1.05E-04	down	TMEM50A	chr1	20357549	TMEM50A
520189	1.63E-06	up	HYOU1	chr11	13699861	HYOU1
940497	0.005605	down	GRK7	chr3	51896040	GRK7
3310047	0.009964	down	PKD1P1		239745438	PKD1P1
1940463	0.014108	down	LOC729156	chr7	157671954	LOC729156
6370273	9.28E-07	up	CTPS	chr1	4503132	CTPS
6550594	5.86E-04	down	LOC391157	chr1	113411635	LOC391157
2190414	1.40E-09	up	ACACA	chr17	38679966	ACACA
6180487	1.15E-06	down	FOXRED2	chr22	34303916	FOXRED2
2710438	0.001457	up	LOC728711		169168299	LOC728711
4640500	9.73E-12	up	UCHL1	chr4	34147658	UCHL1
610427	1.38E-04	up	KLF8	chrX	89111942	KLF8
3460537	0.001275	down	LOC100132488		169162132	LOC100132488
2900255	1.48E-05	up	ZBTB45	chr19	21314759	ZBTB45
1580220	0.003519	up	LOC649466		88945886	LOC649466
1740291	9.39E-05	down	TMSB15A	chrX	72255577	TMSB15A
4810497	2.27E-05	down	DMKN	chr19	78486557	DMKN
2600309	2.63E-06	up	AADACL4	chr1	61966716	AADACL4
5720541	0.004956	up	LOC100128485		169206193	LOC100128485

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510725	3.95E-04	down	NDUFA10	chr2	33519462	NDUFA10
430333	6.65E-09	up	NLRX1	chr11	25777609	NLRX1
7650286	1.52E-07	up	EXPH5	chr11	21359817	EXPH5
6550274	8.64E-06	down	LOC649679		88981262	LOC649679
2630133	0.009462	up	LOC729389		169213728	LOC729389
10433	0.005649	up	ITGA10	chr1	38569397	ITGA10
430192	9.25E-05	down	PDSS1	chr10	50659085	PDSS1
7040670	3.12E-08	down	DHRS3	chr1	62988332	DHRS3
4260138	0.017142	down	LOC654116		89034481	LOC654116
7210546	0.001736	up	BHLHB9	chrX	39930462	BHLHB9
3120521	5.54E-04	up	NFATC4	chr14	37595561	NFATC4
4060754	0.001138	up	ZNF552	chr19	99028877	ZNF552
6270131	0.016389	down	LOC100129141	chr2	169163171	LOC100129141
5570747	0.017686	up	C21orf49	chr21	219277614	C21ORF49
6280189	0.005298	up	HPS4	chr22	23110969	HPS4
3890167	0.003908	down	SLC39A3	chr19	47080101	SLC39A3
6510220	0.008568	down	LOC650407		88999379	LOC650407
6290021	9.03E-07	down	PSMD7	chr16	34335279	PSMD7
6250154	9.23E-04	up	LZTFL1	chr3	56676319	LZTFL1
2810767	0.010155	up	EBI3	chr19	14577916	EBI3
870669	9.66E-06	up	LRRC56	chr11	142363978	LRRC56
5490347	0.009744	up	CCNL2	chr1	24475708	CCNL2
2490259	0.0022	up	PINK1	chr1	112382374	PINK1
510451	0.01591	up	TIRAP	chr11	89111123	TIRAP
2350730	2.98E-08	up	CGA	chr6	10800407	CGA
7380338	5.78E-05	up	UST	chr6	5032218	UST
3120136	2.21E-04	up	NFAT5	chr16	27886525	NFAT5
4050228	0.00411	up	MEX3A	chr1	147902745	MEX3A
5360202	5.61E-04	up	MRFAP1L1	chr4	44921607	MRFAP1L1
4290358	0.003442	down	CCT7	chr2	58331184	CCT7
520497	0.00382	up	LOC729786	chr15	113425046	LOC729786
7150196	0.001204	up	TMEM87A	chr15	31377764	TMEM87A
3170184	1.50E-10	down	RPL36AL	chr14	34335143	RPL36AL
380762	4.70E-04	down	LOC646300		113415240	LOC646300
3940632	0.007287	down	C8orf79	chr8	153251912	C8ORF79
7570754	0.016603	up		chr13	8905106	HS.539599
1820682	0.013799	down	RNASEL	chr1	30795246	RNASEL
5340528	0.008648	down		chr16	1108803	HS.557218
6580184	0.006146	down	SERHL		113429616	SERHL
1110730	0.001148	up	WWTR1	chr3	34147583	WWTR1
4920286	0.003392	up	C15orf44		113425683	C15ORF44
780762	8.60E-06	up	CSNK1D	chr17	20544143	CSNK1D
540639	0.002492	up	MIR197		262206094	MIR197
1580301	0.008457	down	ATP10A	chr15	157649070	ATP10A

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6650639	0.003236	up	VAMP1	chr12	40549445	VAMP1
6520576	3.90E-06	down	BOLA3	chr2	78486585	BOLA3
5570114	5.24E-09	up	GADD45G	chr9	9790905	GADD45G
5490139	6.13E-05	up	HSPA13	chr21	48928055	HSPA13
2850364	2.95E-04	up	KIAA1333	chr14	33620748	KIAA1333
2490128	0.017746	up	SIK2	chr11	38569459	SIK2
2650682	0.001963	down	RBMX2	chrX	7706315	RBMX2
460170	1.02E-05	down	LOC728368	chr11	169202179	LOC728368
4390646	1.94E-09	up	ZHX2	chr8	63079684	ZHX2
1170528	0.012861	down	FANCB	chrX	66528784	FANCB
2230201	0.015463	up	TARBP1	chr1	110825987	TARBP1
6130138	7.47E-06	down	POLR3C	chr1	141801742	POLR3C
2900184	0.016813	up	LOC653163	chr9	89030014	LOC653163
4860358	1.34E-04	down	VBP1	chrX	66346740	VBP1
1710139	0.008976	down	LOC642362	chr11	89034189	LOC642362
3520438	0.006134	down	ARAP2	chr4	21264591	ARAP2
2140288	2.71E-04	down	FLJ22662	chr12	55743115	FLJ22662
3140639	0.007504	down	LOC133874	chr5	113416861	LOC133874
1940167	1.24E-04	up	PDPR		113426487	PDPR
7330364	0.002109	down	LOC647065	chr2	113413275	LOC647065
1940576	1.64E-12	up	RPS6KB1	chr17	55925648	RPS6KB1
1230047	7.05E-04	up	CBS	chr21	4557414	CBS
840349	1.36E-04	up	OVOL1	chr11	38570157	OVOL1
4060131	0.005606	up	C3orf58	chr3	34222231	C3ORF58
110523	0.006601	down		chr3	10302685	HS.188979
780598	8.67E-05	up	TBK1	chr12	19743810	TBK1
4860500	0.001974	down	LOC652968	chr22	83415180	LOC652968
3440672	0.001161	up	KIAA0261	chr10	42734324	KIAA0261
7160612	7.51E-06	up	USP36	chr17	35250685	USP36
2000128	1.57E-04	up	C4BPA	chr1	62912459	C4BPA
7550707	2.93E-05	up	ING3	chr7	38201654	ING3
460021	0.002601	up	SLC39A7	chr6	117553618	SLC39A7
2120082	0.005283	down	LOC651302		113417962	LOC651302
2900193	0.012448	up	TAF5	chr10	50363367	TAF5
2690270	6.61E-04	up	SNIP1	chr1	21314719	SNIP1
1780446	6.89E-05	up	PCK2	chr14	66346720	PCK2
1070280	0.005095	up		chr21	6711912	HS.542575
5570669	0.009916	up	POFUT1	chr20	27436890	POFUT1
2070477	0.006292	down	C12orf71	chr12	122937208	C12ORF71
510358	0.008557	down	LOC651952		89067026	LOC651952
7100639	4.06E-10	up	ERRFI1	chr1	21314673	ERRFI1
2970397	7.76E-07	down	ZNF296	chr19	21687251	ZNF296
3310451	0.014032	down	PIGO	chr9	38045916	PIGO
4220246	6.63E-05	up	CCL20	chr2	4759075	CCL20

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1780482	7.56E-05	up	CACHD1	chr1	110578648	CACHD1
2120689	1.84E-04	up	UBXN6	chr19	13376853	UBXN6
5390451	3.64E-10	up	DKK3	chr11	66346687	DKK3
270019	1.46E-04	up	GOSR1	chr17	55774986	GOSR1
5090424	2.29E-04	down	RPS27	chr1	68160923	RPS27
2140239	1.47E-11	down	CMTM7	chr3	31657098	CMTM7
5670296	1.67E-04	up	UBTF	chr17	115529450	UBTF
2640341	7.98E-06	down	FKBP5	chr6	17149847	FKBP5
4830433	0.005753	up	LARP6	chr15	37537709	LARP6
3780156	0.002557	up	AMH	chr19	6138973	AMH
1450634	2.89E-08	up	TINAGL1	chr1	11545917	TINAGL1
5310379	0.004393	up	TMEM115	chr3	40795669	TMEM115
1430152	2.15E-05	up	SCML1	chrX	82830431	SCML1
7570142	0.011886	down	DGCR11	chr22	205830444	DGCR11
6130343	0.010677	up	MIR557		262205768	MIR557
1010674	4.01E-04	up	LOC400352		169208770	LOC400352
2000615	0.011791	down	WWP1	chr8	33946331	WWP1
5900593	3.04E-04	up	THTPA	chr14	54607163	THTPA
160072	0.011453	up			10373073	HS.574023
2970402	4.92E-04	up	TUBGCP6	chr22	56788363	TUBGCP6
1070471	0.008473	down	CLU	chr8	42740906	CLU
2490168	1.67E-08	up	MAGEH1	chrX	18105051	MAGEH1
5490131	1.92E-04	up	NIPSNAP1	chr22	4505398	NIPSNAP1
6510403	6.56E-05	up	LGSN	chr6	7705581	LGSN
4050056	7.47E-07	down	TBC1D16	chr17	33563375	TBC1D16
4570730	0.01199	down	LOC652797		89064519	LOC652797
1740343	1.56E-08	up	PCSK5	chr9	20336245	PCSK5
2630554	0.007179	up	JMY	chr5	94721314	JMY
6420674	5.18E-09	down	LOC647954		113417184	LOC647954
3780382	0.005964	up	DKFZp761E198	chr11	149999370	DKFZP761E198
3120681	0.001755	up	AFM	chr4	27754774	AFM
1850437	3.71E-04	up	UBR5	chr8	41352716	UBR5
870402	9.00E-04	up	MAGI1	chr3	74272283	MAGI1
1450241	2.57E-07	up	CLEC4GP1	chr19	89886424	CLEC4GP1
5220528	0.006098	down	LOC646627	chr1	88943942	LOC646627
1570685	0.003351	up	KIAA0415	chr7	82546846	KIAA0415
6960445	0.013041	down	LOC653104	chr8	89028013	LOC653104
5270446	0.013446	down	RBM41	chrX	38454187	RBM41
2030672	0.006982	up	LOC158160	chr10	72534773	LOC158160
6290463	0.004669	up	FALZ	chr17	38788259	FALZ
4880021	0.003245	down	RBM12	chr20	33469952	RBM12
2810138	0.008587	up	PMEPA1	chr20	40317614	PMEPA1
4060598	0.01285	down		chr17	2140439	HS.98815
5290037	0.001491	down	HEATR2	chr7	31377743	HEATR2

