EXPLORING THE EFFECT OF CHRONIC ASCORBIC ACID DEFICIENCY IN THE MMTV-NEU MOUSE MODEL OF HER2-POSTIVE BREAST CANCER

EXPLORING THE EFFECT OF CHRONIC ASCORBIC ACID DEFICIENCY IN THE MMTV-NEU MOUSE MODEL OF HER2-POSTIVE BREAST CANCER

BY

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

McMaster University

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October 25, 2016

MASTER OF SCIENCE (2016) McMaster University

BIOLOGY Hamilton, Ontario

TITLE: Exploring the effect of chronic ascorbic acid deficiency in the MMTV-Neu mouse model of Her-2 positive breast cancer

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Number of Pages: x, 92

Lay Abstract

Vitamin C is an antioxidant that humans must include in their diet to prevent the disease scurvy. Taking a high level of vitamin C may reduce the incidence or severity of some types of cancer. In certain cases, very high doses of vitamin C injected intravenously can be used as an alternative cancer treatment. To date, most studies of the role of vitamin C in cancer have been performed using laboratory animals, like mice, that naturally make vitamin C within their own body, which humans cannot do. In this thesis, we describe the development and use of a genetically engineered mouse (GULO MMTV-Neu) that does not produce vitamin C to investigate the role of long-term vitamin C insufficiency on breast cancer development and growth. We maintained the GULO MMTV-Neu mouse on normal rodent chow supplemented with either (1) low amounts of vitamin C or (2) normal amounts of vitamin C in their drinking water. If vitamin C had an effect on breast cancer development or progression, we expected that vitamin C supplementation would delay tumour formation or slow tumour growth. We found that there was no difference in the age at tumour development, number of tumours per mouse or average size of tumours between the mice that received low-dose versus normal supplementation. The results of this study suggest that chronic deficiency of vitamin C may not have an effect in one specific type of breast cancer. This study can be replicated in other mouse models of cancer to provide more insight into the effectiveness of using vitamin C in the prevention and treatment of cancer,

Abstract

Vitamin C (VC) is an essential nutrient in humans mostly commonly known for its role in the prevention of scurvy. VC is an antioxidant that quenches free radicals naturally generated in the body by respiration and metabolism. The failure to maintain a balance between the production and removal of reactive oxygen species (ROS) may play a role in the pathogenesis of degenerative diseases like cancer and cardiovascular disease. Because of its ability to prevent the oxidation of biological molecules, VC may play a protective role in preventing oxidative damage to cells and tissues. The role of VC in cancer is highly debated, with evidence that VC has anti-tumorigenic effects on cancer cells in a HIF-dependent manner, yet meta-analyses of VC in human cancer have not shown any significant effect of VC intake on cancer incidence or progression. Transgenic mouse models have provided some insight into the role of VC in cancer, however, the continued use of VC-synthesizing animal models complicates the generalization of results to humans who cannot synthesize VC.

This thesis presents the development and testing of a novel animal model of human HER2-positive breast cancer. Unlike previous models, our bitransgenic mouse is both VC-deficient and spontaneously develops mammary adenocarcinomas with HER2 overexpression as the driving factor. In Experiment 1 we introgressed the GULO knockout allele into the FVB/N mouse background and characterized the FVB/N GULO knockout mouse. Subsequently, in Experiment 2, we generated the bitransgenic FVB/N MMTV-Neu GULO knockout mouse as a vitamin C-deficient model of HER2-positive breast cancer. We maintained a control group of MMTV-Neu GULO knockout mouse on

normal VC supplementation and a treatment group on low-dose VC supplementation. We then characterized the effect of chronic VC deficiency on the latency to palpable tumours, the number of tumours per mouse and the size of tumours. We found that there was no difference in average latency to palpable tumours, the number of palpable tumours or the size of each tumour between the control and treatment groups. These results suggest that there is no effect of chronic VC-deficiency on tumorigenesis in HER2-positive breast cancer. This study can be replicated in other mouse models of cancer to provide further insight into the role of VC in disease etiology and progression.

Acknowledgements

I would like to thank Dr. Schellhorn for the guidance, supervision and patience he has afforded me throughout my undergraduate and graduate degree. I would also like to thank Dr. Igdoura for his contributions to the VC mouse project.

Thank you to Chantel, Dallas, Chris and Julia for all of the study and writing sessions at 1516. Thank you also to Kasia and Alex for their support and encouragement.

A special thanks to Cathy for all of her hard work on this project. Your assistance was much appreciated. Thank you to the other members of the Schellhorn lab for their feedback and guidance. Lastly, thank you Rosemary Yu and Daniel Li for your work that lay the foundations for this project.

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Abbreviations

AA	Ascorbic Acid
DAD	Diode Array Detector
DHA	Dehydroascorbic acid
ECD	Electrochemical Detection
GLUT	Sodium-independent vitamin C transporters
HIF	Hypoxia Indicible Factor
HPLC	High-performance Liquid Chromatography
MDA	Malondialdehyde
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SE	Standard Error
SVCT	Sodium-dependent vitamin C transporters
TBARS	Thiobarbituic acid reactive substance
VC	Vitamin C

Chapter 1: Vitamin C in cancer

1.1 Introduction

The generation of Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS) and other free radicals occurs as a byproduct of anaerobic respiration and increases during times of oxidative stress (Halliwell and Gutteridge, 2007). Elevated levels of these reactive species cause oxidative damage to essential macromolecules including proteins, lipids and nucleic acids thus damaging cells and tissues. These highly reactive species have been implicated in aging as well as in the etiology of chronic illnesses like atherosclerosis, cancer and neurodegenerative disease (Halliwell and Gutteridge, 2007). Animals, including humans, have numerous antioxidant defenses that can quench free radicals and lessen the extent of oxidative damage. Determining the antioxidant status of an organism, or its balance of antioxidants and proxidants, is crucial to understanding the pathogenesis of the many chronic diseases linked to oxidative stress.

Laboratory animal models are used extensively to study the genetic basis, etiology and manifestation of symptoms of human disease. Rodents are some of the most commonly used models due to their rapid generation times, ease to maintain and the developments in genetic manipulation. The use of rodents allows researchers to model the complex pathways and processes that interact to manifest human disease. In many aspects, mice mirror human genetics and physiology remarkably well with only approximately 300 genes being unique to one species or the other (Mouse Genome Sequencing et al., 2002). Despite the similarities between the species, there are

biological, physiological and immunological differences that must be taken into account when modeling human disease in rodents. As the preventative measures and treatments of human disease become more sophisticated and target-specific, the genetic and physiological discrepancies between mice and humans must be resolved.

One important difference between rodents and humans regarding antioxidant status is the ability to endogenously synthesize vitamin C (VC). VC (ascorbic acid) is an essential nutrient in the human diet and plays several physiological roles in the body. Chronic deficiency in vitamin C leads to scurvy with symptoms including lethargy, bruising, bleeding gums, deficiencies in wound healing, generalized edema and death. Intravenous administration of high doses of vitamin C is used as an alternative therapy for cancer, cardiovascular disease and degenerative disease. Unlike most mammals, humans have lost the ability to synthesize VC due to a series of inactivating mutations in the gene coding for ι-GULOno-γ-lactone oxidase (*GULO*) (Nishikimi et al., 1994). The recent development of the *GULO* knockout mouse (Maeda et al., 2000) has generated the means by which other mouse models can be modified to better represent the redox systems of humans (Arrigoni and De Tullio, 2002; Li and Schellhorn, 2007a; Maeda et al., 2000).

In this project, we investigate the manipulation of animal models to better mimic the antioxidant status of humans. My project focuses on two aspects: (1) introgression of the mutant GULO allele into the FVB background and characterization of the FVB GULO knockout mouse; and (2) determination of the effect of chronic VC deficiency on the Her2-positive breast cancer phenotype.

1.2 Vitamin C

Vitamin C (VC) is a water-soluble antioxidant that is best known for its antiscorbutic properties. While scurvy has been described since ancient times (Fain, 2005) the recognition that the disease is the result of a deficient diet can be a considered a relatively recent development. The first evidence that the disease may be diet-related was obtained in 1753 when James Lind, a surgeon in the Royal Navy, conducted a series of experiments on sailors that proved scorbutic symptoms could be alleviated with the ingestion of citrus fruits (Lind, 1980; Packer, 2002). Over a century later, Axel Holst and Theodor Frolich reported that scurvy could be experimentally induced in guinea pigs fed a diet deficient in fresh fruits and vegetables (Packer, 2002). The antiscorbutic factor contained in these foods was isolated and named “hexuronic acid” by Albert Szent-Györgyi in the late 1920s (Davies et al., 1991). In 1933, Edmund Hirst and Norman Haworth determined the chemical structure and synthesized hexuronic acid, which had since been renamed ascorbic acid in recognition of its antiscorbutic properties (Packer, 2002). In 1937, Szent-Gyorgi and Haworth were awarded the Nobel Prize in Physiology or Medicine and Chemistry, respectively, for their work in isolating and synthesizing vitamin C (Davies et al., 1991).

Today the term vitamin C refers collectively to ascorbic acid (AA) and its reduced form dehydroascorbic acid (DHA). VC is a water-soluble vitamin that quenches free radicals generated by cellular respiration. VC donates a single electron to radical species, generating the ascorbyl radical semidehydroascorbate. The ascorbyl radical is an inert single-electron radical that can be excreted in the urine, or further oxidized to DHA.

1.2.1 Physiological roles of VC

At physiological pH, VC is an important antioxidant that acts as a reducing agent to quench dangerous free radicals that are naturally produced through cellular metabolism and mitochondrial respiration (Arrigoni and De Tullio, 2002). VC in the form of ascorbic acid can donate two electrons (Padayatty et al., 2003). The loss of one radical forms the relatively stable free radical semidehydroascorbic acid, which can reduce another molecule to become dehydroascorbic acid (DHA). Both semidehydroascorbic acid and DHA can be reduced back to ascorbic acid through several enzymatic pathways or other reducing agents (Padayatty et al., 2004). VC scavenges both ROS and NOS and can regenerate vitamin E, urate, glutathione and β-carotene *in vitro* (Halliwell, 1996).

VC is involved in the metabolism of iron and folate. VC promotes the absorption of iron in the small intestine and the mobilization of iron from bodily stores (Lee et al., 1967). VC is also required for the biosynthesis of collagen, which is an essential component of connective tissue (Geesin et al., 1988). Deficiencies in collagen biosynthesis results in insufficiencies in wound healing. As a cofactor in several enzymatic reactions VC is involved in a range of processes including tyrosine metabolism, the conversion of cholesterol to bile acids, and the synthesis of norepinephrine (Ausman, 1999; Ginter, 1975; La Du and Zannoni, 1961).

1.2.2 Biochemistry, Bioavailability, Metabolism and Intake

Most terrestrial vertebrates synthesize VC endogenously in the liver through the glucuronic acid pathway and are not significantly affected by exogenous VC (Maeda et al., 2000). Humans, primates, bats and guinea pigs are unable to synthesize AA and rely on dietary intake (Nishikimi et al., 1994). This inability to synthesize the six-carbon lactone endogenously is the result of a series of inactivating mutations within the gene coding for ι-GULOno-γ-lactone oxidase (*GULO*), which occurred approximately 40 million years ago (Nishikimi et al., 1994). *GULO* is the enzyme that catalyzes the terminal step in AA synthesis, which is the oxidation of L-GULOnolactone to L-2-ketoGULOnolactone, which spontaneously isomerizes to form VC (Nishikimi et al., 1994). In mammals that lack functional *GULO*, the precursor L-GULOnolactone is decarboxylated to form xylulose that can be shunted into the pentose phosphate pathway of carbohydrate metabolism (Dayton et al., 1959). Without adequate dietary intake of VC, humans develop scurvy with symptoms including weight loss, anemia, muscle weakness, damage to the cardiovasculature and eventually death (Williams and Deason, 1967).

Dietary vitamin C consists of both ascorbic acid and its oxidized form dehydroascorbic acid (DHA). Plasma AA concentrations are tightly regulated in humans through intestinal absorption and renal excretion (Li and Schellhorn, 2007b). Two of the major classes of transporters that dictate tissue-specific VC levels are the sodium-independent facilitative glucose transporters (GLUT) and sodium-dependent vitamin C cotransporters (SVCT) (Rumsey et al., 1997). While GLUT transporters play an

important role in facilitated diffusion of the oxidized form of VC, DHA, SVCT1 is considered the bulk transporter of the vitamin (Rumsey et al., 1997). Both of the SVCT isoforms are down regulated by high concentrations of AA, which limits the maximum achievable plasma concentrations of AA through oral ingestion to 70 to 85 μmol/L (MacDonald et al., 2002; Padayatty et al., 2004).