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4570672	0.014288	up	TEKT2	chr1	16507949	TEKT2
630474	6.62E-09	down	ZP3	chr7	38327648	ZP3
830274	0.002439	up	CD55	chr1	40788009	CD55
580433	7.00E-04	up	DENND1A	chr9	55749778	DENND1A
3990278	1.10E-05	up	SCGN	chr6	59814903	SCGN
4830747	8.60E-05	up	ERI3	chr1	74136558	ERI3
7380504	0.003195	up	ZNF607	chr19	47271463	ZNF607
430450	0.010878	down	LOC649891		89042418	LOC649891
3130541	0.013872	down	CCNF	chr16	4502620	CCNF
6270468	0.014574	down	LOC643000	chrX	89060141	LOC643000
2690603	0.008168	up	RNF5	chr6	34305290	RNF5
6420743	1.26E-04	up	DYSFIP1	chr17	116235449	DYSFIP1
7000332	4.58E-07	up	CCDC126	chr7	93277090	CCDC126
3830577	0.001709	down	LOC100133478		169215724	LOC100133478
5420538	1.79E-05	up	TP53INP1	chr8	20127661	TP53INP1
270170	1.38E-05	up	GLYATL2	chr11	31542212	GLYATL2
5340615	0.008778	down	TRPV4	chr12	22547179	TRPV4
3130438	0.010258	up	LOC100133167	chr22	169215504	LOC100133167
2690639	0.013847	down	AQR	chr15	58374127	AQR
540609	0.00115	down	LOC653746	chr8	89028158	LOC653746
3930577	0.001781	up	HMGN2	chr1	148922918	HMGN2
4180243	0.006362	down	YIPF5	chr5	68226421	YIPF5
4120192	0.010301	up	CES3	chr16	38455414	CES3
5670095	1.10E-09	down	ZNF239	chr10	149999359	ZNF239
290242	0.004851	down	POTEC	chr18	212549545	POTEC
4730309	9.02E-08	up	PCCA	chr13	65506441	PCCA
6220037	1.42E-04	up	UTP23	chr8	14150117	UTP23
2030537	0.014889	up		chr5	3430558	HS.543983
4120671	2.38E-04	down	SSBP3	chr1	58218978	SSBP3
4120750	1.36E-07	down	OBFC1	chr10	34147613	OBFC1
110056	1.67E-04	up	NRP2	chr2	41872561	NRP2
4180180	0.014362	up	LOC100133950		169160865	LOC100133950
6770639	1.38E-04	up	NDEL1	chr17	71284428	NDEL1
1740753	1.36E-05	down	CBWD3	chr9	148727350	CBWD3
7210594	1.79E-07	up	ZNF650	chr2	40255162	ZNF650
4810064	3.43E-04	up	LOC730041		169218223	LOC730041
3140110	1.05E-06	down	C18orf10	chr18	68534956	C18ORF10
3400400	0.002126	down	LOC100131660		169204872	LOC100131660
6900154	0.006386	up	ZNF704	chr8	76253909	ZNF704
6110561	0.006359	down	MRPS27	chr5	16950608	MRPS27
6980746	0.00417	down	SSX3	chrX	28559008	SSX3
1980706	0.005878	down	ATP6V0D2	chr8	22749164	ATP6V0D2
6650747	1.66E-07	up	ZNF641	chr12	22748700	ZNF641
5820091	4.38E-04	up	LOC650612		88947033	LOC650612

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4070059	0.00693	down	LOC729197	chr15	113425458	LOC729197
4860196	0.014239	up	FLJ44124	chr7	89886184	FLJ44124
7050180	2.73E-08	up	SLC22A5	chr5	24497491	SLC22A5
7000142	3.50E-06	up	KIAA0430	chr16	85797659	KIAA0430
3610424	0.006239	up	TRIM67	chr1	134288905	TRIM67
5900270	0.005496	up	FOXD4L4	chr9	76880471	FOXD4L4
670403	3.23E-06	down	B4GALNT4	chr11	40789264	B4GALNT4
630059	0.010897	down	LOC648167		89038845	LOC648167
6220451	1.14E-06	down	SLC25A4	chr4	55749576	SLC25A4
3130220	4.35E-04	up	TMEM158	chr3	116805333	TMEM158
1940026	0.008344	down	LOC642797	chr12	89035773	LOC642797
270670	0.012951	down	LOC100133444		169217923	LOC100133444
1170072	1.70E-06	up	ZSWIM4	chr19	65301125	ZSWIM4
20553	1.06E-05	down	C1orf124	chr1	58331106	C1ORF124
5670037	0.014606	down	MUTYH	chr1	115298649	MUTYH
3780497	0.009768	down	LOC100129465	chr10	169190996	LOC100129465
3460309	6.01E-08	up	ATF4	chr22	33469975	ATF4
730725	9.82E-06	up	AOF2	chr1	58761545	AOF2
3360377	2.84E-06	down	UTP14A	chrX	21361347	UTP14A
6980369	0.001303	down		chr2	27878421	HS.131259
1470521	1.55E-05	up	NR2C1	chr12	73808083	NR2C1
1580093	0.001128	up	SCYL1	chr11	115430240	SCYL1
540390	1.19E-04	up	ALPK1	chr4	21361968	ALPK1
7330446	0.001311	up	LOC729764	chr5	113416915	LOC729764
2100521	0.002569	up	STARD8	chrX	31543658	STARD8
2810739	0.016226	up	PDE4B	chr1	82799483	PDE4B
3390392	0.006185	up	SUGT1P		89029227	SUGT1P
3840193	3.50E-06	up	KIAA0649	chr9	45387957	KIAA0649
2470603	3.09E-07	up	ARID4B	chr1	118136292	ARID4B
6450402	0.003296	up	LOC643699	chr15	113425118	LOC643699
5260343	7.67E-04	up	SCG5	chr15	4506916	SCG5
130070	0.001371	up	IFT172	chr2	46358427	IFT172
2260133	0.006305	up	RPS6KA3	chrX	56243494	RPS6KA3
7200608	6.26E-08	up	HSPB1	chr7	4996892	HSPB1
5570315	1.15E-04	up	C2orf58	chr2	27734974	C2ORF58
610592	5.63E-06	up	ADCY1	chr7	31083192	ADCY1
6100441	9.94E-09	down	PRPF4	chr9	34222192	PRPF4
7400402	0.003026	up	HIST1H4D	chr6	21071023	HIST1H4D
6760026	0.002391	up	LRRC57	chr15	23397553	LRRC57
4280739	4.39E-09	down	APCDD1	chr18	30387616	APCDD1
2260070	5.54E-06	up	MTERF	chr7	68448540	MTERF
4730019	0.015204	up	EFNB1	chrX	31317225	EFNB1
650301	1.72E-04	down	SNRPF	chr12	83776586	SNRPF
1740091	0.001627	down	LOC388458	chr18	113427693	LOC388458

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6040148	0.016378	down	PML		89039091	PML
1170468	0.008586	up	UBE2MP1	chr16	84872128	UBE2MP1
4180255	0.005662	down	C1QTNF9B	chr13	56847615	C1QTNF9B
1070019	0.001327	up	FBXO17	chr19	22325386	FBXO17
1980202	0.002838	up	CAPRIN2	chr12	50428932	CAPRIN2
7610050	8.10E-04	up	ABCC5	chr3	66529092	ABCC5
2750139	1.47E-06	down	RNF216L	chr7	190194405	RNF216L
4280327	7.74E-04	up	C2orf82	chr2	46047466	C2ORF82
4860553	1.67E-05	down	INTS12	chr4	21361850	INTS12
5220014	0.00341	up	LOC96610	chr22	18426889	LOC96610
3890397	1.61E-06	down	C14orf112	chr14	142349815	C14ORF112
2070605	0.001837	down	ACAT1	chr11	31563501	ACAT1
1710332	0.003893	down	FAHD1	chr16	66348061	FAHD1
6940619	0.001528	up	Sep-02	chr2	56549635	Sep-02
5360402	0.013099	up	RTEL1	chr20	30089967	RTEL1
5810121	0.004301	down	LOC652786		89064437	LOC652786
2650161	0.015031	down	LOC644986	chr8	89028116	LOC644986
2490452	9.26E-07	up	GAA	chr17	119393890	GAA
2600487	0.008811	down		chr13	24724856	HS.550139
1340358	0.014793	up	CREB1	chr2	22219459	CREB1
6270347	1.55E-09	up	TRAK1	chr3	111074531	TRAK1
5690692	0.006578	up		chr20	6398275	HS.542384
5550553	0.008251	up	DHRS13	chr17	146231949	DHRS13
1090132	0.001036	up	PARP10		113420558	PARP10
1820504	1.92E-08	down	NME1	chr17	38045911	NME1
3710598	0.004219	up	PSTK	chr10	23503260	PSTK
7560019	0.003415	down	MIR15A		262205622	MIR15A
6550026	1.65E-06	up	TICAM2	chr5	48675828	TICAM2
6860300	1.21E-04	up	SMARCA4	chr19	21071055	SMARCA4
5670475	8.81E-04	down	GCHFR	chr15	6382072	GCHFR
4560202	0.003465	up	AAA1	chr7	46402501	AAA1
5340053	3.07E-06	up	MAK10	chr9	39725954	MAK10
4200270	0.010042	up	TMUB2	chr17	115527089	TMUB2
7000609	1.41E-06	up	ARRDC2	chr19	18373304	ARRDC2
7150671	0.004794	up	ARL3	chr10	38569402	ARL3
5260132	9.23E-04	up	LOC100190938	chr17	213385284	LOC100190938
870475	1.27E-08	up	SYCP2	chr20	38373672	SYCP2
290296	6.35E-06	up	FAM113A	chr20	21362095	FAM113A
7610431	0.001262	down	FLJ40330	chr2	113413041	FLJ40330
7100300	0.003488	up	ZNF200	chr16	37675269	ZNF200
3990465	1.22E-06	down	FASTKD3	chr5	40068496	FASTKD3
7160730	0.010109	up	NCRNA00120	chr6	84872053	NCRNA00120
7510537	0.00104	down	SCO2	chr22	4826991	SCO2
6020482	5.45E-11	up	KHDRBS3	chr8	5730072	KHDRBS3

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3830070	0.015925	up		chr7	34526302	HS.193767
2510477	0.011417	down	B3GALT6	chr1	116268096	B3GALT6
6840100	1.07E-04	down	C11orf51	chr11	7661621	C11ORF51
4780193	0.012002	down	POFUT2	chr21	116734661	POFUT2
6380047	0.00327	down	MIR586		262205906	MIR586
6480682	0.012575	up	FSHB	chr11	66528900	FSHB
2970296	8.89E-10	down	ALG8	chr11	91984777	ALG8
2070470	7.56E-04	up	C1orf21	chr1	58761542	C1ORF21
3610427	0.002925	up	LOC100128098	chr10	169191377	LOC100128098
3390093	1.13E-05	down	BOLA3	chr2	78486577	BOLA3
2690040	0.01101	down	ABHD12B	chr14	32451491	ABHD12B
1770630	3.86E-05	down	ALOX12P2	chr17	117940057	ALOX12P2
6130088	0.00251	up	MED1	chr17	154813205	MED1
510044	4.09E-06	up	KLF4	chr9	34916057	KLF4
7160167	9.10E-04	down	ALKBH1	chr14	87298839	ALKBH1
6290717	5.48E-04	down	DMRTA1	chr9	46195736	DMRTA1
6510598	1.35E-06	up	NHLRC3	chr13	62821784	NHLRC3
5340327	2.39E-04	up	EIF4ENIF1	chr22	10947034	EIF4ENIF1
5270747	3.41E-09	up	HSDL1	chr16	24432036	HSDL1
6650438	2.02E-05	up	SYNGR1	chr22	39777617	SYNGR1
2100224	3.99E-05	up	RHBDD3	chr22	11072100	RHBDD3
5310161	0.005654	up	MTHFD2L	chr4	62243411	MTHFD2L
5720180	1.10E-06	up	FZD2	chr17	5922012	FZD2
110026	0.015352	down	LOC286239		89030280	LOC286239
2650021	6.15E-05	up	NRP2	chr2	41872543	NRP2
1570382	9.93E-04	up	ADAMTS9	chr3	33624895	ADAMTS9
6940719	1.14E-05	up	PAPOLA	chr14	47834324	PAPOLA
620433	0.011961	down	C1orf122	chr1	38348207	C1ORF122
1190674	2.69E-04	down	C19orf69	chr19	194473672	C19ORF69
6060458	3.22E-05	up	GFRA3	chr5	45593145	GFRA3
2450020	6.61E-04	up	SNORD56	chr20	84872023	SNORD56
5270209	0.007488	up		chr12	27830880	HS.436006
4040674	0.008165	up	UGT1A4	chr2	45827763	UGT1A4
1070050	0.002206	up	C9orf126	chr9	27735124	C9ORF126
7160100	0.004027	up	PPRC1	chr10	40807451	PPRC1
1260544	3.15E-05	up	AKIRIN1	chr1	13375790	AKIRIN1
6100010	7.63E-04	up	THEM4	chr1	76159292	THEM4
6510754	6.11E-12	down	ALDH1A1	chr9	25777722	ALDH1A1
1410519	0.003505	up		chr2	2185354	HS.66072
4060095	0.01236	down	CD84	chr1	4502686	CD84
830446	7.86E-04	down	RUNDC1	chr17	27436874	RUNDC1
4920014	0.001667	up	GIF	chr11	32189397	GIF
270630	0.006462	up	LOC642213		89038794	LOC642213
2760113	0.005586	up	RANBP6	chr9	52486449	RANBP6

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6770202	1.49E-06	up	IPO11	chr5	39725949	IPO11
4730427	0.014516	down	MYLC2PL	chr7	40286635	MYLC2PL
780170	0.013616	up	LOC100128505		169177891	LOC100128505
3310487	0.011462	up	RPL18A	chr19	15431299	RPL18A
5390041	4.41E-07	up	TRIM3	chr11	32454738	TRIM3
1990692	0.00753	down	LOC100131895		169202635	LOC100131895
3120520	1.07E-07	down	GABARAPL2	chr16	27374999	GABARAPL2
730291	6.76E-06	up	OXR1	chr8	63055068	OXR1
5860347	0.002367	up	AKR1D1	chr7	66348134	AKR1D1
5290075	0.002583	down	GAS5	chr1	88943597	GAS5
4230653	1.68E-06	up	CCNC	chr6	61676090	CCNC
5570678	3.47E-09	down	RPS27L	chr15	76563938	RPS27L
2340441	5.18E-05	up	SLC22A18AS	chr11	6005877	SLC22A18AS
5490376	3.71E-05	down	NCOR2	chr12	116256452	NCOR2
840392	0.00106	up	PCYOX1	chr2	33620750	PCYOX1
5890735	0.004648	down	SMURF2	chr17	56550041	SMURF2
3870408	0.001584	up	DUSP18	chr22	51093844	DUSP18
50373	0.008038	up	PKP3		89034409	PKP3
2060703	0.007976	down	LOC643304	chr12	89035736	LOC643304
2900270	0.005195	up	LOC729090	chr5	169167519	LOC729090
1570056	2.68E-08	up	MYO5A	chr15	115511013	MYO5A
3420544	0.005806	down	LOC653584	chr3	88965597	LOC653584
1230746	1.69E-07	up	ZCCHC6	chr9	58331271	ZCCHC6
4640484	2.71E-05	up	CNOT8	chr5	31542314	CNOT8
4590608	1.53E-04	down	POLD3	chr11	38492355	POLD3
5270717	0.002061	up	CCT3	chr1	58761483	CCT3
6380112	0.006262	up	GRAMD4	chr22	67782363	GRAMD4
2760070	0.00274	down	FLJ36492	chr17	50979285	FLJ36492
1980138	7.77E-05	down	MRRF	chr9	40317613	MRRF
4830551	0.009629	up	HDX	chrX	142357374	HDX
4280209	0.013667	up	LOC442546	chr7	169170379	LOC442546
4290196	0.009606	up	SCRIB	chr8	115527071	SCRIB
4050242	0.009557	down	LOC642356	chr8	89028010	LOC642356
150181	0.012188	down	RUVBL1		88971019	RUVBL1
3850687	7.83E-04	up	FICD	chr12	42794619	FICD
7330753	1.20E-07	down	ACAT2	chr6	148539871	ACAT2
770326	7.56E-06	down	LDHA	chr11	207028493	LDHA
1050112	0.013148	down	LOC643879	chr2	88953376	LOC643879
3710253	1.96E-07	up	CAPN12	chr19	46852396	CAPN12
6040307	0.014748	up	MORN3	chr12	50355989	MORN3
2510088	0.012578	up	GPR64	chrX	119943128	GPR64
5670411	0.00747	down		chr2	80804776	HS.580480
2810437	0.001525	down	LOC648834		89035927	LOC648834
7650441	1.51E-07	down	FGFBP1	chr4	49574208	FGFBP1

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-1.166888		LOC646198	646198	chrX	89059491	LOC646198
1.105352		KIAA1571	57683		113414229	KIAA1571
-1.1341	Hs.578698			chr16	28444660	HS.578698
1.038292		CCDC18	343099	chr1	62243483	CCDC18
1.104407		TBC1D2	55357	chr9	62198224	TBC1D2
-1.108194		EPHA1	2041	chr7	56119206	EPHA1
-1.10814		HS6ST2	90161	chrX	116295255	HS6ST2
1.100031		YARS	8565	chr1	38202242	YARS
1.046394		RNF128	79589	chrX	37588872	RNF128
-1.187303		DCDC2B	149069	chr1	150456470	DCDC2B
1.16733		LOC652627	652627		169218179	LOC652627
-1.128098	Hs.98466			chr16	15939282	HS.98466
1.12353	Hs.187293			chrX	11593467	HS.187293
1.171721		MSX2	4488	chr5	84452153	MSX2
1.087886		LOC650851	650851		89042527	LOC650851
1.109211		ZC3H12A	80149	chr1	13376631	ZC3H12A
1.087393		LOC401357	401357	chr7	61966820	LOC401357
-1.094295		LOC641846	641846		89027586	LOC641846
1.084805		LOC728728	728728	chr1	169162773	LOC728728
1.091768		LOC100134420	100134420		169168352	LOC100134420
-1.157601		EGFLAM	133584	chr5	33469934	EGFLAM
1.130594		C2orf42	54980	chr2	8923527	C2ORF42
-1.101714		RBMY1E	378950	chrY	56090526	RBMY1E
-1.236282		LOC647012	647012	chr2	113413206	LOC647012
1.10317		LOC642325	642325	chr15	113425388	LOC642325
-1.097124		LOC644749	644749	chrX	89060510	LOC644749
-1.101309		LOC387703	387703	chr10	169188349	LOC387703
-1.133106		LOC100131774	100131774		169169846	LOC100131774
-1.08602		LOC649060	649060		89037132	LOC649060
-1.078223		ZNF713	349075	chr7	33438599	ZNF713
1.174702		ZNF658	26149	chr9	55769536	ZNF658
-1.09019		MITF	4286	chr3	38156696	MITF
1.066027		APP	351	chr21	41406053	APP
-1.072007		SPP2	6694	chr2	54262133	SPP2
-1.160614		SBDSP	155370	chr7	38348442	SBDSP
1.135423		FLJ13224	79857	chr12	113423275	FLJ13224
1.223264		ATP2B4	493	chr1	48255958	ATP2B4
-1.095717		LOC392335	392335	chr9	169177392	LOC392335
1.082646		SNX32	254122	chr11	72534837	SNX32
-1.127806	Hs.211182			chr9	27837068	HS.211182
-1.059878		LOC100129055	100129055	chr10	214831164	LOC100129055

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3060008	5.18E-04	up	C2CD4B	chr15	157278360	C2CD4B
6560091	1.70E-11	down	TGFBR2	chr3	133908632	TGFBR2
1780156	4.54E-04	up	SAMD4B	chr19	55742694	SAMD4B
2370017	0.010312	up	MCM8	chr20	33469925	MCM8
7550192	2.06E-11	up	SLC16A10	chr6	45433547	SLC16A10
5080020	0.007012	up	UBE2J1	chr6	37577121	UBE2J1
2100450	0.010103	down	LOC653651	chr10	89031348	LOC653651
3780270	0.001178	up	HBP1	chr7	47834345	HBP1
6270307	2.61E-06	down	LOC644934	chr15	113425249	LOC644934
6400711	4.21E-04	up	ZNF573	chr19	40789269	ZNF573

Appendix B: Genes affected by resveratrol treatment in MCF-7 cell line

ProbeID	Regulation	Fold change	Unigene_ID	Symbol	Entrez_Gene_ID	Accession
6380242	down	-1.101846		TNFSF11	8600	NM_033012.2
2810327	up	1.1271994		GIT2	9815	NM_014776.2
5360398	down	-1.161789		ERI1	90459	NM_153332.3
2690528	up	1.1861886		SHMT1	6470	NM_004169.3
110427	up	1.1791819		TCEA1	6917	NM_201437.1
6200615	down	-1.138269		SAPS2	9701	XM_942540.1
3420279	up	1.091244	Hs.427229			AI820955
2940435	down	-1.173766		TFRC	7037	NM_003234.1
3360592	up	1.1556085		LOC388925	388925	XR_016133.2
4390703	up	1.1696571		PTPN1	5770	NM_002827.2
7610403	up	1.1212988		HAO1	54363	NM_017545.2
4260382	down	-1.166522		LOC651609	651609	XM_940790.1
2630619	down	-1.198774		RNF20	56254	NM_019592.5
4590026	down	-1.208029		IMPDH2	3615	NM_000884.2
6520026	down	-1.211906		NUCB1	4924	NM_006184.3
3840372	down	-1.152212		LOC440225	440225	XR_042392.1
2570091	down	-1.144436		PHF19	26147	NM_015651.1
4260291	down	-1.141839		BEND4	389206	NM_207406.2
620136	up	1.1948407		LILRA5	353514	NM_181879.1
2510324	up	1.1385332		NUDT2	318	NM_147173.1
1660403	down	-1.095697		FLJ41327	401045	NM_207485.1
130554	up	1.0999346		MIR620	693205	NR_030351.1
3800487	up	1.1551031		ZNFX1	57169	NM_021035.2
4540682	down	-1.146227		PALM	5064	NM_002579.2
940754	down	-1.097704		TMEM183A	92703	NM_138391.4
110121	up	1.200783		UBIAD1	29914	NM_013319.1
3870184	up	1.2462937		LOC441131	441131	XR_038026.1
7000719	down	-1.132033		LOC650254	650254	XM_499385.2
5310086	down	-1.132955		ZBED3	84327	NM_032367.2
3870747	down	-1.164346		LOC645478	645478	XM_932922.2
50470	down	-1.154932		PM20D2	135293	NM_001010853.1
3850398	up	1.1297903		STX2	2054	NM_194356.1
5720053	up	1.1915785		KRTHB6	3892	NM_002284.2
6560445	up	1.1450862		DNAJA3	9093	NM_005147.3
130725	up	1.068769		VWDE	221806	NM_001135924.1
6290037	up	1.0788046		LOC100130938	100130938	XM_001720237.1
3870671	up	1.1964148		SUPT7L	9913	NM_014860.1
2350373	up	1.176096		C1orf9	51430	NM_014283.2
6770192	up	1.1502244		LOC100134584	100134584	XM_001725215.1
2480259	down	-1.085064		FLJ37201	283011	XR_000539.1
2600608	down	-1.199936		LOC652436	652436	XM_941879.1
6280201	down	-1.15571		PTPRK	5796	NM_002844.2
2810181	up	1.1928123		LOC645663	645663	XM_933036.1