At moderate intakes of 30-180mg/day, approximately 70-90% of VC is absorbed. At doses higher than 1g/day, the efficiency of VC absorption falls below 50%, due to saturated transport mechanisms, and most VC is excreted in the urine (Jacob and Sotoudeh, 2002). The dose-concentration relationship is sigmoidal, with the steep portion of the curve falling between 30-100mg/day (Levine et al., 1996). Intravenous administration of AA bypasses the limiting absorptive mechanisms and can achieve pharmacological blood plasma concentrations up to 15 000μmol/L (Padayatty et al., 2004). Figure 1, below, displays the typical tissue and fluid distribution of VC. High levels of VC are maintained in the eye, adrenal glands, pituitary glands and brain (Hornig, 1975; Shils and Shike, 2006). Lower levels of VC are maintained in the extracellular fluids such as the plasma and saliva (Hornig, 1975; Shils and Shike, 2006). The total pool of VC in the body is 1500-2500mg with a daily turnover of approximately 3% of total body stores (Baker et al., 1971). Clinical signs of scurvy begin to manifest when VC intake is chronically below 10mg/day or body stores fall below 300mg (1948)

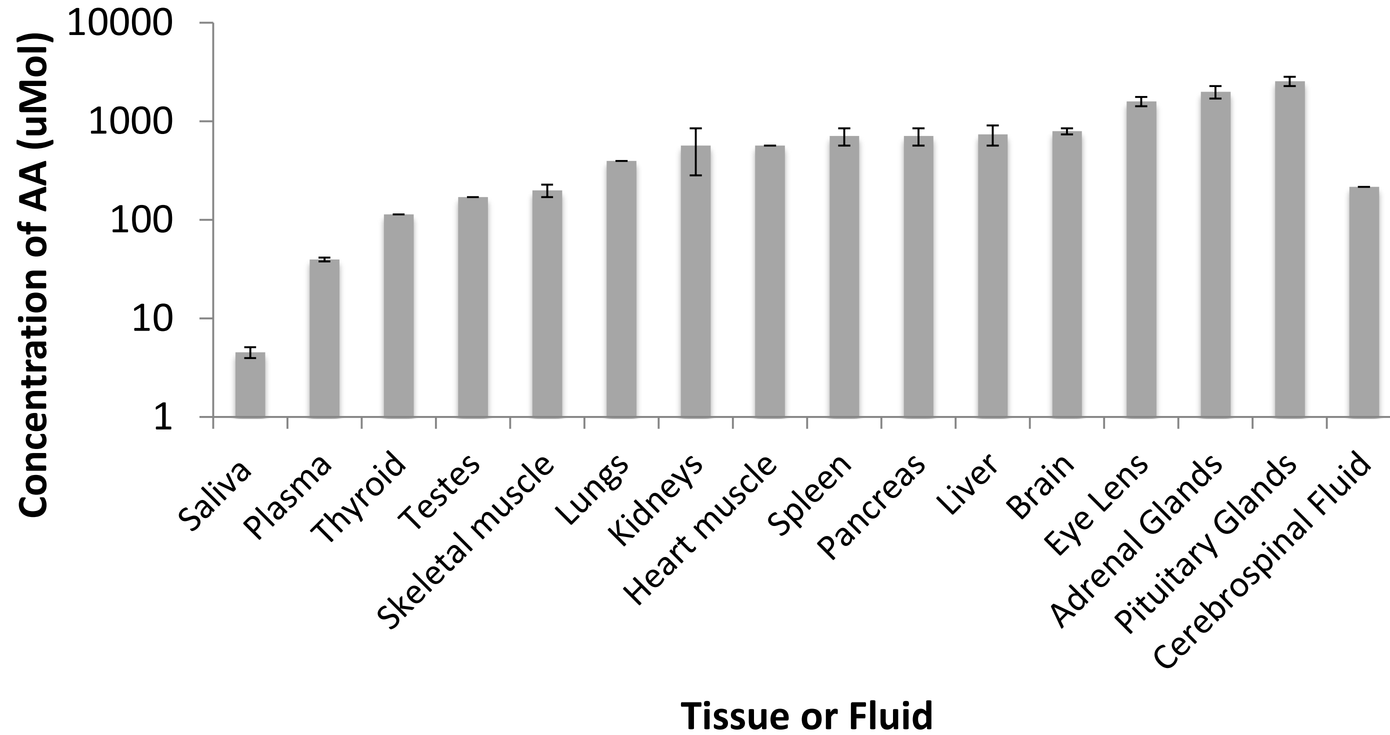

Figure 1: Human tissue and fluid distribution of AA.

Error bars indicate normal range of concentration values. Data from (Shils and Shike, 2006) and (Hornig, 1975).

The Recommended Daily Allowance (RDA) of vitamin C varies significantly between health agencies. The United States Food and Drug Administration (FDA) RDA for VC are 90mg/day for men and 75mg/day for women. The Estimated Average Requirement (EAR) for VC are even lower at 75mg/day and 60mg/day for men and women, respectively (Monsen, 2000a). The RDA denotes the intake at which the risk of inadequacy is 2-3% while the EAR represents an increased risk of 50%.

VC is primarily found in fresh fruits and vegetables, with particularly high concentrations in citrus fruits and leafy green vegetables (Sinha et al., 1993). A 250mL serving of many common fruits and vegetables, see Table 1 below, contains over 100mg of VC, which is above the RDA (Verdier and Beare-Rogers, 1984). Cooking processes such as boiling, microwaving, and frying inactivates VC and can drastically reduce the content of prepared food (Yuan et al., 2009).

Table 1: Average Vitamin C content of a selection of common fruits and vegetables (per 1 cup or 250mL serving) (Verdier and Beare-Rogers, 1984)

Food, preparation	**Vitamin C (mg)**
Sweet red peppers, raw	202-288
Broccoli, cooked	108
Cabbage, cooked	108
Kiwi, raw	168
Orange, raw	108-166

AA is considered to be non-toxic as it has few side effects and usually only at very high doses (above 3g/day) (Johnston, 1999). AA absorption in the small intestine and reabsorption in the kidney are saturable processes, thus preventing excessive buildup in the body. Doses above the tolerable upper limit of 2g/day can lead to diarrhea, bloating and indigestion due to the osmotic effect of unabsorbed AA passing through the gastrointestinal tract (Monsen, 2000b). As AA can increase iron absorption in the small intestine, chronic ingestion of high doses of vitamin C can lead to iron overload in at-risk populations, including those with hemochromatosis, but not in healthy patients. Doses of AA higher than 1g/day may increase oxalate excretion and facilitate the formation of calcium oxalate kidney stones in patients with kidney disease or renal failure (Auer et al., 1998; Lamarche et al., 2011; Ono, 1986).

1.2.3 Populations at-risk for inadequacy

The median intake of VC for Canadians is estimated to be between 120 and 133mg/day, which is well above the RDA (Canada, 2004). Populations at risk of developing scurvy include those with poor nutrition including the impoverished, homeless and elderly. Although scurvy is relatively rare in Western cultures, no subset of the Canadian population is exempt. In a study of young Canadian adults (ages 20-29), almost 50% of participants were marginally to severely deficient in VC with 14% having deficient serum concentrations of VC and 33% of the subjects having suboptimal serum levels of VC (Cahill et al., 2009). Serum VC concentrations are considered adequate if above 28umol, suboptimal if between 11-28umol and deficient if lower than 11umol

(Cahill et al., 2009). Figure 2, below, displays the classification of serum VC and their potential effects on human health.

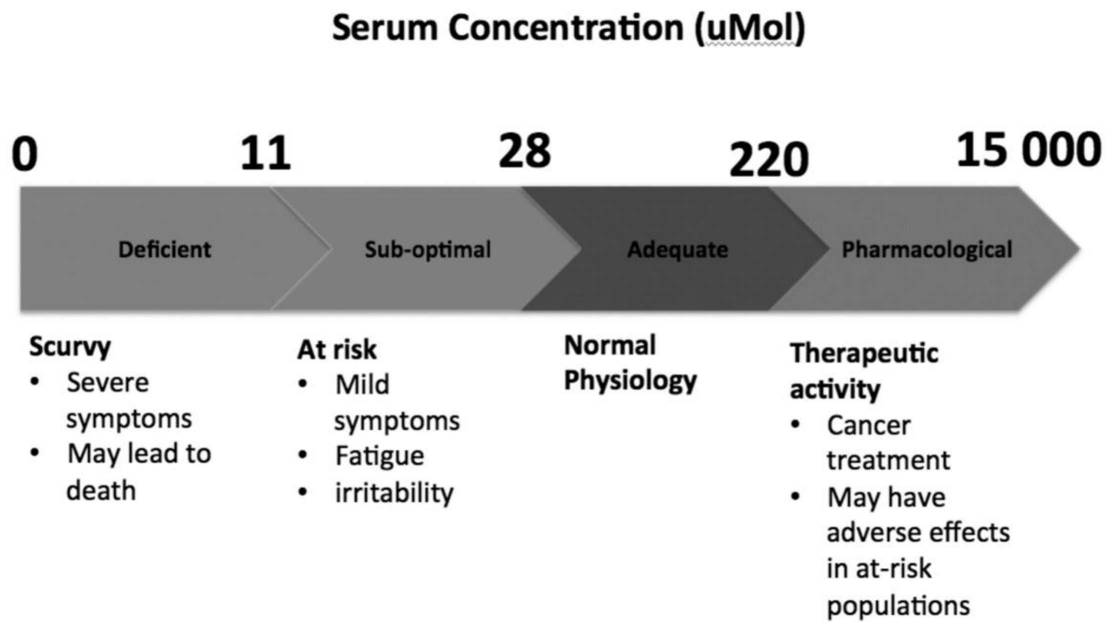

Figure 2: The classifications of serum levels of AA and the associated potential effects on health

While dietary VC is the main determinant of tissue and fluid concentrations, other factors that affect VC status include age, gender, smoking, body weight, activity level, season, dietary iron and serum lipids (Block et al., 1999; Cahill et al., 2009; Galan et al., 2005; Jacob, 1990; Lynch and Cook, 1980). A significantly at-risk population for VC deficiency is tobacco smokers. Smoking decreases the intestinal absorption of VC and increases the rate at which VC is catabolized. Smoking more than 20 cigarettes per day is associated with a 40% increase in the turnover of VC compared to non-smokers, which may contribute to the low serum VC levels reported in smokers (Kallner et al., 1981). Smokers also report a lower intake of VC compared to non-smokers, and are less likely to use supplements (Palaniappan et al., 2001; Subar et al., 1990). Only 36.2% and 39.2% of Canadian male and female smokers, respectively, meet the RDA for VC compared to 72.7% and 77.2% of non-smokers (Palaniappan et al., 2001).

Pregnancy denotes another population of those at-risk for VC inadequacy. Plasma VC concentrations are lower in pregnant women compared to non-pregnant women (Mikhail et al., 1994). Plasma VC concentrations also progressively decrease from first to third trimester (Kalaiselvi et al., 2014). During pregnancy, oxygen consumption and metabolism increase in response to increased energy demands. Increased ROS production also occurs, which depletes body stores of VC because of increased utilization of antioxidants to maintain oxidative homeostasis (Kalaiselvi et al., 2014). Other factors that may contribute to decreased plasma VC concentrations during pregnancy include hemodilution, active transfer across the placenta to the fetus and increased renal loss (Dayton et al., 1959). Loss of VC through the breast milk during lactation extends VC-

deficiency postpartum. VC deficiency during pregnancy is associated with an increased risk of infection, premature delivery, gestational hypertension, intrauterine growth retardation and pre-eclampsia (Mikhail et al., 1994). To prevent the adverse effects of VC deficiency, the RDA for VC increases to 85mg/day and 120mg/day during pregnancy and breastfeeding, respectively (Monsen, 2000b).

Therapeutic Significance

Oxidative damage has been implicated in many chronic diseases such as cardiovascular disease, cancer and neurodegenerative disease. Due to its role in protecting the body from oxidative stress and DNA damage by quenching reactive oxygen species (ROS), pharmacological doses of VC may have a therapeutic role in these age-related degenerative diseases (Gao et al., 2007; Li and Schellhorn, 2007a).

Cancer

Several studies have associated dietary antioxidants (i.e. tocopherol, ascorbate and β-carotene) with reduced incidence of cancer in humans (Block, 1991; Byers and Perry, 1992; Enstrom et al., 1992). In rodents, antioxidants have been seen to counteract the induction of cancer by several carcinogens including cigarette smoke (Daoud and Griffin, 1980; Kushida et al., 1992; Leuchtenberger and Leuchtenberger, 1977). VC and other antioxidants have been shown to have various anti- tumorigenic effects *in vivo* although the mechanisms by which this occurs is under debate (Gao et al., 2007) Oxidants are one class of agents that fuel cellular proliferation in cancer thus antioxidants can reduce mutagenesis both by decreasing cell division stimulated oxidants as well as decreasing potentially mutative oxidative damage to DNA (Ames et al., 1993). Extracellular

concentrations of VC greater than 1000μmol/L induce hydrogen peroxide-dependent cytotoxicity in malignant cells while treatment with 1-2 mM of AA was seen *in vitro* to have pro-apoptotic effects on several cancer lines (Fromberg et al., 2011; Padayatty et al., 2004). VC is also associated with decreased cell proliferation by inducing G_0/G_1 arrest, which inhibits progression of the cell cycle (Fromberg et al., 2011). There is also evidence that pre-treatment with VC may increase the efficacy of other cancer treatments including cytostatics (Fromberg et al., 2011)

AA can lower the basal invasiveness in some tumors by regulating the Hypoxia Inducible Factor (HIF)-1 signal transduction pathway (Lu et al., 2005). HIF-1 is a heterodimeric transcription factor that plays a role in adaptation to hypoxia and can stimulate angiogenesis and promote survival in hypoxic tumor cells (Lu et al., 2005). Under normoxic conditions, HIF-1α is hydroxylated at conserved proline residues by HIF-prolyl hydroxylases (HPHs) and is degraded in an ubiquitin-dependent manner by the proteasome (Gao et al., 2007; Lu et al., 2005). Under hypoxic conditions, or in the presence of ROS or nitric oxide (NO), HPHs are inactivated and HIF-1α is not degraded and accumulates in the cell where it dimerizes with HIF-1β (Lu et al., 2005; Pugh and Ratcliffe, 2003). The HIF dimer recruits the co-activator p300 and translocates to the nucleus where it activates the expression of HIF-mediated genes that promote angiogenesis, erythropoiesis and tumorigenesis (Lu et al., 2005; Yasinska and Sumbayev, 2003) AA regulates the HIF-1 signal transduction pathway by reversing HPH inactivation by hypoxia, ROS or NO which promotes degradation of HIF-1α by the proteasome (Lu et al., 2005; Yasinska and Sumbayev, 2003). Dosing of 100μM of AA

was seen to lower basal HIF-1α levels in human head and neck cancer 22B cells (Lu et al., 2005).