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6650026	up	1.138121		ASTE1	28990	NM_014065.2
3060278	up	1.165285		MGC15763	92106	NM_138381.1
3170632	down	-1.090755		LOC100132373	100132373	XR_038782.1
1740674	down	-1.173845		TRAPPC3	27095	NM_014408.3
6980368	down	-1.13592		LOC100131335	100131335	XM_001718069.1
2140152	down	-1.088221	Hs.130639			BX109627
240152	up	1.1372197		MGC57346	401884	XM_377476.4
2570189	up	1.1536074		NTF4	4909	NM_006179.4
240484	up	1.0792688		ZNF182	7569	NM_001007088.1
240392	down	-1.076115	Hs.202577			AK091904
3290025	up	1.1511773		LOC730074	730074	XM_001713717.1
460259	up	1.1365315		LAT	27040	NM_001014987.1
6380747	up	1.2090231		ANKRD32	84250	NM_032290.2
6370315	up	1.143988		HLA-DRB5	3127	NM_002125.3
990671	down	-1.139834		TMEM150C	441027	NM_001080506.1
4120278	up	1.2168293		KIF23	9493	NM_004856.4
1170437	up	1.185572		FAM115C	285966	NM_001130026.1
3360139	up	1.1297554		NUDCD1	84955	NM_032869.2
6980707	down	-1.101264	Hs.566307			AW296281
3940136	down	-1.190212		NUDT15	55270	NM_018283.1
4070064	down	-1.227426		LOC100133017	100133017	XM_001725861.1
990711	up	1.0892527		FAM45A	404636	NM_207009.2
6980097	up	1.1785063		NANP	140838	NM_152667.1
6840315	down	-1.164836		LOC644968	644968	XM_928045.1
1030022	up	1.0780691		PDCD11	22984	NM_014976.1
5220445	up	1.158647		QKI	9444	NM_006775.1
4890112	up	1.2324053		ZNRD1	30834	NM_014596.4
3890215	up	1.2472177		KIAA0258	9827	XM_941746.1
3460762	up	1.0891758		LOC653441	653441	XM_934161.1
4390014	up	1.1294407		C1orf58	148362	NM_144695.2
4120451	down	-1.172609		LOC654032	654032	XM_939494.1
7330750	up	1.0786141		ZNF491	126069	NM_152356.3
4250091	down	-1.160961	Hs.543683			AI494041
2100050	up	1.1440773		IGHMBP2	3508	NM_002180.2
4010195	up	1.125784		COL6A2	1292	NM_058175.2
730259	down	-1.141625		CACNA1H	8912	NM_001005407.1
160343	up	1.1662397		ALDH4A1	8659	NM_003748.2
2680044	up	1.0811857		RAB27A	5873	NM_183236.1
6270754	up	1.14812		LHX3	8022	NM_014564.2
6220246	down	-1.111788		LOC647726	647726	XM_936788.1
4230356	up	1.1693654		C17orf56	146705	NM_144679.1
6250553	down	-1.156608		ITFG3	83986	NM_032039.1
270068	up	1.0794486		SMOX	54498	NM_175842.1
4850538	up	1.1937208		PAOX	196743	NM_207128.1

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510274	up	1.1062914		LOC401010	401010	NR_002826.1
6180600	up	1.1627448		NLRP7	199713	NM_139176.2
6510437	up	1.172108		HNRNPM	4670	NM_005968.3
6250180	up	1.2303317		HMG20A	10363	NM_018200.2
580670	up	1.1504056		CCM2	83605	NM_031443.3
5670594	up	1.1357732		NMB	4828	NM_021077.3
940148	down	-1.168866		ABTB2	25841	NM_145804.1
1470181	down	-1.203757		KIAA1712	80817	NM_030633.1
3830678	down	-1.06753		CD207	50489	NM_015717.2
5560273	down	-1.081719		BTBD9	114781	NM_052893.1
6020280	up	1.1831449		NRAS	4893	NM_002524.2
2630370	up	1.1182835		MAD1L1	8379	NM_001013836.1
6020612	down	-1.140802		FOXN2	3344	NM_002158.3
670367	up	1.1702397		RAB5A	5868	NM_004162.3
4260075	up	1.1414013		LSM3	27258	NM_014463.1
4570735	down	-1.141777		ARG99	83857	NM_031920.2
1820739	down	-1.128086		CCRK	23552	NM_012119.3
6270619	up	1.1851883		ZNF689	115509	NM_138447.1
2970100	up	1.2290854		LOC652773	652773	XM_942415.1
70070	down	-1.223366		GRINA	2907	NM_000837.1
2360446	up	1.1962687		OSBPL3	26031	NM_145321.1
4640706	down	-1.199041	Hs.579522			CR744191
7210465	down	-1.087286	Hs.543364			AI392610
4640722	down	-1.108859		LOC653581	653581	XM_932712.1
1940082	up	1.1404402	Hs.40289			BX538337
3610594	up	1.0752829		LOC401602	401602	XR_038621.1
6200215	up	1.1300117		TTC13	79573	NM_024525.2
1990687	up	1.1811662		LOC285307	285307	XM_211837.2
3130445	up	1.1414944	Hs.536451			BX105743
3830739	up	1.1539812		TCEAL1	9338	NM_001006640.1
1090072	down	-1.10742		KIAA0494	9813	NM_014774.1
3710719	up	1.1377243		MRPL45	84311	NM_032351.3
3830112	down	-1.130088		LOC644150	644150	XM_933686.1
6960026	down	-1.232799		STXBP2	6813	NM_006949.1
6220048	up	1.118372		LOC392501	392501	XR_016267.2
3610019	down	-1.101024		DSEL	92126	NM_032160.2
7160474	down	-1.146197		HLA-DQB1	3119	NM_002123.2
1820154	up	1.1883807	Hs.334093			BG184196
5360463	down	-1.1561		LOC651991	651991	XM_945086.1
2810746	down	-1.163429		DNAJB2	3300	NM_006736.5
3290671	down	-1.112728		LOC647042	647042	XM_294765.7
4290270	up	1.1540604		LOC645445	645445	XM_932902.1
730605	up	1.1977056		MRPS22	56945	NM_020191.2
2060220	up	1.1460649		FAM174A	345757	NM_198507.1

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1090753	down	-1.358616		OAZ2	4947	NM_002537.2
4150392	up	1.1726096		NR6A1	2649	NM_001489.3
7050010	up	1.1139034		BNC2	54796	NM_017637.5
4210408	down	-1.150428		GPR157	80045	NM_024980.4
270341	up	1.2323076		NUDT9	53343	NM_198038.1
5960162	down	-1.15478		ATP6V1G3	127124	NM_133262.2
4670020	up	1.1310441		NIPA2	81614	NM_030922.5
2360189	up	1.1435916		AFF4	27125	NM_014423.3
4610411	up	1.1183329		MTERFD1	51001	NM_015942.3
7320717	up	1.1143837		GALNT4	8693	NM_003774.3
4290041	down	-1.090744	Hs.146614			DB032405
3930228	down	-1.101705		LOC730274	730274	XM_001125976.1
4810372	down	-1.094232	Hs.87066			BF448257
5080561	down	-1.156813		BARD1	580	NM_000465.1
7150326	up	1.0703942		ZNF253	56242	NM_021047.2
1690647	down	-1.194795		WASF1	8936	NM_001024936.1
2140082	down	-1.169753		PALMD	54873	NM_017734.3
2680750	down	-1.114575		PTK2	5747	NM_153831.2
6620669	down	-1.114098		C3orf23	285343	NM_173826.3
3830072	up	1.0999687		HIF3A	64344	NM_022462.3
2230343	up	1.23422		RABIF	5877	NM_002871.3
50176	up	1.0749401		TMEM16B	57101	NM_020373.1
1010246	up	1.4021088		IFI6	2537	NM_022872.2
2510463	up	1.1417383		METTL13	51603	NM_001007239.1
7160523	down	-1.165254		LOC650439	650439	XM_944199.1
5700484	down	-1.08405		RHBG	57127	NM_020407.2
2140370	up	1.0898942		RPS29	6235	NM_001030001.1
1850184	up	1.1209373		PMS2CL	441194	NR_002217.1
1660341	down	-1.191054		FLJ39632	642477	XR_015133.1
5810068	down	-1.112694		SIPA1	6494	NM_006747.2
3190592	up	1.173245		KIAA1644	85352	XM_936510.2
780204	down	-1.170127		ZNF669	79862	NM_024804.1
3310039	down	-1.135884		KIAA0754	643314	NM_015038.1
1450408	up	1.161646		SIX1	6495	NM_005982.2
1780440	up	1.067443		CD79A	973	NM_001783.3
6760008	down	-1.211301		DBN1	1627	NM_004395.2
4830706	down	-1.130678	Hs.561633			BM674971
1570300	up	1.1124007		SPATA13	221178	NM_153023.1
5690400	down	-1.30657		GSTTP2	653399	NR_003082.1
5720474	up	1.1827844		TMCO6	55374	NM_018502.3
6510661	down	-1.221892		SS18L1	26039	NM_198935.1
20056	up	1.0747428		TPT1	7178	NM_003295.1
1230382	down	-1.129323		LOC644285	644285	XM_929631.1
2900129	up	1.1805732		FTO	79068	NM_001080432.1

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4060035	up	1.1900021		WDR20	91833	NM_181308.1
2340193	down	-1.165052		KRT80	144501	NM_182507.2
5420475	down	-1.127245		LOC100128056	100128056	XM_001722552.1
6520440	down	-1.149656		KIF1A	547	NM_004321.4
2060400	up	1.1352553		NKTR	4820	NM_001012651.1
510112	up	1.1547036		PTAFR	5724	NM_000952.3
1470170	up	1.1055858		C18orf8	29919	NM_013326.3
1820187	down	-1.11035		TMX3	54495	NM_019022.3
630524	up	1.1387056		PCTK2	5128	NM_002595.2
2260349	down	-1.447008		MIR1974	100302207	NR_031738.1
4560110	up	1.1937718		ARMET	7873	NM_006010.2
2260181	down	-1.173932		SLC38A4	55089	NM_018018.2
290154	down	-1.123759		LOC127406	127406	XM_497679.2
7320291	up	1.1901416		IQCK	124152	NM_153208.1
770717	up	1.0971276		LOC646079	646079	XM_929039.2
4150291	up	1.1540513		LOC643466	643466	XM_926789.1
780435	down	-1.180221		LSAMP	4045	NM_002338.2
1850348	up	1.1251006		ERCC4	2072	NM_005236.1
2190156	up	1.200798		ASB7	140460	NM_198243.1
4010386	up	1.127643		PGBD1	84547	NM_032507.2
2060095	up	1.1251669		SNRK	54861	NM_017719.3
5960438	down	-1.164907		MASP1	5648	NM_139125.2
450021	up	1.1029086		LOC338797	338797	NM_001025466.1
4120187	down	-1.084005		EFNA3	1944	NM_004952.3
5690017	down	-1.086775		LOC100132288	100132288	NM_001033515.1
6660736	up	1.1210369	Hs.577713			BI830379
1980424	up	1.2212617		LOC647349	647349	XR_019572.1
160364	down	-1.165164		ZNF282	8427	NM_003575.2
2810487	up	1.1136636		OTUB2	78990	NM_023112.2
990719	down	-1.171091	Hs.579530			CD522953
1580731	up	1.1475848		LOC441896	441896	XR_038885.1
7320246	up	1.0889354		HEMGN	55363	NM_197978.1
5890491	down	-1.133837	Hs.542687			AK125234
1580079	up	1.153013		LOC648665	648665	XR_042292.1
4150673	down	-1.093552		LOC646617	646617	XM_929557.1
20239	down	-1.153654		REPS2	9185	NM_004726.2
380594	up	1.1134462		ZFYVE21	79038	NM_024071.2
6110768	down	-1.229162		ZNF334	55713	NM_199441.1
7400468	down	-1.147194		AP1G2	8906	NM_003917.2
6020504	down	-1.141693		DSCR5	51227	NM_153681.1
4390326	up	1.1470286		SDR42E1	93517	NM_145168.2
4830653	down	-1.122231	Hs.551358			BF057027
5260100	down	-1.109773		LOC100128548	100128548	XR_037705.1
4490358	up	1.175183		KIF12	113220	NM_138424.1

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3780435	up	1.1220481		GART	2618	NM_175085.1
6400088	up	1.2453427		C6orf148	80759	NM_030568.2
4230626	down	-1.185336		PRKCD	5580	NM_006254.3
7050138	up	1.1063979		RQCD1	9125	NM_005444.1
4480112	down	-1.201179		PGM5	5239	NM_021965.3
2030253	up	1.1219461		THSD1P	374500	NR_002816.1
630332	down	-1.205303		ZNF773	374928	NM_198542.1
4920768	up	1.1533679		LOC647691	647691	XM_936748.1
2710114	down	-1.142388		GNB1	2782	NM_002074.2
2570300	up	1.1195511		IFI44	10561	NM_006417.3
4280292	up	1.1600114		RNF126	55658	NM_194460.1
4120523	up	1.1541446		LOC100131673	100131673	XM_001724387.1
1470397	down	-1.164193		C8orf33	65265	NM_023080.1
540301	down	-1.160606		WHSC1	7468	NM_133330.1
6400646	up	1.1914828		CCDC85B	11007	NM_006848.2
3450020	up	1.1057111		PTK7	5754	NM_152882.2
4250497	down	-1.167817		ABCC13	150000	NM_138726.2
3840593	up	1.116043		SP110	3431	NM_004510.2
290372	down	-1.271676	Hs.125395			BX110351
4490156	down	-1.14282		LOC390367	390367	XM_374352.3
4480091	up	1.1935943		ASB9	140462	NM_024087.1
5050403	up	1.0921485		TDH	157739	NR_001578.1
6940524	down	-1.130081		EXD1	161829	NM_152596.2
5890768	down	-1.118049		NFIC	4782	NM_005597.2
1400037	down	-1.143737		OXCT2	64064	NM_022120.1
4920364	up	1.1595012		LOC728654	728654	XM_001128037.1
360192	up	1.2140545		INSIG1	3638	NM_198336.1
6280577	down	-1.183386		LOC284620	284620	XR_039089.1
7610129	up	1.1245514		RAD51C	5889	NM_058216.1
160424	down	-1.12283		SCARNA6	677772	NR_003006.1
1980039	up	1.1396345		NEIL3	55247	NM_018248.1
5260477	down	-1.204522		LOC100133163	100133163	XM_001726385.1
1510468	up	1.1456934		GRPEL1	80273	NM_025196.2
2450338	up	1.1336782		TUSC1	286319	NM_001004125.2
1980563	down	-1.165844		FANCA	2175	NM_001018112.1
1450072	up	1.1505334		PIGF	5281	NM_002643.3
6180088	up	1.1098685		ATP6V0E2	155066	NM_145230.2
730035	up	1.1653159		RAP1GDS1	5910	NM_021159.3
2070386	down	-1.122579		GABRA5	2558	NM_000810.2
4260066	down	-1.17216		LOC646111	646111	XM_929073.1
4830577	up	1.241573		MRPS16	51021	NM_016065.3
5910215	up	1.2241374		RAD51C	5889	NM_002876.2
2000291	up	1.2037286		PCGF3	10336	NM_006315.4
1780035	down	-1.170406		SNORD19	692089	NR_003047.1

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150386	up	1.0920098		LOC642624	642624	XM_926095.1
2120717	down	-1.121327		BMP10	27302	NM_014482.1
2900246	up	1.1266797		TRIOBP	11078	NM_138632.2
1090546	up	1.1745024		PPTC7	160760	NM_139283.1
4860747	up	1.0886035		LOC654342	654342	XM_946374.1
4390195	up	1.1022699		HMGN1	3150	NM_004965.6
2230204	up	1.1496263		OAS2	4939	NM_001032731.1
1470176	up	1.1264374		SYT17	51760	NM_016524.2
6620315	down	-1.132426		COQ6	51004	NM_182476.1
2370301	down	-1.161003		PELI3	246330	NM_145065.1
3800692	up	1.2002835		LOC652683	652683	XM_942283.2
2230059	up	1.1651405		NAT12	122830	NM_001011713.1
770040	up	1.1363423		CBWD7	728013	XM_001717050.1
7510170	down	-1.273866		CD79A	973	NM_021601.3
1110280	down	-1.167376		MAP6	4135	NM_207577.1
1260056	down	-1.127988		LOC643265	643265	XM_926627.1
4730634	up	1.1757776		SLC31A2	1318	NM_001860.2
7330114	up	1.1184865		ZNF366	167465	NM_152625.1
160162	down	-1.156233		LOC652721	652721	XM_942346.2
1820717	down	-1.146642		LOC100129767	100129767	XR_039365.1
2320603	down	-1.199532		FLJ40113	374650	NR_003246.1
3130300	up	1.1497453		NRN1	51299	NM_016588.2
7210209	up	1.0830995		CENTD2	116985	NM_001040118.1
5720086	down	-1.125677	Hs.137466			AL137470
4250184	down	-1.091055		LOC653184	653184	XM_927570.1
6840184	up	1.3923239		GRN	2896	NM_002087.2
5560398	up	1.1731099		DEGS1	8560	NM_003676.2
3120400	up	1.2459776		KANK2	25959	NM_015493.4
2230113	up	1.1942986		TANK	10010	NM_004180.2

down	-1.13842		LOC646198	646198	89059491	LOC646198
up	1.115448		KIAA1571	57683	113414229	KIAA1571
up	1.129676	Hs.578698			28444660	HS.578698
down	-1.051351		CCDC18	343099	62243483	CCDC18
down	-1.096435		TBC1D2	55357	62198224	TBC1D2
down	-1.12665		EPHA1	2041	56119206	EPHA1
down	-1.09942		HS6ST2	90161	116295255	HS6ST2
up	1.068824		YARS	8565	38202242	YARS
down	-1.063068		RNF128	79589	37588872	RNF128
up	1.215158		DCDC2B	149069	150456470	DCDC2B
up	1.171807		LOC652627	652627	169218179	LOC652627
up	1.129679	Hs.98466			15939282	HS.98466
up	1.156498	Hs.187293			11593467	HS.187293
up	1.119788		MSX2	4488	84452153	MSX2
up	1.082557		LOC650851	650851	89042527	LOC650851
up	1.099799		ZC3H12A	80149	13376631	ZC3H12A
down	-1.08052		LOC401357	401357	61966820	LOC401357
down	-1.068555		LOC641846	641846	89027586	LOC641846
down	-1.118542		LOC728728	728728	169162773	LOC728728
up	1.080354		LOC100134420	100134420	169168352	LOC100134420
down	-1.173822		EGFLAM	133584	33469934	EGFLAM
up	1.101918		C2orf42	54980	8923527	C2ORF42
down	-1.120504		RBMY1E	378950	56090526	RBMY1E
up	1.21002		LOC647012	647012	113413206	LOC647012
down	-1.104548		LOC642325	642325	113425388	LOC642325
up	1.073368		LOC644749	644749	89060510	LOC644749
down	-1.17688		LOC387703	387703	169188349	LOC387703
down	-1.10599		LOC100131774	100131774	169169846	LOC100131774
down	-1.075613		LOC649060	649060	89037132	LOC649060
up	1.082465		ZNF713	349075	33438599	ZNF713
up	1.123446		ZNF658	26149	55769536	ZNF658
up	1.092025		MITF	4286	38156696	MITF
down	-1.121361		APP	351	41406053	APP
down	-1.073675		SPP2	6694	54262133	SPP2
up	1.171518		SBDSP	155370	38348442	SBDSP
down	-1.143442		FLJ13224	79857	113423275	FLJ13224
up	1.161505		ATP2B4	493	48255958	ATP2B4
down	-1.065657		LOC392335	392335	169177392	LOC392335
up	1.081459		SNX32	254122	72534837	SNX32
down	-1.136309	Hs.211182			27837068	HS.211182
up	1.064002		LOC100129055	100129055	214831164	LOC100129055

Appendix C: Genes affected by *Sirt1* knockdown in Caco-2

ProbeID	Regulation	Symbol	Entrez_Gene_ID
3930132	up	TUBA3D	113457
5960328	down	LOC644844	644844
150706	up	UGP2	7360
5560747	up	LOC100133803	100133803
770743	down	MIR1978	100302173
10176	up	TAF15	8148
7320722	up	LOC727984	727984
5260035	down	LGALS14	56891
2690561	up	RPLP1	6176
70286	up	LOC441743	441743
6840072	down	PTEN	5728
2760564	up	LOC389322	389322
620047	up	BNIP3L	665
5290220	up	LOC100129599	100129599
1660477	down	CALM1	801
5860689	down	PRODH	5625
830593	up	VIM	7431
7050019	up	VIM	7431
6650603	up	LOC100131905	100131905
7040286	down	ACSL5	51703
5360156	down	IFITM1	8519
3310050	down	GNRH2	2797
780309	down	CIDEC	63924
4880678	down	GNRH2	2797
7100424	down	N-PAC	84656
4570044	up	ODAM	54959
4150014	down	CEACAM1	634
5700753	down	CEACAM1	634
4050070	up	UBE2E1	7324
3060408	up	LOC645231	645231
5960324	up	ARL6IP1	23204
3060477	down	RPL8	6132
3930370	up	LOC100131672	100131672
6960411	down	RCN2	5955
730626	down	LOC285074	285074
2810093	down	LOC285074	285074
2030736	down	SEPHS2	22928
3140521	down	SCARNA14	692149
2190128	down	RAB32	10981
2060674	up	CA2	760
870630	up	CA2	760
240576	down	C5orf28	64417
4670487	down	SIVA	10572
4260397	up	LOC727821	727821
5340129	down	AKR1C2	1646
5490341	up	CLEC2D	29121
290600	up	ZNF738	148203
6250408	up	RPL12P6	440176
5090278	down	GPX2	2877
5360347	down	NQO1	1728