Although there are many studies that suggest VC may be an effective cancer treatment, there are also many studies that suggest the opposite. VC supplementation decreases the efficacy of some antineoplastic agents by preventing mitochondrial membrane depolarization in RL cell-derived xenogeneic tumors (Heaney et al., 2008). The controversy concerning the value of VC in cancer treatment requires more *in vivo* studies, which can be performed on well-established mouse models.

Cardiovascular Disease

Oxidative damage within the components of the cardiovasculature has been linked to atherosclerosis, stroke, and other cardiovascular disease (Frei, 1997). Antioxidants can prevent atherosclerotic plaque formation by preventing oxidation modifications of low density lipoproteins by free radicals (Gale et al., 1995). VC may play a role in protecting nitric oxide (NO) from oxidation and increasing its synthesis, which is beneficial to vascular endothelial function. In a study by Gale et al., both VC intake and plasma ascorbate concentrations were inversely correlated with mortality by stroke in a sample of older adults (1995). Of the 730 subjects, those with VC intake in the highest third had a 30% reduced risk of dying from stroke compared with those with VC intake in the lowest third (Gale et al., 1995). In a pooled analysis of 9 cohorts, VC intake of greater than 700mg/day was correlated with a 25% reduction in risk of developing coronary heart disease (Knekt et al., 2004).

Animal Models of VC-deficiency

Rodents have been used extensively as models to study diseases associated with oxidative stress. The use of rodents allows researchers to model the complex pathways and processes that interact to manifest human disease. In many aspects, mice mirror human genetics and physiology remarkably well with only approximately 300 genes being unique to one species or the other (Mouse Genome Sequencing et al., 2002). Despite the similarities between the species, there are biological, physiological and immunological differences that must be taken into account when modeling human disease in rodents. The mouse's ability to endogenously synthesize VC results in differences between the redox systems of mice and humans (Maeda et al., 2000). The development and use of VC-deficient animal models have been pivotal in providing insight into the complex processes of oxidative damage and aging. These models are reviewed in (Yu and Schellhorn, 2013).

The GULO knockout mouse is one of the primary small animal model of vitamin C deficiency. The *GULO*$^{-/-}$ mouse, which cannot endogenously produce VC, was constructed in the B6 background by replacing GULO exons 3 and 4 with a neomycin resistance gene to disturb the reading frame and eliminate all gene activity (Maeda et al., 2000). *GULO* $^{-/-}$ homozygous mutants require dietary VC supplementation in order to facilitate normal growth and reproduction (Maeda et al., 2000). Supplementation of VC in the drinking water (330mg/L) is sufficient to support normal growth, development and reproduction of homozygous and heterozygous *GULO* knockout mice (Maeda et al., 2000). If VC supplementation is withdrawn from adult mice for more than five weeks,

plasma AA levels drop to 10% of normal levels, weight loss occurs and the mice do not live longer than six weeks (Maeda et al., 2000). Unsupplemented *GULO* $^{-/-}$ mutants have significantly decreased plasma, liver and brain concentrations of AA as compared to the wildtype *GULO* $^{+/+}$ (Maeda et al., 2000). Heterozygous mutants have lower plasma concentrations than the wildtype but their liver and brain concentrations are not significantly different from the wildtype (Maeda et al., 2000). *GULO* knockout mice also display altered morphology in the cardiovasculature, reduced plasma antioxidant capacity and increased plasma total cholesterol (Maeda et al., 2000).

Breast Cancer

Breast cancer is the second most common cancer and the leading cause of cancer death in women (Siegel et al., 2014). With over two hundred thousand newly diagnosed cases in the US each year, one in eight American women are expected to develop breast cancer during their lifetime(Siegel et al., 2014). Breast cancer alone accounts for approximately one third of new diagnoses in women (Siegel et al., 2014).

Her2 Positive breast cancer

Her2 (mouse orthologue *ErbB2*, rat orthologue *neu*) is a receptor tyrosine kinase (RTK) that belongs to the epidermal growth factor receptor (EGFR) family. In addition to *Her2*, the EGFR family contains three other RTKs (EGFR/Her1, Her3 and Her4) that are involved in signal transduction at the cell membrane. Their structure consists of a ligand-binding and cysteine-rich extracellular domain, a transmembrane domain and an intracellular tyrosine kinase domain. In response to mitogenic stimulation, the EGFR receptors form homo- or hetero-dimers that are stabilized by transphosphorylation of their

intracellular domains. These phosphorylated tyrosines act as docks for members of downstream signaling pathways involved with the regulation of cell growth, survival, differentiation and angiogenesis. Figure 3, below, displays the mechanism of *Her2* dimerization and activation in response to ligand binding. Two oncogenic pathways that are activated by *Her* signaling are the P13K/Akt and MAPK pathways, which increase survival and proliferation in cancer cells (Tokunaga et al., 2006; Vadlamudi et al., 2003; Viglietto et al., 2002; Wallasch et al., 1995). Dimer partner selection is a key determinant of downstream signaling (Tzahar et al., 1996). *Her2* has the strongest tyrosine kinase activity and *Her2*-containing dimers have the greatest signaling activity. *Her2* is also the only EGFR-like receptor that does not bind a ligand, and constitutively exists in an active conformation.

Her2 is amplified or overexpressed in 15-30% of breast cancers (Slamon et al., 1987; Slamon et al., 2001). *Her2* amplification-positive breast cancer is associated with a more aggressive cancer, increased potential to metastasize, high recurrence rates and poor patient outcome (Chia et al., 2008; Ross et al., 2003; Ross et al., 2009). The development of Her2-targeted therapeutics like trastuzumab have improved patient outcome, however, increased incidence of drug resistance in *Her2*-positive cancers drives the need for further study of the role of *Her2* in breast cancer (Nahta et al., 2006).

The rapid progress in cancer-genome sequencing studies has led to the identification of many novel cancer-associated genetic alterations. Many of these mutations have not yet been functionally characterized. Table 5, in the Appendix, lists all

of the *Her2* somatic mutations that have been catalogued in the major online cancer databases. While the majority of these mutations have not been functionally characterized, a select few have been characterized as activating (Bose et al., 2013).

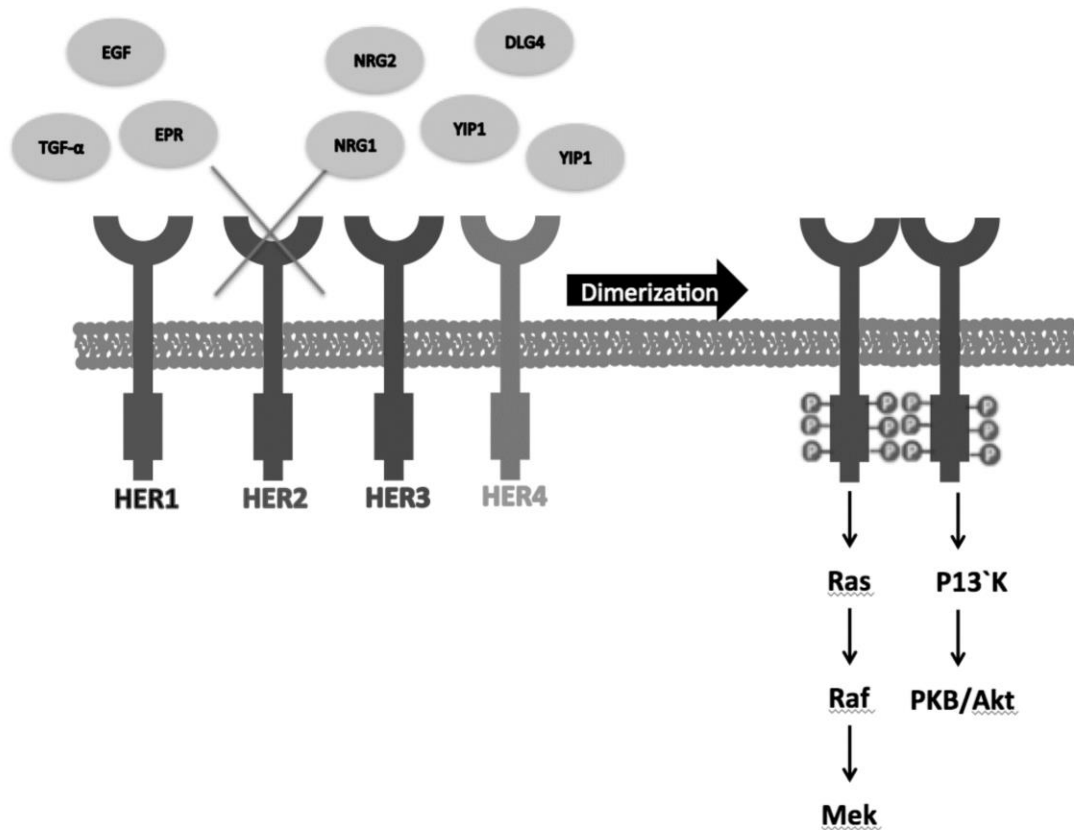

Figure 3: Receptor activation in response to mitogenic stimulation.

Ligand binding to Her3 results in the formation of the Her2/Her3 dimer stabilized by disulfide bonds. Dimerization stimulates the receptors' tyrosine kinase activity and trans-phosphorylation occurs. These phosphorylated residues act as docking sites for downstream signaling molecules.

Transgenic Mouse Models of Human Breast Cancer

There are many well-characterized transgenic mouse models that heritably develop mammary tumours (Ursini-Siegel et al., 2007). Mouse models of cancer have been pivotal in furthering our understanding of oncogenes, transformation and progression of cancer. The mouse models of breast cancer have been reviewed in (Fantozzi and Christofori, 2006) and (Kim and Baek, 2010). Specifically, the development of transgenic ErbB2 mouse models has been pivotal in developing our understanding of the role of this receptor in the pathogenesis of breast cancer and its potent transforming activity. The mouse models of *ErbB2*-induced breast cancer are reviewed in (Ursini-Siegel et al., 2007). Many of the transgenic mouse models of breast cancer express the transgene under the control of the murine mammary tumour virus-long terminal repeat (MMTV-LTR) promoter. The MMTV-LTR promoter is a strong promoter that can drive the high-level expression of several oncogenes specifically in the mammary epithelium. The MMTV promoter is hormonally regulated (Otten et al., 1988), and while also expressed in the virgin mammary gland, expression is increased during pregnancy and lactation. MMTV is selective for the mammary gland but is also expressed in the lungs, kidneys, salivary glands, and other tissues (Wagner et al., 2001).

The development of the MMTV-Neu-NT transgenic line, which expresses an activated form of *neu* ((rat *ErbB2* orthologue) under the control of the MMTV-LTR promoter, provided the first *in vivo* evidence that overexpression of activated *neu* is sufficient to transform mammary epithelium (Muller et al., 1988). These mice rapidly developed mammary tumors of the entire mammary epithelium with an average latency

of 3 months (Muller et al., 1988). While this model provided evidence that activated neu has potent transforming capability, there was no evidence for comparable activating mutations in human breast cancer. In addition, the primary mechanism of *Her2*-induced tumorigenesis is through gene amplification and overexpression rather than mutational activation. To determine if wildtype *neu* could still induce tumours, the MMTV-Neu transgenic line was established, which expresses wildtype *neu* under the control of the MMTV-LTR promoter. MMTV-Neu mice develop focal mammary tumours with a latency of 7 months. Many of the mammary tumours harbor in-frame insertions or deletions of cysteine residues within the cysteine-rich region of the extracellular domain (Siegel et al., 1994). A disruption in the cysteine balance of *neu* can promoter receptor dimerization and activation through constitutive dimerization stabilized through disulphide bonds (Siegel et al., 1994) (Siegel and Muller, 1996). The role of the reducing agent ascorbic acid in disrupting the disulphide bond has not yet been established.

Chapter 2: Introgression of the GULO$^{-/-}$ allele into the FVB/N background

2.1 Introduction

Vitamin C (VC) is a ubiquitous antioxidant that quenches free radicals naturally generated in the body by respiration and metabolism. The failure to maintain a balance between the production and removal of reactive oxygen species (ROS) may play a role in the pathogenesis of degenerative diseases like cancer and cardiovascular disease. Because of its ability to prevent the oxidation of biological molecules, VC has the potential to play a protective role in preventing oxidative damage to cells and tissues. Recent evidence indicates that VC has anti-tumorigenic effects on human cancer cells in a HIF-dependent manner (Gao et al., 2007) and pharmacological levels of VC have also been seen to increase the efficacy of other chemotherapeutics (Arrigoni and De Tullio, 2002; Cha et al., 2013). The efficacy of VC as a cancer treatment is still controversial due to studies that report VC may antagonize the cytotoxic effects of antineoplastic drugs (Heaney et al., 2008). This controversy (Li and Schellhorn, 2007a) requires further studies exploring the therapeutic value of VC, which can be accomplished through comprehensive animal models.