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650519	down	TMBIM6	7009
270609	up	ANXA2P1	303
4640445	up	AIDA	64853
4480128	up	LOC100134273	100134273
990768	down	OAS3	4940
1980176	down	LOC644799	644799
520240	up	SERPINI1	5274
4070037	down	TMED10	10972
6450524	up	LOC653737	653737
3780056	down	GGCT	79017
7400377	down	CEACAM6	4680
3890541	down	ITGA2	3673
2760452	down	RPS29	6235
6370703	down	UGT1A6	54578
2230008	up	FGG	2266
2100196	down	ISG15	9636
5670497	down	LOC644363	644363
6840014	up	TULP3	7289
5130255	up	FGA	2243
2850703	up	FGA	2243
4860463	up	TAF15	8148
4260338	up	LOC647307	647307
6200068	up	CYP51A1	1595
160615	up	GGH	8836
5260176	down	GSTA1	2938
1090561	down	GSTA1	2938
7210259	down	ANAPC1	64682
830039	down	ERGIC1	57222
6290537	up	HRSP12	10247
1660181	down	LOC440145	440145
6520273	up	LOC646527	646527
130228	down	HDHD1A	8226
1690066	down	MX1	4599
3450735	down	UGT1A6	54578
4200152	down	IFRD2	7866
6860593	down		
6760017	up	YY1	7528
5260047	up	FABP5	2171
650685	up	LOC390735	390735
2750670	down	SCARNA18	677765
2340577	down	AQP10	89872
2070437	up	LOC653631	653631
6620368	down	NAT13	80218
4210041	down	UQCRQ	27089
830682	up	LOC387934	387934
3290435	down	TFDP1	7027
6370538	down	WBSCR22	114049
6960735	up	UQCRH	7388
1340192	down	REEP1	65055
1260129	down	TMED10P	286102
3990170	down	IFI27	3429

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3140202	down	MYPOP	339344
2750088	up	LOC730996	730996
2970577	up	LOC730996	730996
2120021	up	COL3A1	1281
5820601	down	CCND1	595
780041	down	LAMP2	3920
3120743	down	LAMP2	3920
4640086	down	FOXQ1	94234
3400215	down	B3GNT1	11041
3170594	down	MAL2	114569
2000148	down	IFIT1	3434
3940358	down	OSBPL8	114882
6770202	down	IPO11	51194
4010086	down	OKL38	29948
2060280	up	LOC646966	646966
3180025	up	FGG	2266
1470435	up	LOC642738	642738
6250672	up	CCNO	10309
5360307	up	LOC644937	644937
1260341	down	IL13RA1	3597
1710142	up	RGS4	5999
7400072	up	FGA	2243
4900343	down	MORF4L1	10933
7000224	down	SIRT1	23411
1240440	down	TXNIP	10628
840554	down	RYBP	23429
620615	down	NDUFA7	4701
4070075	up	OSTCL	202459
1820253	down	PRKRIR	5612
2360594	down	TPT1	7178
4220259	up	CTSZ	1522
110181	up	KIAA1199	57214

Appendix D: Genes affected by dietary restriction

ENSMUSG00000000001	ENSMUSG00000006574	ENSMUSG00000019969
ENSMUSG00000000056	ENSMUSG00000006599	ENSMUSG00000019970
ENSMUSG00000000078	ENSMUSG00000006611	ENSMUSG00000019977
ENSMUSG00000000088	ENSMUSG00000006641	ENSMUSG00000019979
ENSMUSG00000000168	ENSMUSG00000006676	ENSMUSG00000019997
ENSMUSG00000000171	ENSMUSG00000006715	ENSMUSG00000020010
ENSMUSG00000000184	ENSMUSG00000006728	ENSMUSG00000020017
ENSMUSG00000000194	ENSMUSG00000006740	ENSMUSG00000020027
ENSMUSG00000000275	ENSMUSG00000006782	ENSMUSG00000020029
ENSMUSG00000000278	ENSMUSG00000006932	ENSMUSG00000020034
ENSMUSG00000000296	ENSMUSG00000007476	ENSMUSG00000020038
ENSMUSG00000000303	ENSMUSG00000007564	ENSMUSG00000020044
ENSMUSG00000000326	ENSMUSG00000007613	ENSMUSG00000020048
ENSMUSG00000000374	ENSMUSG00000007617	ENSMUSG00000020051
ENSMUSG00000000420	ENSMUSG00000007656	ENSMUSG00000020076
ENSMUSG00000000440	ENSMUSG00000007659	ENSMUSG00000020078
ENSMUSG00000000530	ENSMUSG00000007739	ENSMUSG00000020085
ENSMUSG00000000531	ENSMUSG00000007815	ENSMUSG00000020089
ENSMUSG00000000532	ENSMUSG00000008035	ENSMUSG00000020091
ENSMUSG00000000562	ENSMUSG00000008090	ENSMUSG00000020097
ENSMUSG00000000581	ENSMUSG00000008301	ENSMUSG00000020098
ENSMUSG00000000594	ENSMUSG00000008348	ENSMUSG00000020102
ENSMUSG00000000686	ENSMUSG00000008398	ENSMUSG00000020108
ENSMUSG00000000708	ENSMUSG00000008976	ENSMUSG00000020114
ENSMUSG00000000711	ENSMUSG00000009013	ENSMUSG00000020122
ENSMUSG00000000751	ENSMUSG00000009079	ENSMUSG00000020123
ENSMUSG00000000753	ENSMUSG00000009097	ENSMUSG00000020130
ENSMUSG00000000787	ENSMUSG00000009112	ENSMUSG00000020131
ENSMUSG00000000792	ENSMUSG00000009378	ENSMUSG00000020149
ENSMUSG00000000817	ENSMUSG00000009549	ENSMUSG00000020163
ENSMUSG00000000826	ENSMUSG00000009575	ENSMUSG00000020173
ENSMUSG00000000861	ENSMUSG00000009646	ENSMUSG00000020176
ENSMUSG00000000876	ENSMUSG00000009739	ENSMUSG00000020182
ENSMUSG00000000901	ENSMUSG00000009828	ENSMUSG00000020190
ENSMUSG00000001014	ENSMUSG00000009927	ENSMUSG00000020205
ENSMUSG00000001016	ENSMUSG00000010097	ENSMUSG00000020211
ENSMUSG00000001029	ENSMUSG00000010358	ENSMUSG00000020241
ENSMUSG00000001095	ENSMUSG00000010376	ENSMUSG00000020250
ENSMUSG00000001119	ENSMUSG00000010435	ENSMUSG00000020260
ENSMUSG00000001123	ENSMUSG00000010517	ENSMUSG00000020262
ENSMUSG00000001128	ENSMUSG00000010660	ENSMUSG00000020265
ENSMUSG00000001131	ENSMUSG00000010830	ENSMUSG00000020271
ENSMUSG00000001225	ENSMUSG00000011148	ENSMUSG00000020277
ENSMUSG00000001240	ENSMUSG00000011179	ENSMUSG00000020288
ENSMUSG00000001247	ENSMUSG00000011257	ENSMUSG00000020290
ENSMUSG00000001249	ENSMUSG00000011305	ENSMUSG00000020300
ENSMUSG00000001270	ENSMUSG00000011958	ENSMUSG00000020309
ENSMUSG00000001323	ENSMUSG00000012123	ENSMUSG00000020330
ENSMUSG00000001366	ENSMUSG00000012422	ENSMUSG00000020361
ENSMUSG00000001403	ENSMUSG00000012443	ENSMUSG00000020380
ENSMUSG00000001416	ENSMUSG00000012705	ENSMUSG00000020381
ENSMUSG00000001435	ENSMUSG00000012848	ENSMUSG00000020385
ENSMUSG00000001467	ENSMUSG00000013275	ENSMUSG00000020400
ENSMUSG00000001472	ENSMUSG00000013593	ENSMUSG00000020402

ENSMUSG00000001506	ENSMUSG00000013663	ENSMUSG00000020405
ENSMUSG00000001517	ENSMUSG00000013698	ENSMUSG00000020427
ENSMUSG00000001524	ENSMUSG00000013822	ENSMUSG00000020432
ENSMUSG00000001525	ENSMUSG00000013973	ENSMUSG00000020458
ENSMUSG00000001542	ENSMUSG00000014077	ENSMUSG00000020459
ENSMUSG00000001576	ENSMUSG00000014177	ENSMUSG00000020463
ENSMUSG00000001604	ENSMUSG00000014195	ENSMUSG00000020484
ENSMUSG00000001627	ENSMUSG00000014226	ENSMUSG00000020486
ENSMUSG00000001663	ENSMUSG00000014313	ENSMUSG00000020514
ENSMUSG00000001670	ENSMUSG00000014503	ENSMUSG00000020534
ENSMUSG00000001729	ENSMUSG00000014542	ENSMUSG00000020538
ENSMUSG00000001774	ENSMUSG00000014551	ENSMUSG00000020541
ENSMUSG00000001794	ENSMUSG00000014599	ENSMUSG00000020570
ENSMUSG00000001829	ENSMUSG00000014606	ENSMUSG00000020571
ENSMUSG00000001891	ENSMUSG00000014725	ENSMUSG00000020572
ENSMUSG00000001930	ENSMUSG00000014748	ENSMUSG00000020585
ENSMUSG00000001942	ENSMUSG00000014771	ENSMUSG00000020591
ENSMUSG00000001962	ENSMUSG00000014773	ENSMUSG00000020593
ENSMUSG00000002017	ENSMUSG00000014859	ENSMUSG00000020599
ENSMUSG00000002031	ENSMUSG00000014905	ENSMUSG00000020609
ENSMUSG00000002032	ENSMUSG00000014956	ENSMUSG00000020610
ENSMUSG00000002055	ENSMUSG00000015002	ENSMUSG00000020611
ENSMUSG00000002129	ENSMUSG00000015053	ENSMUSG00000020614
ENSMUSG00000002147	ENSMUSG00000015083	ENSMUSG00000020629
ENSMUSG00000002204	ENSMUSG00000015112	ENSMUSG00000020641
ENSMUSG00000002222	ENSMUSG00000015143	ENSMUSG00000020644
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ENSMUSG00000029178	ENSMUSG00000031446	ENSMUSG00000034484
ENSMUSG00000029188	ENSMUSG00000031453	ENSMUSG00000034486
ENSMUSG00000029191	ENSMUSG00000031458	ENSMUSG00000034528
ENSMUSG00000029198	ENSMUSG00000031461	ENSMUSG00000034563
ENSMUSG00000029217	ENSMUSG00000031482	ENSMUSG00000034566
ENSMUSG00000029229	ENSMUSG00000031489	ENSMUSG00000034570
ENSMUSG00000029231	ENSMUSG00000031490	ENSMUSG00000034579
ENSMUSG00000029234	ENSMUSG00000031502	ENSMUSG00000034584
ENSMUSG00000029238	ENSMUSG00000031520	ENSMUSG00000034614
ENSMUSG00000029247	ENSMUSG00000031529	ENSMUSG00000034620
ENSMUSG00000029249	ENSMUSG00000031536	ENSMUSG00000034647
ENSMUSG00000029253	ENSMUSG00000031537	ENSMUSG00000034648
ENSMUSG00000029267	ENSMUSG00000031538	ENSMUSG00000034659
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ENSMUSG00000029273	ENSMUSG00000031555	ENSMUSG00000034681
ENSMUSG00000029275	ENSMUSG00000031561	ENSMUSG00000034707
ENSMUSG00000029283	ENSMUSG00000031568	ENSMUSG00000034708
ENSMUSG00000029287	ENSMUSG00000031578	ENSMUSG00000034724
ENSMUSG00000029304	ENSMUSG00000031584	ENSMUSG00000034744
ENSMUSG00000029314	ENSMUSG00000031586	ENSMUSG00000034785
ENSMUSG00000029321	ENSMUSG00000031591	ENSMUSG00000034801
ENSMUSG00000029328	ENSMUSG00000031596	ENSMUSG00000034868
ENSMUSG00000029330	ENSMUSG00000031613	ENSMUSG00000034875
ENSMUSG00000029359	ENSMUSG00000031628	ENSMUSG00000034880
ENSMUSG00000029380	ENSMUSG00000031654	ENSMUSG00000034903
ENSMUSG00000029385	ENSMUSG00000031660	ENSMUSG00000034906
ENSMUSG00000029390	ENSMUSG00000031673	ENSMUSG00000034917
ENSMUSG00000029405	ENSMUSG00000031696	ENSMUSG00000034928
ENSMUSG00000029426	ENSMUSG00000031700	ENSMUSG00000034932
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ENSMUSG00000029440	ENSMUSG00000031714	ENSMUSG00000034981
ENSMUSG00000029452	ENSMUSG00000031725	ENSMUSG00000034993
ENSMUSG00000029455	ENSMUSG00000031731	ENSMUSG00000034994
ENSMUSG00000029467	ENSMUSG00000031734	ENSMUSG00000035000
ENSMUSG00000029513	ENSMUSG00000031762	ENSMUSG00000035104
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ENSMUSG00000029545	ENSMUSG00000031767	ENSMUSG00000035133
ENSMUSG00000029550	ENSMUSG00000031770	ENSMUSG00000035150
ENSMUSG00000029571	ENSMUSG00000031774	ENSMUSG00000035212
ENSMUSG00000029575	ENSMUSG00000031778	ENSMUSG00000035237
ENSMUSG00000029580	ENSMUSG00000031782	ENSMUSG00000035248
ENSMUSG00000029581	ENSMUSG00000031790	ENSMUSG00000035268
ENSMUSG00000029597	ENSMUSG00000031799	ENSMUSG00000035351
ENSMUSG00000029608	ENSMUSG00000031808	ENSMUSG00000035372
ENSMUSG00000029616	ENSMUSG00000031811	ENSMUSG00000035373
ENSMUSG00000029617	ENSMUSG00000031819	ENSMUSG00000035376
ENSMUSG00000029629	ENSMUSG00000031838	ENSMUSG00000035383
ENSMUSG00000029630	ENSMUSG00000031840	ENSMUSG00000035385
ENSMUSG00000029635	ENSMUSG00000031844	ENSMUSG00000035443
ENSMUSG00000029650	ENSMUSG00000031851	ENSMUSG00000035451
ENSMUSG00000029651	ENSMUSG00000031858	ENSMUSG00000035458
ENSMUSG00000029656	ENSMUSG00000031880	ENSMUSG00000035469

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ENSMUSG00000029669	ENSMUSG00000031924	ENSMUSG00000035530
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ENSMUSG00000029718	ENSMUSG00000031960	ENSMUSG00000035561
ENSMUSG00000029720	ENSMUSG00000031967	ENSMUSG00000035585
ENSMUSG00000029723	ENSMUSG00000031970	ENSMUSG00000035637
ENSMUSG00000029727	ENSMUSG00000031980	ENSMUSG00000035649
ENSMUSG00000029730	ENSMUSG00000031987	ENSMUSG00000035683
ENSMUSG00000029763	ENSMUSG00000031996	ENSMUSG00000035686
ENSMUSG00000029771	ENSMUSG00000031997	ENSMUSG00000035692
ENSMUSG00000029772	ENSMUSG00000032000	ENSMUSG00000035769
ENSMUSG00000029781	ENSMUSG00000032010	ENSMUSG00000035828
ENSMUSG00000029802	ENSMUSG00000032018	ENSMUSG00000035878
ENSMUSG00000029833	ENSMUSG00000032026	ENSMUSG00000035896
ENSMUSG00000029836|ENSMUSG00000059647	ENSMUSG00000032035	ENSMUSG00000035901
ENSMUSG00000029840	ENSMUSG00000032038	ENSMUSG00000035992
ENSMUSG00000029922	ENSMUSG00000032041	ENSMUSG00000036019
ENSMUSG00000029999	ENSMUSG00000032047	ENSMUSG00000036053
ENSMUSG00000030008	ENSMUSG00000032051	ENSMUSG00000036073
ENSMUSG00000036292	ENSMUSG00000032060	ENSMUSG00000036078
ENSMUSG00000036295	ENSMUSG00000032066	ENSMUSG00000036083
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ENSMUSG00000036241	ENSMUSG00000032076	ENSMUSG00000036120
ENSMUSG00000036199	ENSMUSG00000036144	ENSMUSG00000036123
ENSMUSG00000036216	ENSMUSG00000036181	ENSMUSG00000039994
ENSMUSG00000040006	ENSMUSG00000040562	ENSMUSG00000041020
ENSMUSG00000040010	ENSMUSG00000040565	ENSMUSG00000041028
ENSMUSG00000040093	ENSMUSG00000040584	ENSMUSG00000041058
ENSMUSG00000040127	ENSMUSG00000040600	ENSMUSG00000041119
ENSMUSG00000040158	ENSMUSG00000040652	ENSMUSG00000041132
ENSMUSG00000040170	ENSMUSG00000040659	ENSMUSG00000041220
ENSMUSG00000040181	ENSMUSG00000040660	ENSMUSG00000041261
ENSMUSG00000040204	ENSMUSG00000040666	ENSMUSG00000041263
ENSMUSG00000040213	ENSMUSG00000040694	ENSMUSG00000041333
ENSMUSG00000040242	ENSMUSG00000040701	ENSMUSG00000041378
ENSMUSG00000040249	ENSMUSG00000040706	ENSMUSG00000041390
ENSMUSG00000040282	ENSMUSG00000040715	ENSMUSG00000041417
ENSMUSG00000040325	ENSMUSG00000040746	ENSMUSG00000041426
ENSMUSG00000040328	ENSMUSG00000040759	ENSMUSG00000041431
ENSMUSG00000040413	ENSMUSG00000040843	ENSMUSG00000041459
ENSMUSG00000040446	ENSMUSG00000040857	ENSMUSG00000041483
ENSMUSG00000040447	ENSMUSG00000040891	ENSMUSG00000041488
ENSMUSG00000040479	ENSMUSG00000040935	ENSMUSG00000041540
ENSMUSG00000040505	ENSMUSG00000040940	ENSMUSG00000041559
ENSMUSG00000040521	ENSMUSG00000040963	ENSMUSG00000041567
ENSMUSG00000040548	ENSMUSG00000040997	ENSMUSG00000041571
ENSMUSG00000040550	ENSMUSG00000040998	ENSMUSG00000041625
ENSMUSG00000041733	ENSMUSG00000041773	ENSMUSG00000041638
ENSMUSG00000041750	ENSMUSG00000041774	ENSMUSG00000041654
ENSMUSG00000051113	ENSMUSG00000052837	ENSMUSG00000053931
ENSMUSG00000051236	ENSMUSG00000052974	ENSMUSG00000053935
ENSMUSG00000051314	ENSMUSG00000052997	ENSMUSG00000054072

ENSMUSG00000051391	ENSMUSG00000053044	ENSMUSG00000054128
ENSMUSG00000051469	ENSMUSG00000053093	ENSMUSG00000054206
ENSMUSG00000051483	ENSMUSG00000053113	ENSMUSG00000054280
ENSMUSG00000051592	ENSMUSG00000053175	ENSMUSG00000054309
ENSMUSG00000051671	ENSMUSG00000053205	ENSMUSG00000054364
ENSMUSG00000051768	ENSMUSG00000053279	ENSMUSG00000054422
ENSMUSG00000051802	ENSMUSG00000053289	ENSMUSG00000054435
ENSMUSG00000051817	ENSMUSG00000053302	ENSMUSG00000054545
ENSMUSG00000051864	ENSMUSG00000053303	ENSMUSG00000054619
ENSMUSG00000051910	ENSMUSG00000053317	ENSMUSG00000054717
ENSMUSG00000052040	ENSMUSG00000053329	ENSMUSG00000054757
ENSMUSG00000052102	ENSMUSG00000053460	ENSMUSG00000054889
ENSMUSG00000052117	ENSMUSG00000053477	ENSMUSG00000054942
ENSMUSG00000052146	ENSMUSG00000053483	ENSMUSG00000055053
ENSMUSG00000052151	ENSMUSG00000053510	ENSMUSG00000055065
ENSMUSG00000052160	ENSMUSG00000053559	ENSMUSG00000055116
ENSMUSG00000052293	ENSMUSG00000053581	ENSMUSG00000055128
ENSMUSG00000052392	ENSMUSG00000053600	ENSMUSG00000055148
ENSMUSG00000052516	ENSMUSG00000053644	ENSMUSG00000055172
ENSMUSG00000052520	ENSMUSG00000053654	ENSMUSG00000055296
ENSMUSG00000052566	ENSMUSG00000053768	ENSMUSG00000055301
ENSMUSG00000052593	ENSMUSG00000053799	ENSMUSG00000055401
ENSMUSG00000052684	ENSMUSG00000053819	ENSMUSG00000055436
ENSMUSG00000052712	ENSMUSG00000053846	ENSMUSG00000055491
ENSMUSG00000052751	ENSMUSG00000053862	ENSMUSG00000055531
ENSMUSG00000052798	ENSMUSG00000053870	ENSMUSG00000055612
ENSMUSG00000052819	ENSMUSG00000053898	ENSMUSG00000055675
ENSMUSG00000052833	ENSMUSG00000053916	ENSMUSG00000055692
ENSMUSG00000064023	ENSMUSG00000067847	ENSMUSG00000069919
ENSMUSG00000064145	ENSMUSG00000067889	ENSMUSG00000069922
ENSMUSG00000064215	ENSMUSG00000068086	ENSMUSG00000070436
ENSMUSG00000064225	ENSMUSG00000068205	ENSMUSG00000070561
ENSMUSG00000064254	ENSMUSG00000068220	ENSMUSG00000070702
ENSMUSG00000064294	ENSMUSG00000068328	ENSMUSG00000070730
ENSMUSG00000064326	ENSMUSG00000068329	ENSMUSG00000070953
ENSMUSG00000066026	ENSMUSG00000068335	ENSMUSG00000071177
ENSMUSG00000066036	ENSMUSG00000068391	ENSMUSG00000071369
ENSMUSG00000066072	ENSMUSG00000068614	ENSMUSG00000071650
ENSMUSG00000066149	ENSMUSG00000068742	ENSMUSG00000071655
ENSMUSG00000066232	ENSMUSG00000068874	ENSMUSG00000071713
ENSMUSG00000066324	ENSMUSG00000069020	ENSMUSG00000072082
ENSMUSG00000066516	ENSMUSG00000069089	ENSMUSG00000072235
ENSMUSG00000066637	ENSMUSG00000069456	ENSMUSG00000072620
ENSMUSG00000066860	ENSMUSG00000069515	ENSMUSG00000072849
ENSMUSG00000067279	ENSMUSG00000069601	ENSMUSG00000072949
ENSMUSG00000067283	ENSMUSG00000069662	ENSMUSG00000073057
ENSMUSG00000067338	ENSMUSG00000069668	ENSMUSG00000073126
ENSMUSG00000067653	ENSMUSG00000069743	ENSMUSG00000073418
ENSMUSG00000067713	ENSMUSG00000069899	ENSMUSG00000073421