The development of the GULO knockout mouse has provided a VC-deficient laboratory model (Maeda et al., 2000) that can be used to study the interaction of endogenous and exogenous redox systems and their role in the pathogenesis of disease. This inactivated *GULO* can be integrated into different mouse models to explore the specific effects of VC on well-characterized animal models of human disease, including breast cancer.

Breast cancer is the most commonly diagnosed cancer and second leading cause of cancer death in women (Siegel et al., 2015). In 2015, over 230 000 Americans are expected to be diagnosed with breast cancer and 40 430 American breast cancer patients will die from the disease (Siegel et al., 2015). The lifetime probability of a woman developing breast cancer is 1 in 8 (Siegel et al., 2015). Approximately 20-30% of breast cancers are Her2-positive (Ross et al., 2009; Slamon et al., 1989), in which amplification of the *Human epidermal growth factor receptor (Her)2* gene and/or overexpression of Her2 at the mRNA or protein level is present. High levels of Her2 expression are an indicator of poor prognosis in breast cancer patients, and are associated with larger tumours (Rilke et al., 1991), metastasis (Braun et al., 2001; Lin and Winer, 2007), increased recurrence rate (Slamon et al., 1987), drug resistance (Nahta et al., 2006), and poor survival (Paik et al., 1990; Slamon et al., 1987).

Her2 encodes a transmembrane tyrosine kinase receptor that belongs to the epidermal growth factor receptor (EGFR) family, which also includes Her1, Her2 and Her4 . In response to extracellular ligand binding, HER proteins change conformation and form homo- or hetero-dimers stabilized by disulphide bonds (Mass, 2004). Subsequent transactivation of these dimers produces phosphorylated tyrosines which dock intracellular signaling molecules to activate downstream signaling cascades (Yarden and Sliwkowski, 2001). The specific tyrosines that are phosphorylated and cascades that are activated depend on both the ligand and dimerization partner (Tzahar et al., 1996). Of the four EGFR family members, Her2 has both the greatest catalytic activity (Tzahar et al., 1996) and is the preferred dimerization partner (Graus-Porta et al., 1997; Tzahar et

al., 1996). *Her2* is also the only EGFR-like receptor that does not bind a ligand, and constitutively exists in an active conformation (Cho et al., 2003). When overexpressed, an excess of Her2 at the membrane results in constitutive activation of downstream signaling cascades including the PI3K/Akt (Yarden and Sliwkowski, 2001), Ras/Raf/MEK (Graus-Porta et al., 1997) and NF-kB canonical (Merkhofer et al., 2010) pathways. While Her2-amplification/overexpression has been well established as a driving force in breast cancer, recent cancer genome sequencing of human tissue isolates (Banerji et al., 2012; Boulbes et al., 2015; Lee et al., 2006; Shah et al., 2009; Shah et al., 2012; Stephens et al., 2012) and functional characterization of Her2 variants (Bose et al., 2013; Boulbes et al., 2015) have also identified activating somatic mutations as an alternative mechanism for Her2-dependent breast cancer. Species that damage DNA and produce mutations, such as reactive oxygen species (ROS), may contribute to the pathogenesis of Her2-driven cancer.

ROS are naturally produced through cellular metabolism and mitochondrial respiration (Halliwell and Gutteridge, 2007). Elevated production of these reactive species can overwhelm antioxidant defenses and lead to oxidative stress, a condition that has been strongly implicated in the etiology of cancer (Halliwell and Gutteridge, 2007). Elevated levels of ROS have been detected in almost all cancer types, where they promote oncogenic transformation and progression by triggering redox-sensitive oncogenic pathways (Catarzi et al., 2002), altering proliferation and apoptosis pathways (Souici et al., 2000), inducing angiogenesis (Hughes et al., 2005), and causing genomic instability leading to mutagenesis and carcinogenesis (Cooke et al., 2003; Marnett, 2000;

Moller and Wallin, 1998). Oxidative stress is implicated specifically in the etiology of breast cancer, as markers of lipid peroxidation and levels of DNA adducts are significantly higher in breast cancer patients than in normal tissues (Wang et al., 1996). Furthermore, levels of oxidative stress increase as breast cancer stage progresses, while plasma levels of the antioxidant ascorbic acid decrease (Khanzode et al., 2004).

Ascorbic acid is a potent antioxidant that acts as a reducing agent, at physiological pH, to quench ROS. Unlike most mammals, humans are unable to endogenously synthesize ascorbic acid due to an inactivating mutation in ι-gulono-γ-lactone oxidase (GULO), the enzyme that catalyzes the terminal step in the ascorbic acid synthesis pathway (Nishikimi et al., 1994). Humans overcome their GULO deficiency by consuming dietary ascorbic acid. Without adequate dietary intake of ascorbic acid, humans develop scurvy with symptoms including weight loss, anemia, muscle weakness, damage to the cardiovasculature and eventually death (Williams and Deason, 1967). Although scurvy is relatively rare in Western cultures, there is still a high prevalence of serum ascorbic acid deficiency in North Americans (Cahill et al., 2009; Schleicher et al., 2009; Villalpando et al., 2003). This high prevalence of serum ascorbic acid deficiency is especially pronounced in cancer patients, (Anthony and Schorah, 1982; Khanzode et al., 2004; Mayland et al., 2005; Nunez Martin and Ortiz de Apodaca y Ruiz, 1995).

Because of its antioxidant capacities, ascorbic acid is a candidate in both the prevention and treatment of cancer. The GULO knockout mouse (Maeda et al., 2000), which expresses a non-functional *GULO* allele and relies on dietary ascorbic acid similar to humans, is used to model the effects of ascorbic acid deficiency on cancer. In tumor

xenograft models, ascorbic acid-deficient GULO knockout mice experience shorter tumor latency as compared to wildtype controls (Kasprzak et al., 2011). Ascorbic-acid deficient mice also exhibit larger tumors with larger darker cores with increased necrotic areas (Cha et al., 2013). Supplementation of GULO knockout mice with ascorbic acid slows tumour growth and decreases HIF-1 activation (Campbell et al., 2015). While this evidence supports the role of ascorbic acid in the development and progression of cancer, the effect of chronic ascorbic acid deficiency on tumorigenesis in a well-characterized cancer model that develops tumours endogenously has not yet been characterized.

In this study, we develop a GULO knockout MMTV-Neu bitransgenic mouse, which relies on dietary intake of ascorbic acid and develops spontaneous mammary tumours in a Her2-dependent manner. We also report the effects of chronic ascorbic acid deficiency on breast cancer latency and tumour number to characterize the effects of chronic ascorbic acid deficiency on *Her2* tumorigenesis.

2.2 Experimental Design

To characterize the role of chronic VC insufficiency in tumorigenesis in HER2-positive breast cancer, we completed two experiments. In Experiment 1, C57 GULO knockout mice were breed with FVB inbred mice in a backcross breeding scheme to introgress the GULO knockout allele into the FVB/N background. All mice were maintained on drinking water supplemented with vitamin C to facilitate normal growth and reproduction. After eight generations of backcrossing, the resultant FVB/N GULO knockout mice were characterized through vitamin C-withdrawal studies. From

Experiment 1, it was determined that FVB/N GULO knockout mice, like C57 BL/6 GULO knockout mice, develop scorbutic symptoms without supplementation with VC.

In Experiment 2, FVB/N GULO knockout mice were bred with MMTV-Neu mice to produce the bitransgenic MMTV-Neu GULO knockout mouse. MMTV-Neu GULO knockout mice cannot synthesize vitamin C endogenously and spontaneously develop mammary tumours. A control group of MMTV-Neu GULO knockout mice was maintained on normal dose VC supplementation (330mg VC/L drinking water) while a treatment group of MMTV-Neu GULO knockout mice was maintained on low dose VC supplementation (33mg VC/L drinking water). Both the treatment and control groups were monitored for tumour development and the characteristics of the breast cancer that developed were compared.

2.3 Methods

2.3.1 Housing

C57BL/6 GULO +/- mice were purchased from the Mutant Mouse Regional Resource Centres (MMRRC; Stock number 000015-UCD). FVB/N mice were purchased from Charles River (CR, strain code 207). FVB MMTV-Neu mice were obtained from John Hassel. Mice were housed in cages on vent racks in the Clean/Vent rooms of the Central Animal Facility at McMaster University. Each cage contained bedding, nesting material and enrichment (i.e. plastic tube). House cages were provided with autoclaved water and low protein rodent chow ad libitum. Breeder cages were provided with autoclaved water and high protein rodent chow ad libitum. Vitamin C-supplemented water was changed once to twice weekly, while non-supplemented water was changed

weekly. Food, nesting materials and cages were changed biweekly. All mice were subject to a 12hr light-dark cycle consisting of alternating 12-hr periods of light and darkness with the light period beginning at 07:00. House cages contained between one and five mice per cage and were separated by gender. Breeder cages contained either a mating pair (i.e. one male and one female) or a mating harem (i.e. two females and one male). Identification of males and females was determined by external appearance. A large anogenital distance (i.e. distance between the anus and genitals) was representative of males while a shorter distance was representative of females.

Euthanasia of the homozygous wildtype and mutant *GULO* mice not needed for breeding, controls or experiments was done according to the methods pre-approved by the Animal Research Ethics Board. Euthanasia by cervical dislocation was performed according to the CAF SOP GEN460.sop. Euthanasia by carbon dioxide was performed according to the CAF SOP GEN755.sop.

Pups were weaned between eighteen and twenty-one days after birth and were separated into house cages by gender. Pups were designated as breeders, experimental or control mice by six weeks. Breeders were replaced if they produced no litter within eight weeks of being paired, if they did not raise two to three consecutive litters or if their litter size decreased rapidly for two consecutive litters. Breeders older than six months were also replaced.

Ear notching was used to distinguish mice in the same cage. Mice were assigned no holes, one hole in the left ear, one hole in the right ear, two holes in the left ear, two holes in the right ear or one hole in each the right and left ear. Mouse tissue for

genotyping was obtained through tail clipping during weaning. Approximately 0.4cm of tail was obtained according to the McMaster University Central Animal Facility SOP GEN485.sop. Silver nitrate cauterizing sticks were used to cauterize the tail wounds. Each tail clipping was divided into two aliquots, with one being used for genotyping and the other stored at -20°C. Eof the mice and DNA was isolated using the Norgen Genomic DNA Isolation Kit (Cat #24700). The *GULO* and MMTV-Neu genotype of the mice was determined using the PCR primers described in Section *2.3.3*. PCR products were run on a 1.5% agarose gel. The expected fragment size for the mutant *GULO* allele, wildtype *GULO* allele, MMTV-Neu transgene and internal control were 230bp, 330bp, 600bp, and 324bp, respectively.

2.3.2 Breeding

Three breeding colonies were maintained in the McMaster Central Animal Facility according to approved Animal Use Protocols that were reviewed annually by the McMaster Animal Research Ethics Board. One breeding colony was used to maintain the *GULO* knockout allele in the C57 BL/6 (B6) background. A second breeding colony was used to integrate the *GULO* knockout allele into the FVB/N (FVB) background using a backcross breeding scheme as shown in Figure 2. A third breeding colony was used to generate the bitransgenic MMTV-Neu GULO knockout mouse, as shown in Figure 3. The heterozygote and homozygote *GULO* knockout mice in these breeding colonies were supplemented with AA in their drinking water (0.33g/L) to facilitate normal growth and reproduction.

2.3.3 Genotyping

Mouse genomic DNA was isolated using the Norgen Genomic DNA Isolation Kit (CAT #24700 and #24750), which uses spin chromatography with a separation matrix. DNA extraction was performed according to the manufacturer's instructions with the addition of an optional RNase A digestion and the substitution of sterile distilled water instead of elution buffer. Genomic DNA concentration was quantified using the Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham MA) according to the manufacturer's instructions. The estimated purity of the DNA samples was determined using the A2g0A280 and A260/A230 ratios.

The genotype of each mouse at the GULO locus was determined using multiplex PCR with primers P2 (5'-CGCGCCTTAATTAAGGATCC-3'), P3 (5'-GTCGTGACAGAATGTCTTGC-3'), and P4 (5'- GCATCCCAGTGACTAAGGAT-3'). Genomic DNA (4uL) was mixed with PCR reagent master mix (14ul ddH20, 2.5uL Thermopol Buffer, 1ul 10mM P2, 1ul 10mM P3, 1ul 10mM P4, 0.5uL 10mM dNTP and 1ul Taq DNA Polymerase) and run in the Thermocycler PCR Machine. PCR Products were run on a 1.7% agarose gel at 90V and 400mA for 45 to 60 minutes. The agarose gels were imaged under ultraviolet light to view the banding pattern. Amplification of a 230-bp fragment from P2 and P3 indicated the presence of the GULO knockout allele. Amplification of a 330bp-fragment from P3 and P4 indicated the presence of the GULO wildtype allele. The presence of both a 230-bp fragment and a 330-bp fragment indicated a GULO heterozygous mouse.

The genotype of each mouse at the MMTV-Neu locus was determined using multiplex PCR with primers oIMR0386 (5'-TTTCCTGCAGCAGCCTACGC-3'), oIMR0387 (5'-CGGAACCCACATCAGGCC-3'), oIMR8744 (5'-CAAATGTTGCTTGTCTGGTG-3'), and oIMR8745 (5'-GTCAGTCGAGTGCACAGTTT-3'). Genomic DNA (4uL) was mixed with PCR reagent master mix (13.5ul ddH20, 2.5uL Thermopol Buffer, 1ul 10mM oIMR0386, 1ul 10mM oIMR0387, 1ul 10mM oIMR8744, 1ul 10mM oIMR8745, 0.5uL 10mM dNTP and 1ul Taq DNA Polymerase) and run in the Thermocycler PCR Machine. PCR Products were run on a 1.7% agarose gel at 90V and 400mA for 60 minutes. The agarose gels were imaged under ultraviolet light to view the banding pattern. Amplification of a 324-bp fragment from oIMR8744 and oIMR8745 was used as an internal positive control. Amplification of a 600bp-fragment from oIMR0386 and oIMR0387 indicated the presence of the MMTV-Neu allele. This protocol did not distinguish between hemizygous and homozygous MMTV-Neu$^+$ mice.

2.3.4 Blood collection

Blood samples were collected from the saphenous vein of mice into heparin-coated blood collecting tubes (Kimble Chase, Vineland, NJ). Blood was expelled from the heparin-coat capillary tubes into microcentrifuge tubes using a microcap bulb assembly (VWR, Radnor, PA) and centrifuged at 3000 x g for 5 minutes. Cell-free plasma (supernatant) was diluted (1:10) in 5% MPA-0.2M EDTA. Diluted plasma was

centrifuged at 13 000 x g for 30 minutes at $4^{o}C$ to eliminate protein precipitation. The protein-free supernatant was extracted and stored at $-20^{o}C$ until use. Sterile NaCl saline was injected subcutaneously as fluid replacement post-blood sampling.

2.3.6 Experiment 1: VC-Withdrawal for Characterization of the FVB/N GULO knockout mouse

To determine if the plasma AA levels in the FVB *GULO* knockout mouse are similar to those reported in the C57 mouse, VC-withdrawal studies were performed on FVB/N GULO knockout mice generated from the introgression in Experiment 1. A treatment group (n=2-4) of homozygous wildtype *GULO*, heterozygous mutant *GULO* and knockout *GULO* mice were deprived of ascorbic acid and baseline plasma ascorbic acid concentrations were determined. A group (n=2-3) of age-matched control *GULO* knockout mice in each background were maintained on a diet supplemented with VC (330mg/L). Mice were monitored for the endpoint signs described in Table 3. Euthanasia at endpoint was performed as described above.

2.3.7 Experiment 2: Low-dose VC supplementation for characterizing the effect of chronic VC insufficiency on breast tumorigenesis

To determine the effect of chronic VC deficiency in HER-2 positive breast cancer, bitransgenic MMTV-Neu GULO knockout mice were maintained on a control normal-dose diet of normal rodent chow with 330mg VC/L drinking water or a treatment low-dose diet of 33mg VC/L drinking water. VC-supplemented water was changed once

or twice weekly. Mice were monitored for palpable tumours in the ten mammary glands and palpable tumours were measured with digital calipers. The volume of each tumour was calculated according to Table 4 in the Appendix. Endpoint was established when tumour volume was $1.7cm^3$, tumour size significantly impaired the ability to move or groom, or if any of the endpoint signs in Table 3 were noted.

2.3.8 HPLC detection of ascorbic acid

Prior to analysis, samples were diluted in 5% MPA-0.2M EDTA to concentrations within the detection limit of the High-performance liquid chromatography with electrochemical detection (Agilent 1200 Series). The HPLC system consisted of a microvacuum degasser, binary pump, autosampler, C18 analytical column (4.6x250mm, particle size 5um), diode array detector (DAD), and ESA electrochemical detector. The ESA electrochemical detector was set with the Guard Cell at -200mV, Channel 1 at -150mV and Channel 2 at +150 mV.

The mobile phase for ascorbic acid detection was 0.2M KH_2PO_4, pH 2.9. The flow rate of mobile phase was 2.0mL/min with a maximum pressure limit of 500 bar. Prior to sample measurement, the Agilent HPLC System was run to equilibration for at least 15 minutes. Output from the DAD (λ=240nm) and ESA (Channel 2) were used to quantify ascorbic acid. Samples of known concentration were used to construct standard curves for ascorbic acid determination in unknown samples.

2.3.7 Data Analysis

HPLC chromatographs were analyzed and exported using EZChrome Elite Software (Agilent) . All data was compiled and analyzed using Microsoft Excel 2013 and statistics were computed using JMP Statistical software. Graphics and charts were produced and edited using Microsoft Office Excel and Powerpoint 2013.

2.4 Results

2.4.1 The congenic FVB/N GULO knockout mouse was generated from ten generations of backcross breeding

Ten generations of backcrossing were used to generate the congenic FVB/N GULO knockout mouse. Figure 4, below, shows the backcrossing breeding scheme used to introgress the knockout GULO allele into the FVB/N background.

2.4.2 The bitransgenic MMTV-Neu GULO knockout mouse was generated from the F8 progeny of the introgression and a wildtype FVB/N MMTV-Neu mouse

F8 progeny from the GULO knockout allele introgression were crossed with a MMTV-Neu mouse obtained from the Hassel laboratory (Central Animal Facility, McMaster) to generate the bitransgenic MMTV-Neu GULO knockout mouse. Figure 5, below, displays the breeding scheme used to generate the bitransgenic MMTV-Neu GULO knockout mouse.

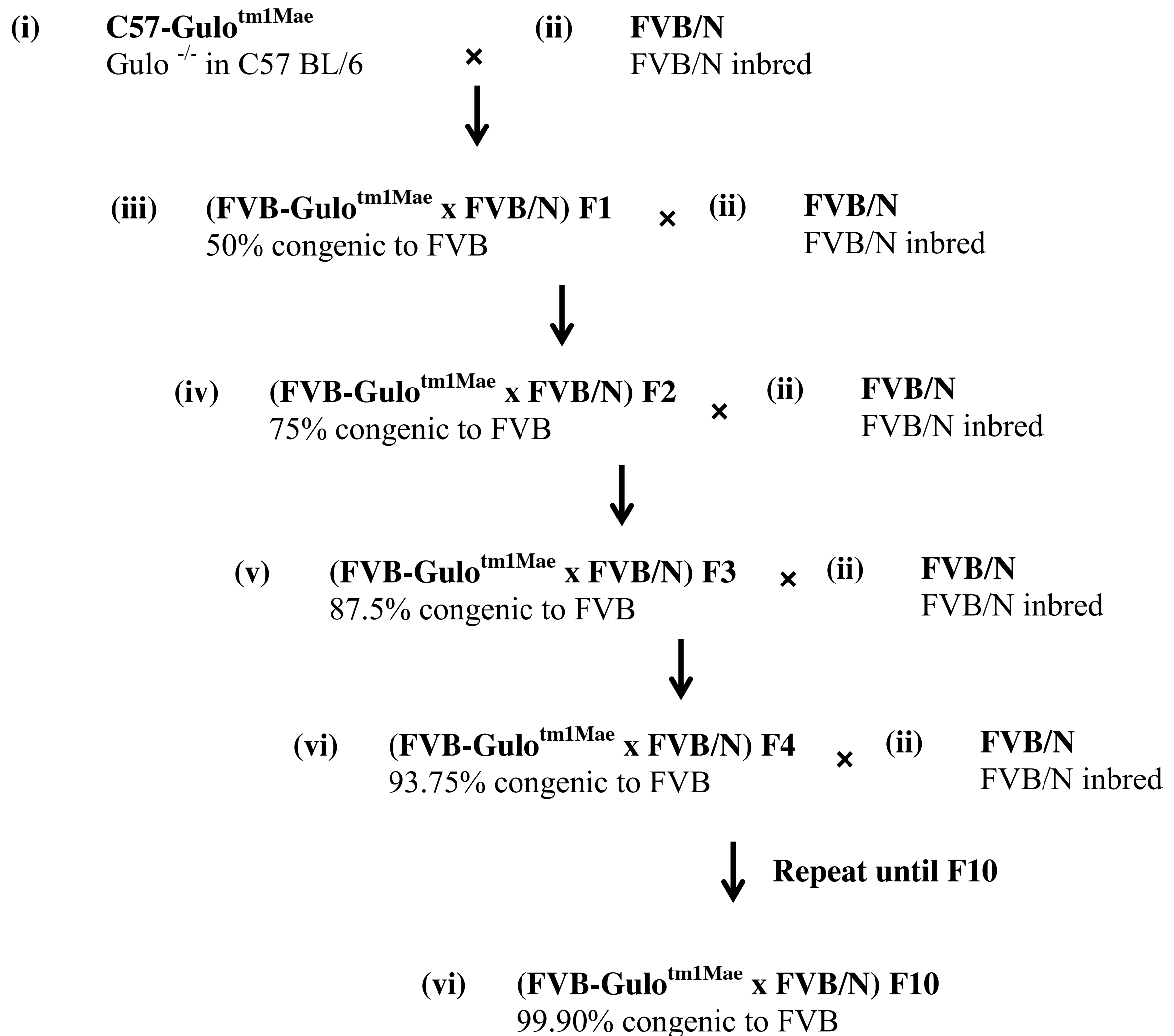

Figure 4: Introgression of the GULO knockout allele into the FVB/N background.

In each generation, a heterozygous Gulo knockout mouse is bred with an inbred FVB/N in a monogamous breeding system. The resulting F10 progeny are 99.90% isogenic to the FVB/N inbred strain, and can be considered congenic.

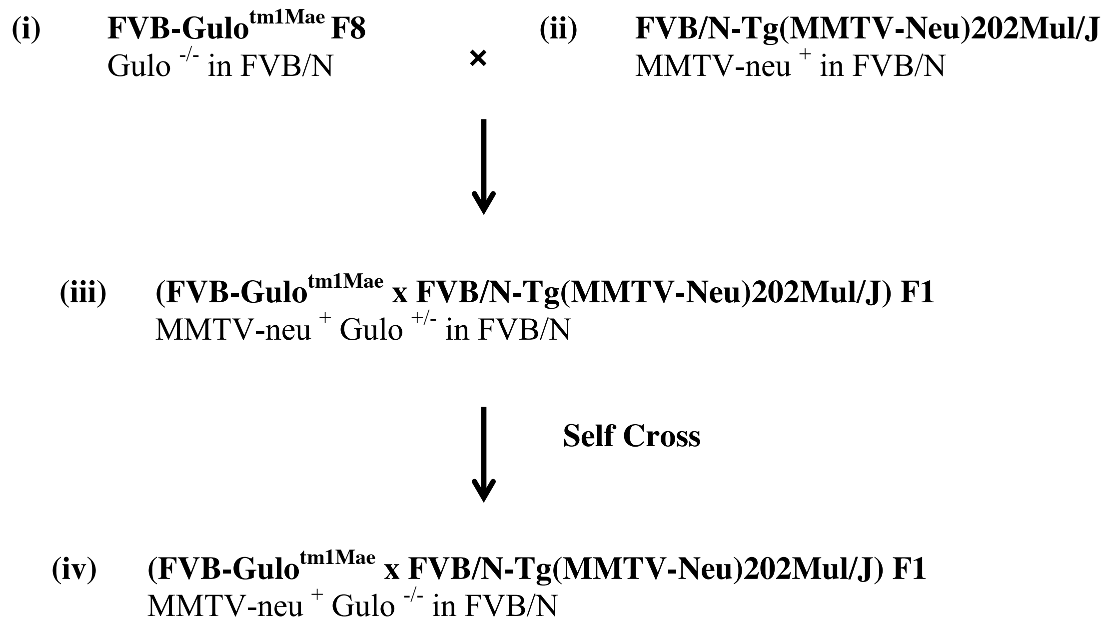

Figure 5: Cross of the Gulo knockout mouse (FVB) with the MMTV-neu (FVB) mouse.

The resultant model can be used to study the role of exogenous vitamin C on breast cancer progression

2.4.3 GULO knockout mice develop scurvy without VC supplementation

In Experiment 1, we withdrew VC supplementation from FVB/N GULO knockout (n=3), heterozygous (n=3) and wildtype (n=4) adult mice. FVB/N GULO knockout mice developed scorbutic symptoms between 5-8 weeks post-supplementation withdrawal. Symptoms observed included sunken eyes, hind leg weakness, skin tenting, lack of grooming, loss of body weight and hunched posture. These symptoms are consistent with the scorbutic symptoms of C57 GULO knockout mice (Maeda et al., 2000). FVB/N GULO heterozygous and wildtype weanlings did not exhibit scorbutic symptoms. Figure 6, below, shows the body weights of FVB/N GULO knockout mice post-withdrawal of VC supplementation.

2.4.5 GULO knockout mice do not develop scurvy when supplemented with 330mg or 33mg VC/L drinking water

When supplemented with low dose (33mg VC/L H_20) or normal dose (330mg VC/L H_20), MMTV-Neu GULO knockout mice did not develop scorbutic symptoms. The plasma VC concentrations for MMTV-Neu GULO knockout, GULO wildtype and GULO heterozygous mice on low dose and normal dose VC supplementation are displayed in Figure 7. The plasma VC concentrations of MMTV GULO knockout ($p < 0.001$, t-test) and GULO heterozygous ($p = 0.027$, t-test) mice on low dose were significantly different than mice supplemented with normal dose. There were no significant differences between plasma VC concentrations between low dose and normal dose MMTV-Neu GULO wildtype mice ($p = 0.646$, t-test).