Appendix E: genes converted into ensemble identifier in Caco-2 and MCF-7 cells

I. genes converted into ensemble identifier in Caco-2 cells

ENSMUSG00000001056	ENSMUSG00000032041
ENSMUSG00000001416	ENSMUSG00000032047
ENSMUSG00000002910	ENSMUSG00000032109
ENSMUSG00000002996	ENSMUSG00000032115
ENSMUSG00000003032	ENSMUSG00000032187
ENSMUSG00000003038	ENSMUSG00000032258
ENSMUSG00000003206	ENSMUSG00000032440
ENSMUSG00000003348	ENSMUSG00000032536
ENSMUSG00000003545	ENSMUSG00000032932
ENSMUSG00000003847	ENSMUSG00000033423
ENSMUSG00000004948	ENSMUSG00000033624
ENSMUSG00000005897	ENSMUSG00000033808
ENSMUSG00000010045	ENSMUSG00000033909
ENSMUSG00000010392	ENSMUSG00000034175
ENSMUSG00000010755	ENSMUSG00000034189
ENSMUSG00000014158	ENSMUSG00000034285
ENSMUSG00000015202	ENSMUSG00000034551
ENSMUSG00000015478	ENSMUSG00000034584
ENSMUSG00000018160	ENSMUSG00000034593
ENSMUSG00000018363	ENSMUSG00000034757
ENSMUSG00000018736	ENSMUSG00000034839
ENSMUSG00000018900	ENSMUSG00000035007
ENSMUSG00000019792	ENSMUSG00000035021
ENSMUSG00000019838	ENSMUSG00000035248
ENSMUSG00000020018	ENSMUSG00000035262
ENSMUSG00000020115	ENSMUSG00000035392
ENSMUSG00000020260	ENSMUSG00000035671
ENSMUSG00000020262	ENSMUSG00000035704
ENSMUSG00000020431	ENSMUSG00000035900
ENSMUSG00000020454	ENSMUSG00000036587
ENSMUSG00000020515	ENSMUSG00000036781
ENSMUSG00000020516	ENSMUSG00000036986
ENSMUSG00000020532	ENSMUSG00000036989
ENSMUSG00000020834	ENSMUSG00000037112
ENSMUSG00000020923	ENSMUSG00000037465
ENSMUSG00000021111	ENSMUSG00000037487
ENSMUSG00000021175	ENSMUSG00000037513
ENSMUSG00000021337	ENSMUSG00000037601
ENSMUSG00000021453	ENSMUSG00000037773
ENSMUSG00000021690	ENSMUSG00000037999
ENSMUSG00000022037	ENSMUSG00000038147
ENSMUSG00000022307	ENSMUSG00000038400
ENSMUSG00000022313	ENSMUSG00000038564
ENSMUSG00000022332	ENSMUSG00000038637
ENSMUSG00000022339	ENSMUSG00000038641
ENSMUSG00000022415	ENSMUSG00000038685
ENSMUSG00000022540	ENSMUSG00000039067
ENSMUSG00000022568	ENSMUSG00000039219
ENSMUSG00000022721	ENSMUSG00000039476
ENSMUSG00000022822	ENSMUSG00000039976

ENSMUSG00000022898	ENSMUSG00000040383
ENSMUSG00000022957	ENSMUSG00000040429
ENSMUSG00000023075	ENSMUSG00000040618
ENSMUSG00000023236	ENSMUSG00000040747
ENSMUSG00000023832	ENSMUSG00000041058
ENSMUSG00000024039	ENSMUSG00000041632
ENSMUSG00000024222	ENSMUSG00000041649
ENSMUSG00000024327	ENSMUSG00000041650
ENSMUSG00000024366	ENSMUSG00000042035
ENSMUSG00000024487	ENSMUSG00000042328
ENSMUSG00000024682	ENSMUSG00000042406
ENSMUSG00000024713	ENSMUSG00000042590
ENSMUSG00000024922	ENSMUSG00000042694
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ENSMUSG00000025035	ENSMUSG00000042997
ENSMUSG00000025049	ENSMUSG00000043430
ENSMUSG00000025129	ENSMUSG00000043753
ENSMUSG00000025162	ENSMUSG00000045095
ENSMUSG00000025245	ENSMUSG00000045160
ENSMUSG00000025324	ENSMUSG00000045316
ENSMUSG00000025395	ENSMUSG00000045691
ENSMUSG00000025579	ENSMUSG00000045954
ENSMUSG00000025958	ENSMUSG00000046020
ENSMUSG00000025969	ENSMUSG00000046314
ENSMUSG00000026166	ENSMUSG00000046814
ENSMUSG00000026260	ENSMUSG00000046822
ENSMUSG00000026399	ENSMUSG00000047205
ENSMUSG00000026730	ENSMUSG00000047562
ENSMUSG00000026810	ENSMUSG00000047712
ENSMUSG00000027120	ENSMUSG00000047757
ENSMUSG00000027176	ENSMUSG00000048373
ENSMUSG00000027353	ENSMUSG00000048826
ENSMUSG00000027803	ENSMUSG00000048970
ENSMUSG00000028016	ENSMUSG00000050213
ENSMUSG00000028099	ENSMUSG00000050217
ENSMUSG00000028238	ENSMUSG00000050288
ENSMUSG00000028252	ENSMUSG00000050786
ENSMUSG00000028277	ENSMUSG00000050796
ENSMUSG00000028298	ENSMUSG00000051786
ENSMUSG00000028454	ENSMUSG00000053279
ENSMUSG00000028525	ENSMUSG00000053334
ENSMUSG00000028532	ENSMUSG00000054065
ENSMUSG00000028633	ENSMUSG00000054083
ENSMUSG00000028687	ENSMUSG00000054871
ENSMUSG00000028756	ENSMUSG00000055125
ENSMUSG00000028776	ENSMUSG00000055491
ENSMUSG00000028822	ENSMUSG00000055629
ENSMUSG00000028967	ENSMUSG00000056130
ENSMUSG00000029068	ENSMUSG00000058586
ENSMUSG00000029369	ENSMUSG00000060445
ENSMUSG00000029477	ENSMUSG00000060726
ENSMUSG00000029478	ENSMUSG00000060962

ENSMUSG00000029504	ENSMUSG00000061244
ENSMUSG00000029670	ENSMUSG00000061482
ENSMUSG00000029998	ENSMUSG00000061887
ENSMUSG00000030007	ENSMUSG00000063229
ENSMUSG00000030079	ENSMUSG00000063268
ENSMUSG00000030218	ENSMUSG00000063785
ENSMUSG00000030309	ENSMUSG00000066026
ENSMUSG00000030337	ENSMUSG00000066148
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ENSMUSG00000030726	ENSMUSG00000071757
ENSMUSG00000030772	ENSMUSG00000071847
ENSMUSG00000030935	ENSMUSG00000072082
ENSMUSG00000031107	ENSMUSG00000072964
ENSMUSG00000031197	ENSMUSG00000074909
ENSMUSG00000031216	ENSMUSG00000021469
ENSMUSG00000031217	ENSMUSG00000022892
ENSMUSG00000031298	ENSMUSG00000026295
ENSMUSG00000031309	ENSMUSG00000026463
ENSMUSG00000031433	ENSMUSG00000028811
ENSMUSG00000031633	ENSMUSG00000029859
ENSMUSG00000031950	ENSMUSG00000031438
ENSMUSG00000042961	ENSMUSG00000035158
ENSMUSG00000056531	ENSMUSG00000039813
ENSMUSG00000062184	ENSMUSG00000042677

II. genes converted into ensemble identifier in MCF-7 cells

ENSMUSG00000000378	ENSMUSG00000038633
ENSMUSG00000001417	ENSMUSG00000038702
ENSMUSG00000003379	ENSMUSG00000038736
ENSMUSG00000004069	ENSMUSG00000039086
ENSMUSG00000004328	ENSMUSG00000039114
ENSMUSG00000004626	ENSMUSG00000039347
ENSMUSG00000006850	ENSMUSG00000039396
ENSMUSG00000007646	ENSMUSG00000039501
ENSMUSG00000014602	ENSMUSG00000040681
ENSMUSG00000017831	ENSMUSG00000040701
ENSMUSG00000018882	ENSMUSG00000040855
ENSMUSG00000019831	ENSMUSG00000041731
ENSMUSG00000019889	ENSMUSG00000041890
ENSMUSG00000020241	ENSMUSG00000041995
ENSMUSG00000020534	ENSMUSG00000042229
ENSMUSG00000021203	ENSMUSG00000042305
ENSMUSG00000021235	ENSMUSG00000045294
ENSMUSG00000021286	ENSMUSG00000047719
ENSMUSG00000021519	ENSMUSG00000047875
ENSMUSG00000021597	ENSMUSG00000048647
ENSMUSG00000021948	ENSMUSG00000049470
ENSMUSG00000021953	ENSMUSG00000049536
ENSMUSG00000022015	ENSMUSG00000049960
ENSMUSG00000022464	ENSMUSG00000050640
ENSMUSG00000022545	ENSMUSG00000051367
ENSMUSG00000022564	ENSMUSG00000053916
ENSMUSG00000022607	ENSMUSG00000054000
ENSMUSG00000022797	ENSMUSG00000055053
ENSMUSG00000022887	ENSMUSG00000055078
ENSMUSG00000022962	ENSMUSG00000056529
ENSMUSG00000023972	ENSMUSG00000056917
ENSMUSG00000024112	ENSMUSG00000057406
ENSMUSG00000024145	ENSMUSG00000058420
ENSMUSG00000024187	ENSMUSG00000059208
ENSMUSG00000024614	ENSMUSG00000060126
ENSMUSG00000024831	ENSMUSG00000061080
ENSMUSG00000024901	ENSMUSG00000062867
ENSMUSG00000024993	ENSMUSG00000063972
ENSMUSG00000025047	ENSMUSG00000064289
ENSMUSG00000025723	ENSMUSG00000066152
ENSMUSG00000026174	ENSMUSG00000070034
ENSMUSG00000026196	ENSMUSG00000021469
ENSMUSG00000026203	ENSMUSG00000022892
ENSMUSG00000026394	ENSMUSG00000026295
ENSMUSG00000026694	ENSMUSG00000026463
ENSMUSG00000026873	ENSMUSG00000028811
ENSMUSG00000026934	ENSMUSG00000029859
ENSMUSG00000027261	ENSMUSG00000031438
ENSMUSG00000027333	ENSMUSG00000035158
ENSMUSG00000027852	ENSMUSG00000039813

ENSMUSG00000028037	ENSMUSG00000042677
ENSMUSG00000028039	ENSMUSG00000042961
ENSMUSG00000028149	ENSMUSG00000056531
ENSMUSG00000028309	ENSMUSG00000062184
ENSMUSG00000028332	ENSMUSG00000032724
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ENSMUSG00000028847	ENSMUSG00000033377
ENSMUSG00000029064	ENSMUSG00000033405
ENSMUSG00000029198	ENSMUSG00000033623
ENSMUSG00000029310	ENSMUSG00000033813
ENSMUSG00000029428	ENSMUSG00000034192
ENSMUSG00000029554	ENSMUSG00000034308
ENSMUSG00000029822	ENSMUSG00000034675
ENSMUSG00000029851	ENSMUSG00000034708
ENSMUSG00000030046	ENSMUSG00000034783
ENSMUSG00000030452	ENSMUSG00000034998
ENSMUSG00000030509	ENSMUSG00000035890
ENSMUSG00000030742	ENSMUSG00000036315
ENSMUSG00000030824	ENSMUSG00000037185
ENSMUSG00000031384	ENSMUSG00000038145
ENSMUSG00000031527	ENSMUSG00000038582
ENSMUSG00000032194	ENSMUSG00000032459
ENSMUSG00000032202	ENSMUSG00000032525
ENSMUSG00000032254	ENSMUSG00000032567
ENSMUSG00000032329	ENSMUSG00000032690