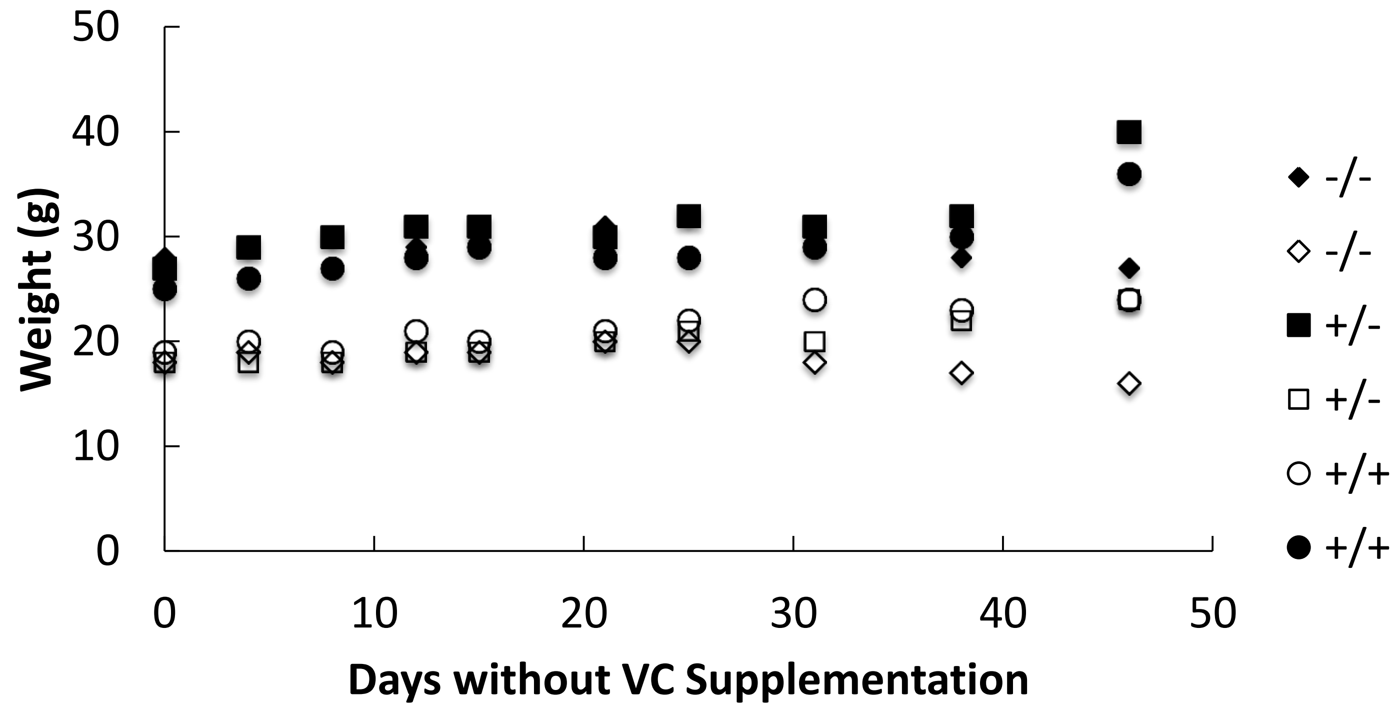

Figure 6: Body weights of FVB/N GULO knockout mice. Closed shapes represent individual male mice, while open shapes represent individual female mice.

GULO knockout, heterozygous and wildtype mice were maintained on a VC-deficient diet and weighed for endpoint monitoring. Weight loss in the GULO knockout, but not the GULO heterozygous or wildtype mice occurred around day 30.

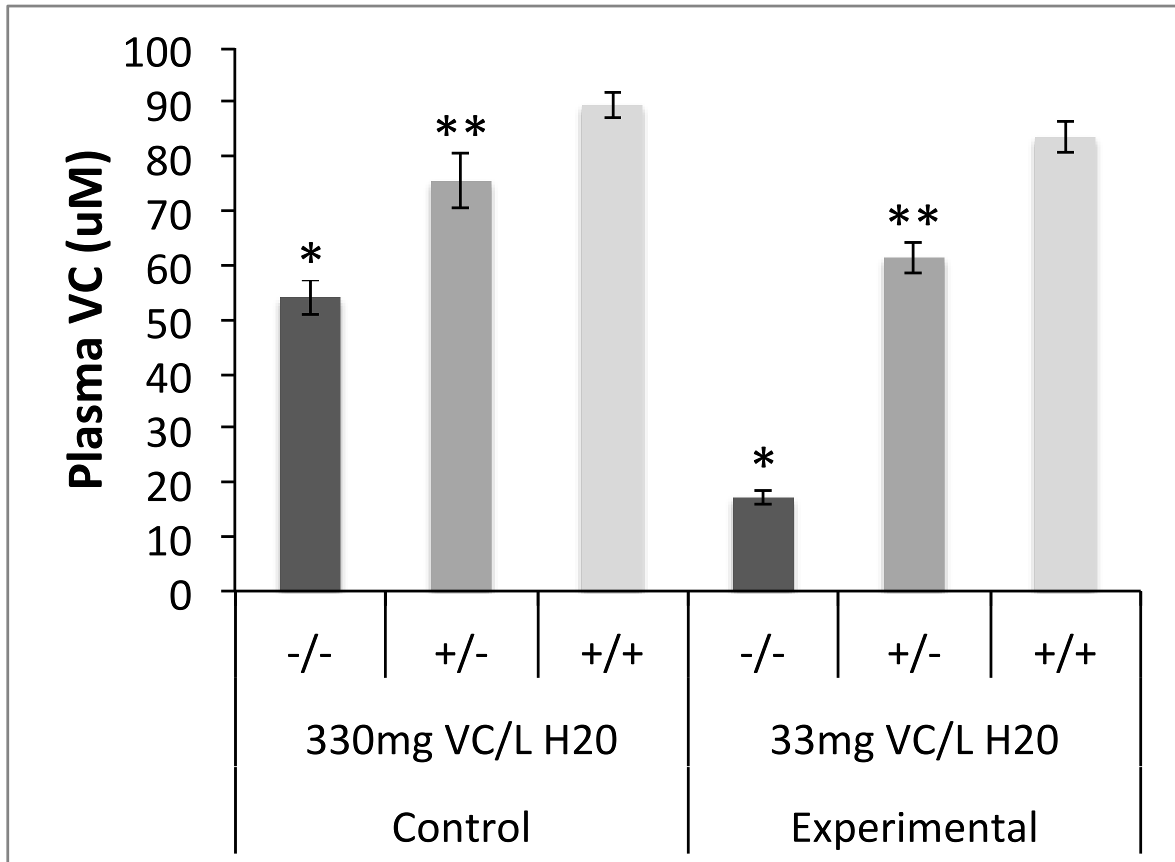

Figure 7: Average plasma ascorbic acid content for MMTV-Neu GULO mice in Experiment 2. Error bars are standard error.

One treatment group of MMTV-Neu GULO knockout mice was maintained on low dose vitamin C supplementation in their drinking water. One control group of MMTV-Neu GULO knockout mice was maintained on normal-dose vitamin C supplementation in their drinking water. *GULO knockout mice on the low-dose diet had significantly lower plasma VC than those on the control diet ($p<0.01$). **GULO heterozygous mice on the low-dose diet had significantly lower plasma VC than those on the control diet ($p=0.027$)

2.4.4 On average, control and treatment GULO knockout mice developed palpable tumours at the same age

In Experiment 2, mice were palpated for tumour development in any of the ten mammary glands. MMTV-Neu$^+$ GULO knockout, GULO heterozygous, and GULO wildtype mice in both the control group (330mg or normal-dose VC in their drinking water) and the experimental group (33mg or low-dose VC in their drinking water) developed tumours. No MMTV$^-$ mice of any GULO genotype developed mammary tumours. The average latency to tumour palpation is shown in Figure 8. The average latency to palpable tumours for each group was between 239 and 337 days. GULO wildtype mice in the experimental group had the lowest average latency to palpable tumours of 238.5 ± 6.5 SE days, while GULO knockout mice in the control group had the highest average latency of 336.6 ± 32.4 SE days. There was no statistically significant difference in latency to palpable tumours between any of the three control groups and any of the three treatment groups ($p \geq 0.57$, Tukey's HSD). There was also no statistically significant difference within either the control ($p = 0.34$, ANOVA) or treatment group ($p = 0.62$, ANOVA).

The average age at endpoint for each group is depicted in Figure 9. Endpoint was reached when the total tumour burden of the mouse was 1.7cm^2, the tumour significantly impaired the ability to eat, move or groom, or any of the other endpoint signs listed in the Appendix were present. The average age at endpoint for each group was between 317 and 467 days. GULO wildtype mice in the experimental group had the lowest age at endpoint of 317.0 ± 30 SE days, while GULO knockout mice in the control group had the greatest

average age at endpoint of 467.0 ± 37.5 SE days. There was no statistically significant difference in the age at endpoint between any of the three control groups and any of the three treatment groups ($p \geq 0.47$, Tukey's HSD). There was also no statistically significant difference between groups within either the control ($p=0.34$, ANOVA) or treatment group ($p = 0.26$, ANOVA).

2.4.5 On average, control and treatment GULO knockout mice developed the same number and the same size of palpable tumours

Once the mice in Experiment 2 developed palpable tumours digital calipers were used to measure tumour size and Table 4, in the Appendix, was used to approximate tumour volume Tumour endpoint was defined as total tumour burden of 1.7cm^3, if a tumour significantly impaired the ability to groom or move, or if the mouse displayed any of the other endpoint signs listed in the Appendix. Figure 10 displays the average number of palpable tumours at endpoint for MMTV-Neu$^+$ GULO knockout, GULO heterozygous, and GULO wildtype mice in both the control group (330mg or normal-dose VC in their drinking water) and the experimental group (33mg or low-dose VC in their drinking water). The average number of tumours at endpoint for each group ranged between 1.0 and 2.1. There was no significant difference in the number of tumours between any control group and any treatment group ($p > 0.69$, Tukey's HSD). There was no difference in the number of tumours between genotypes within either the control ($p = 0.41$, ANOVA) or treatment ($p = 0.31$, ANOVA) groups.

Figure 11 shows the average volume per tumour for Experiment 2 mice at endpoint. The average volume per tumour for each of the three control and three treatment groups ranged between 0.74 ± 0.19 SE and 1.33 ± 0.31 SE cm^3. There was no significant difference in volume per tumour at endpoint between any control group and any treatment group ($p > 0.54$, Tukey's HSD). There was no difference in volume per tumour within either the control ($p = 0.26$, ANOVA) or treatment ($p = 0.96$, ANOVA) groups.

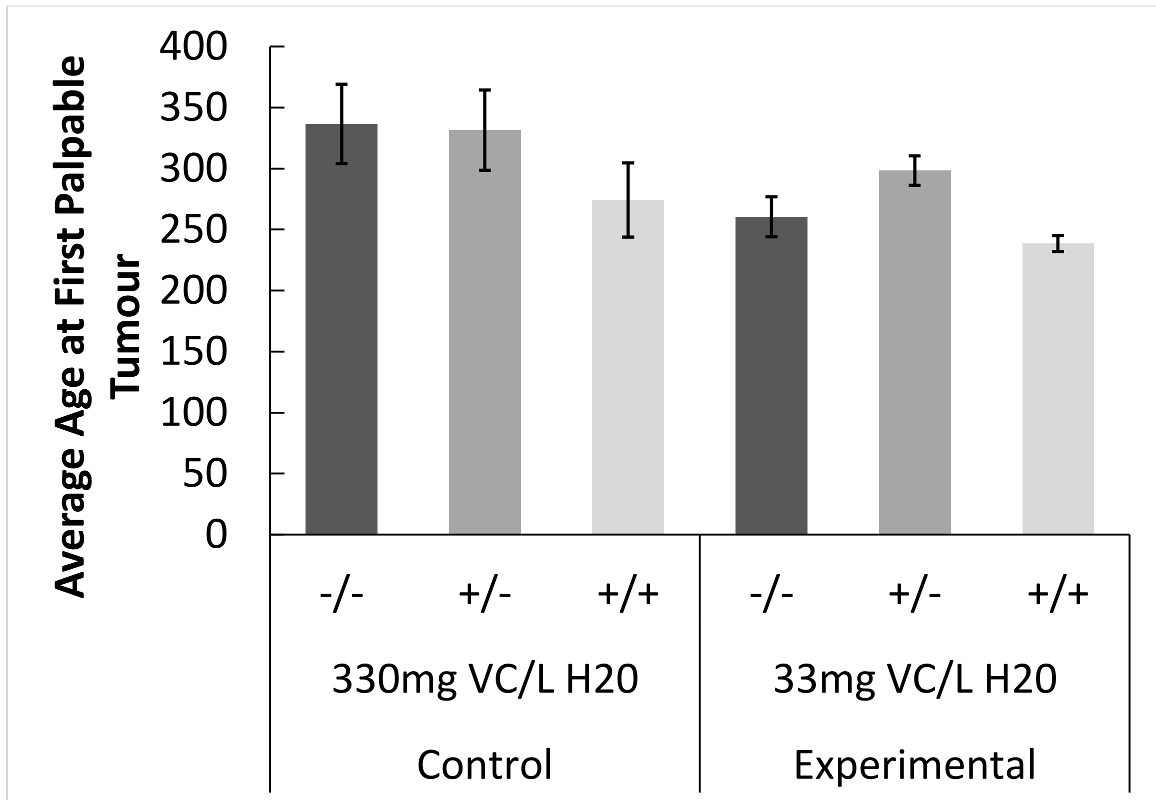

Figure 8: Average age at palpation of the first tumour for MMTV-Neu GULO mice in Experiment 2. Error bars are standard error.

One treatment group of MMTV-Neu GULO knockout mice was maintained on low dose vitamin C supplementation in their drinking water. One control group of MMTV-Neu GULO knockout mice was maintained on normal-dose vitamin C supplementation in their drinking water. There were no significant differences between any of the control or treatment groups ($p>0.57$), within the control group ($p=0.34$) or within the treatment group ($p=0.62$).

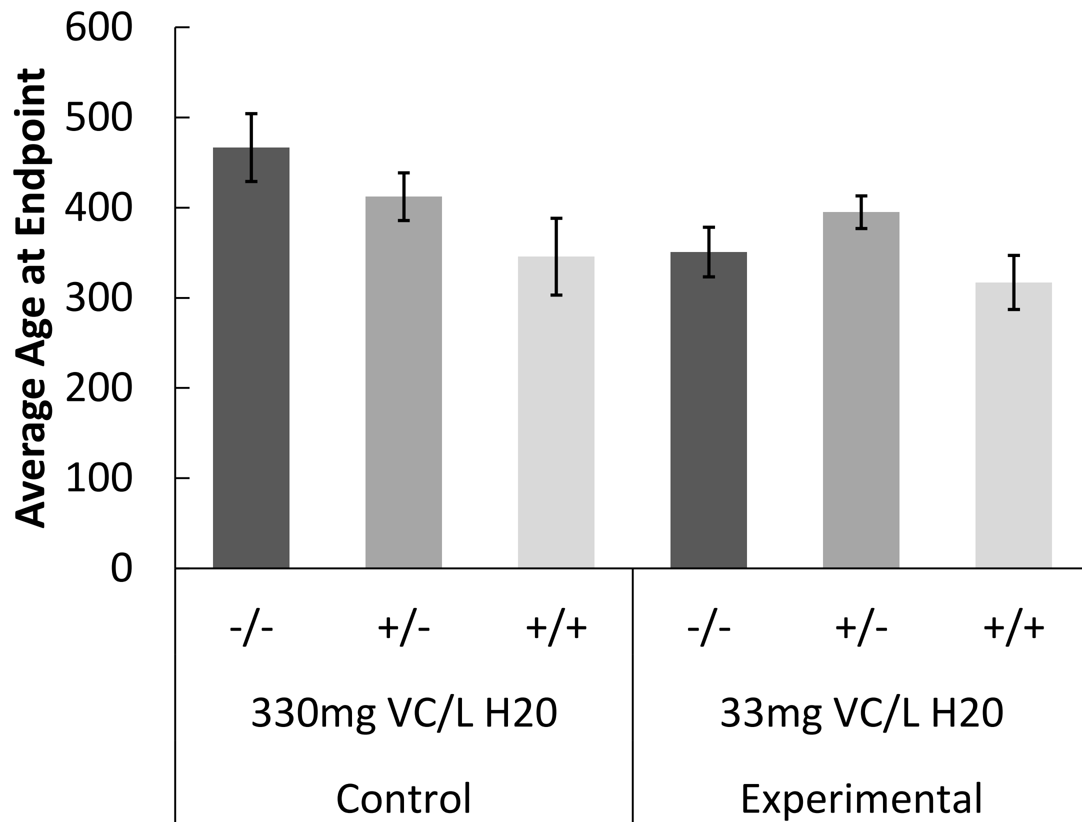

Figure 9: Average age at endpoint for MMTV-Neu GULO mice in Experiment 2. Error bars are standard error.

One treatment group of MMTV-Neu GULO knockout mice was maintained on low dose vitamin C supplementation in their drinking water. One control group of MMTV-Neu GULO knockout mice was maintained on normal-dose vitamin C supplementation in their drinking water. There were no significant differences between any of the control or treatment groups ($p>0.47$), within the control group ($p=0.34$) or within the treatment group ($p=0.26$).

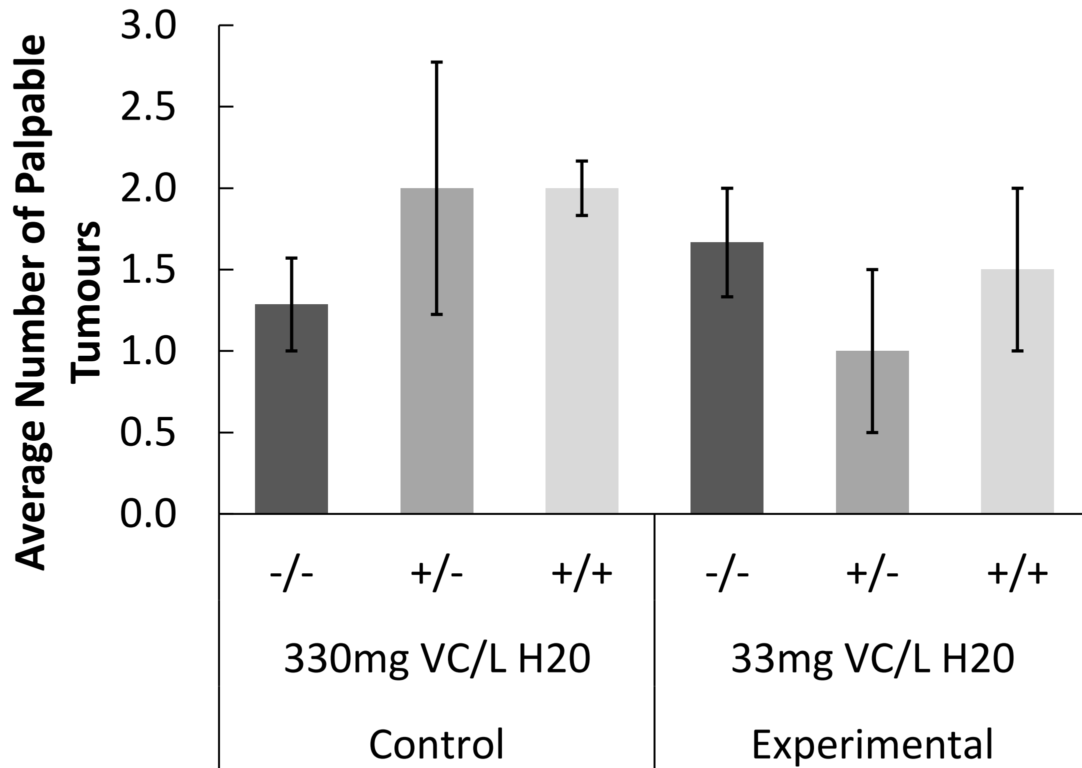

Figure 10: Average tumour number for MMTV-Neu GULO mice in Experiment 2. Error bars are standard error.

One treatment group of MMTV-Neu GULO knockout mice was maintained on low dose vitamin C supplementation in their drinking water. One control group of MMTV-Neu GULO knockout mice was maintained on normal-dose vitamin C supplementation in their drinking water. There were no significant differences between any of the control or treatment groups ($p>0.69$), within the control group ($p=0.41$) or within the treatment group ($p=0.31$).

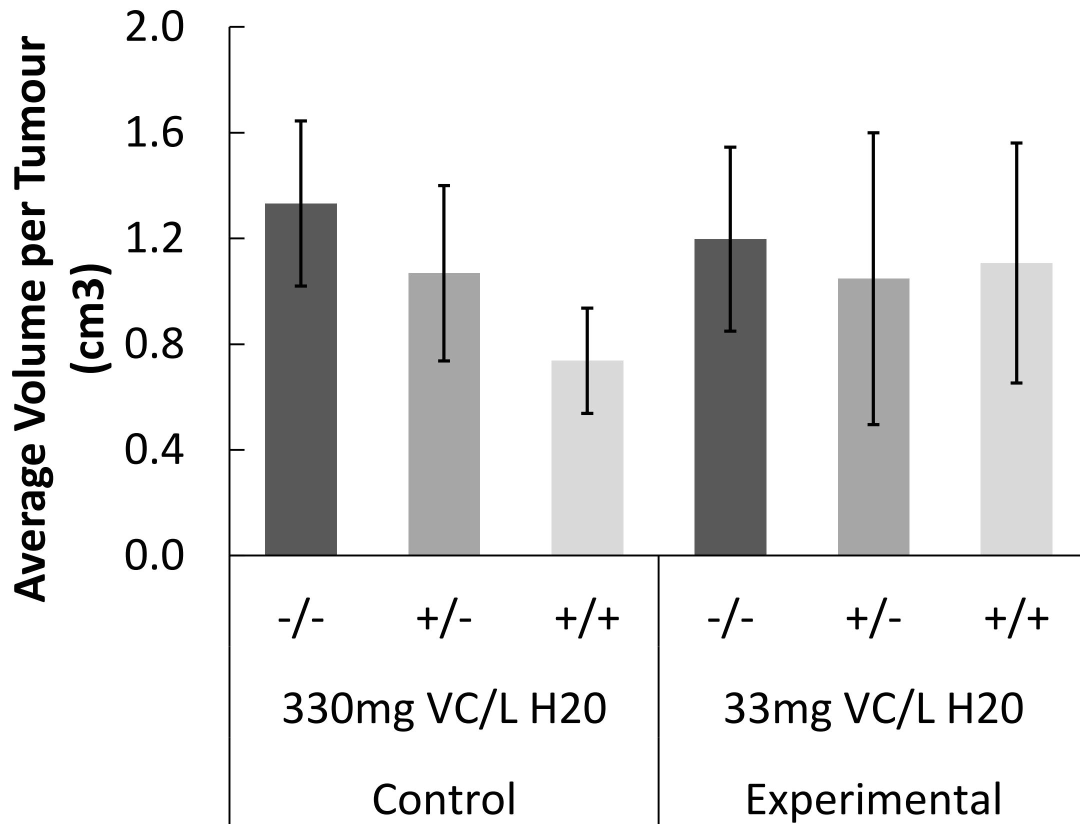

Figure 11: Average tumour size at endpoint for MMTV-Neu GULO mice in Experiment 2.

One treatment group of MMTV-Neu GULO knockout mice was maintained on low dose vitamin C supplementation in their drinking water. One control group of MMTV-Neu GULO knockout mice was maintained on normal-dose vitamin C supplementation in their drinking water. There were no significant differences between any of the control or treatment groups ($p>0.54$), within the control group ($p=0.26$) or within the treatment group ($p=0.96$).

Discussion

The role of VC in cancer prevention, progression and survival rate is highly debated despite over fifty years of research. VC has anti-tumorigenic properties *in vitro* by acting as a cofactor to prolyl hydrolases that inactivate HIF-1α, a potent promoter of tumour progression, as well as through the generation of hydrogen peroxide selectively in cancer cells. The use of sophisticated animal models can provide more definite evidence as to whether VC has anti-cancer properties *in vivo*.

In this study, we demonstrated that using a backcross introgression is a viable way to introduce the GULO knockout allele generated in the C57 BL/6 background to the FVB/N background, which is a common inbred mouse used to model human cancer. We also demonstrated that the FVB/N GULO knockout mouse has a dependency on VC supplementation to facilitate normal growth and respiration. Without VC supplementation, FVB/N GULO knockout mice develop the same scorbutic symptoms previously described in C57 BL/6 mice (Maeda et al., 2000).

In Experiment 2, we examined the effect of chronic vitamin C deficiency on HER2-mediated tumorigenesis, by maintaining a treatment group of mice on the minimum dosage required to maintain viability in the bitransgenic. Without supplementation, GULO knockout mice develop scorbutic symptoms around three to four weeks and reach endpoint between five and six weeks. The standard VC supplementation required to maintain growth and reproduction in the knockout is 330mg/L (Harrison et al., 2010; Maeda et al., 2000). Previous studies determined that *GULO* knockout mice could be maintained on supplementation as low as 33mg/L (Harrison et al., 2008),

however these studies were short in duration. We supplemented MMTV-Neu GULO knockout mice with 33mg/L for up to 600 days without the development of any scorbutic symptoms, despite significant decreases in plasma VC concentration compared to the controls. Therefore, 33mg/L supplementation with VC is sufficient to maintain the viability of GULO knockout mice indefinitely.

If VC is a critical factor in preventing DNA oxidative damage, we expected that MMTV-Neu GULO knockout mice would develop mammary tumours in an accelerated timeframe with reduced latency when maintained on suboptimal VC supplementation. As mutational activation is the primary mechanism of induction of mammary tumours in MMTV-Neu mice, increased oxidative damage from a VC deficient diet would lead to an increased somatic mutation rate and accelerated tumorigenesis. Alternatively, the low-dose treatment mice could have larger tumours and accelerated time to tumour endpoint because VC is a cofactor in HIF-1α inactivation. HIF-1α is a strong promoter of tumour progression, especially in the hypoxic conditions of the tumour microenvironment that would normally lead to cell death.

In Experiment 2, we determined that there was no difference in the average latency to palpable tumours, average tumour size or number between MMTV-Neu GULO knockout mice maintained on normal or low-dose VC supplementation. The tumours that developed had an average tumour volume of 0.74 to 1.3cm^3 per tumour. These results are consistent with previous tumour sizes in MMTV-Neu mice (Maroulakou et al., 2007; Shah et al., 1999; Zhang et al., 2010). The average latency to palpable tumour

development ranged between 238 to 336 days for each group. All six genotypes had an average tumour latency greater than the published median of 205 days (Guy et al., 1992)

One limitation to our study is that we were unable to test if no supplementation with VC affects breast tumorigenesis as GULO knockout mice reach endpoint due to weight loss and other scorbutic symptoms in five to six weeks without supplementation. As the average tumour latency in this study was 300.4±76.0 days, GULO knockout mice did not survive long enough to develop tumours with no VC supplementation.

There are several hypotheses that may explain why there was no difference in the tumour characteristics between normal- and low-dose VC supplementation in MMTV-Neu GULO knockout mice. As the MMTV LTR promoter is a very strong promoter, there may have been no selective pressure for mutational activation of HER2. Alternatively, the plasma VC concentrations in the MMTV-Neu GULO knockout mice in the treatment group may not have been low enough to inhibit HIF-1α inactivation. As plasma VC concentrations of 10umol/L are sufficient to prevent scurvy, these concentrations may also be sufficient for the role of VC as a cofactor in HIF-1α inactivation

In this study, we determined low-doses of VC had no effect on tumorigenesis. Future studies could introduce a high-dose (3300mg VC/L drinking water) treatment group, or introduce intravenous administration of VC to bypass the tightly regulated absorption of VC in the digestive tract and achieve higher plasma VC concentrations. The anti-HIF and hydrogen peroxide generating activities of VC are increased at high concentrations, therefore high-dose or intravenous administration of VC may have anti-

tumorigenic effects. The effect of tumour burden on plasma AA levels in the MMTV-Neu GULO knockout mouse could also be explored. In previous studies C57BL/6 GULO knockout mice with subcutaneously injected melanomas or carcinomas have been shown to have lower plasma VC levels than *GULO* knockout mice with no tumour burden (Cha et al., 2013). This is consistent with clinical studies in which patients display abnormally low levels of plasma VC (Anthony and Schorah, 1982; Nunez Martin and Ortiz de Apodaca y Ruiz, 1995). If this phenomenon is also present in MMTV-Neu GULO knockout mice, it could have implications in the RDA for VC in cancer patients.

Conclusion

The role of vitamin C in the etiology and progression of degenerative disease has been under constant debate, despite over 50 years of research. In this study, we have demonstrated that low-dose versus normal-dose VC supplementation in a VC-deficient model of HER2-positive breast cancer does not affect the latency to palpable tumour development or the number or size of tumours that develop. We also demonstrated that GULO knockout mice can be maintained with low dose supplementation for an extended period of time without the development of scurvy.

Future work could include the study of high dose VC on breast tumorigenesis using higher levels of supplementation or intravenous administration. The effect of tumour burden on plasma VC concentrations could also be explored, to see if the reduced plasma VC in human cancer patients is also present in mice. Additionally, the methodology used in this study could be replicated to generate other VC-deficient mouse

models of human disease to further our understanding of the role of VC in cancer and other degenerative diseases.

Appendix

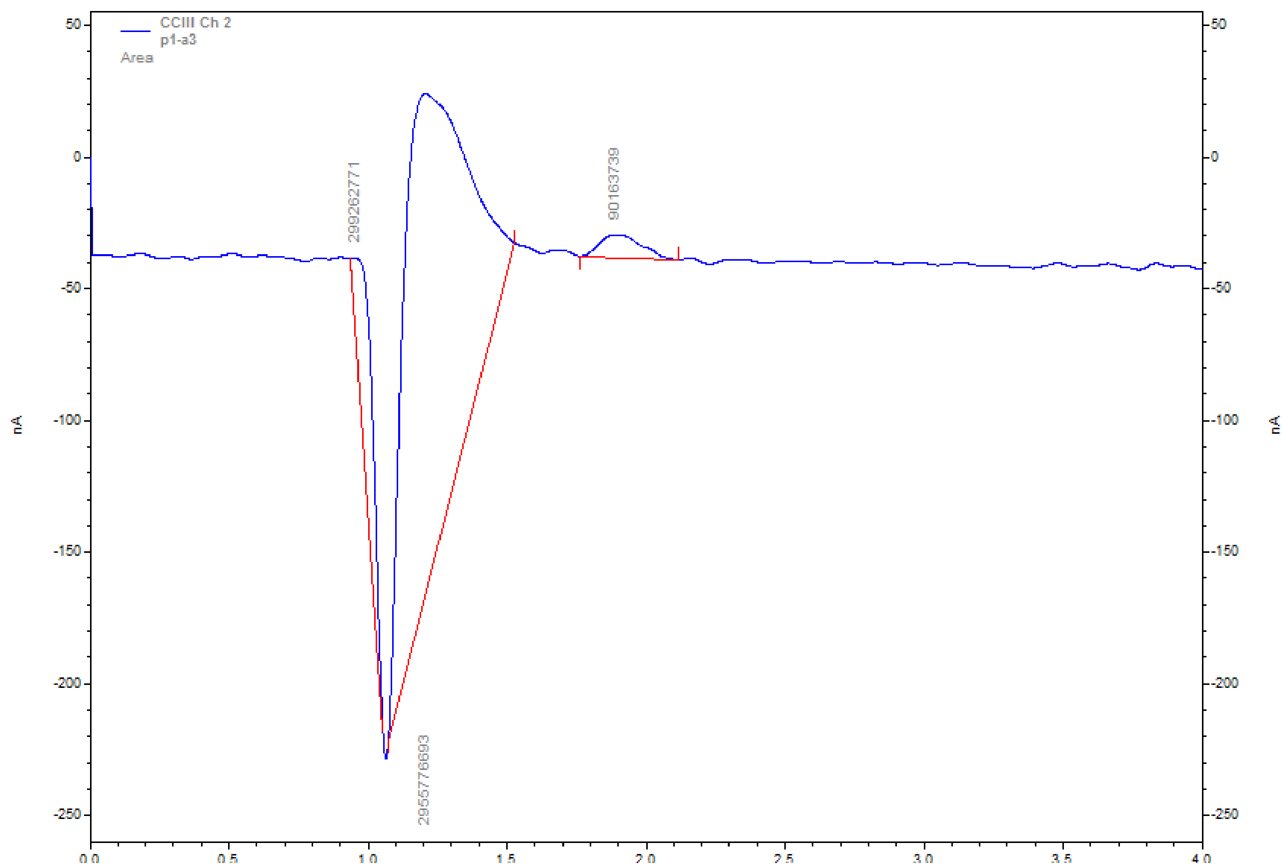

Figure 12: Example chromatogram from HPLC analyses of mouse blood samples.

The chromatogram shows the analysis of a plasma sample from a GULO knockout mouse maintained on vitamin-C free water. Ascorbic acid is eluted at approximately 1.9 minutes and concentration of ascorbic acid is determined by integration from valley to valley.

Table 2: Tumour characteristics for control and treatment group MMTV-Neu GULO knockout, GULO heterozygous and GULO wildtype mice

Group	VC dose	GULO genotype	Average latency to palpable tumours (days)	Average age at endpoint (days)	Average number of tumours at endpoint
Control	Normal (330 mg/L drinking H_20)	-/-	336.6 ± 32.4	467.0 ± 37.5	1.3 ± 0.3
		+/-	331.6 ± 32.9	412.0 ± 26.4.6	2.0 ± 0.8
		+/+	274.2 ± 30.5	345.5 ± 42.6	2.0 ± 0.2
Experimental	Low (33 mg/L drinking H_20)	-/-	260.3 ± 16.4	350.8 ± 27.6	1.7 ± 0.3
		+/-	298.3 ± 12.0	395.0 ± 18.0	1.0 ± 0.5
		+/+	238.5 ± 6.5	317.0 ± 30.0	1.5 ± 0.5

Table 3: Endpoint signs in GULO knockout mice

Category	**Endpoint description**
Body Condition Score	Significantly decreased; see body weight
Weight loss	>10% compared to age-matched control
Hind leg weakness	Inability to reach food or water
Dehydration	Sunken eyes, tented skin
Lethargy	Resistance to movement; hunched posture; no grooming
Righting reflex	Significantly increased; see lethargy
Bleeding gums	May be associated with decreased food/water intake/anorexia

Table 4: Tumour volume (cm^3) estimation.

Formula used for Tumour Volume Calculation:[4/3*(3.14159)*(Length/2)*(Width/2)^2]. Light grey indicates tumour is approaching 1.7cm^3 and dark grey indicated tumour is greater than 1.7cm^3

		Width (smallest measurement) (mm)																	
Length (largest measurement) (mm)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	1	0.00																	
	2	0.00	0.00																
	3	0.00	0.01	0.01															
	4	0.00	0.01	0.02	0.03														
	5	0.00	0.01	0.02	0.04	0.07													
	6	0.00	0.01	0.03	0.05	0.08	0.11												
	7	0.00	0.01	0.03	0.06	0.09	0.13	0.18											
	8	0.00	0.02	0.04	0.07	0.10	0.15	0.21	0.27										
	9	0.00	0.02	0.04	0.08	0.12	0.17	0.23	0.30	0.38									
	10	0.01	0.02	0.05	0.08	0.13	0.19	0.26	0.34	0.42	0.52								
	11	0.01	0.02	0.05	0.09	0.14	0.21	0.28	0.37	0.47	0.58	0.70							
	12	0.01	0.03	0.06	0.10	0.16	0.23	0.31	0.40	0.51	0.63	0.76	0.90						
	13	0.01	0.03	0.06	0.11	0.17	0.25	0.33	0.44	0.55	0.68	0.82	0.98	1.15					
	14	0.01	0.03	0.07	0.12	0.18	0.26	0.36	0.47	0.59	0.73	0.89	1.06	1.24	1.44				
	15	0.01	0.03	0.07	0.13	0.20	0.28	0.38	0.50	0.64	0.79	0.95	1.13	1.33	1.54	1.77			
	16	0.01	0.03	0.08	0.13	0.21	0.30	0.41	0.54	0.68	0.84	1.01	1.21	1.42	1.64	1.88	2.14		
	17	0.01	0.04	0.08	0.14	0.22	0.32	0.44	0.57	0.72	0.89	1.08	1.28	1.50	1.74	2.00	2.28	2.57	
	18	0.01	0.04	0.08	0.15	0.24	0.34	0.46	0.60	0.76	0.94	1.14	1.36	1.59	1.85	2.12	2.41	2.72	3.05

Table 5: Cross-cancer analysis of somatic mutations in the ERBB2/Her2 receptor tyrosine kinase isolated from human primary tumours

Mutation region (Position-Amino Acid)	Tumour histology	Molecular Consequence	Reference (Nucleotide one-letter code)	Variation (Nucleotide one-letter code)	DNA Change (position in coding sequence)	Amino Acid Change (One-letter code)	Domain	Mutation Assessor (Reva et al., 2011)	Clinical Significance
20	Lung (Kan et al., 2010)	Missense	G	A	58G>A	A20T	Extracellular		
39	Stomach (Cancer Genome Atlas Research, 2014b)	Missense	C	T		P39L	Extracellular	Neutral	
49	Glioblastoma (Cancer Genome Atlas Research, 2008)	Missense	T	A		L49H	Extracellular	Low	
75	Glioblastoma (Cancer Genome Atlas Research, 2008)	Splice	T	A		Q75_splice	Extracellular		
87	Pancreas (Cancer Genome Atlas Research)	Missense	G	A		A87T	Extracellular	Low	
92	Head and neck (Agrawal et al., 2011)	Missense	A	G		R92G	Extracellular	Low	
94	Lung (Cancer Genome Atlas Research)	Missense	G	A		V94I	Extracellular	Low	
100	Endometrial (Barretina et al., 2012)	Missense	C	T		R100W	Extracellular	Medium	
101	Uterine (Cancer Genome Atlas Research; Cancer Genome Atlas Research et al., 2013)	Missense	T	G		I101S	Extracellular	Medium	
103	Bladder (Cancer Genome Atlas Research; Cancer Genome Atlas Research et al., 2013) (Cancer Genome Atlas Research, 2014b)	Missense	G	A		R103Q	Extracellular	Medium	
122	Lung (Cancer Genome Atlas Research)	Missense	C	T		P122L	Extracellular	Low	
134	Bladder (Guo et al., 2013)	Synonymous-coding	A	T	402A>T	P134P	Extracellular		
138	Melanoma (Cancer Genome Atlas Research)	Missense	C	T		R138W	Extracellular	Medium	
139	Melanoma (Cancer Genome Atlas Research)	Missense	G	A		E139K	Extracellular	Medium	
152	Breast (Barretina et al.,	Frameshift	G	-		G152_fs	Extracellular		

	2012)	deletion							
152	Lung (Cancer Genome Atlas Research, 2012)	Missense	G	T		G152V	Extracellular	Medium	
156	Melanoma (Cancer Genome Atlas Research)	Nonsense	C	T		Q156*	Extracellular		
182	Head and neck (Cancer Genome Atlas Research)	Missense	C	T		T182I	Extracellular	Medium	
203	Glioma (Cancer Genome Atlas Research)	Missense	C	T		R203C	Extracellular (Cysteine-rich)	Low	
216	Glioblastoma (Cancer Genome Atlas Research, 2008)	Missense	A	T		T216S	Extracellular (Cysteine-rich)	Low	
217	Lung (Barretina et al., 2012)	Missense	G	A		R217H	Extracellular (Cysteine-rich)	Low	
219	Renal (Cancer Genome Atlas Research)	Missense	G	A		V219I	Extracellular (Cysteine-rich)	Neutral	
222	Bladder (Cancer Genome Atlas Research, 2014b) Stomach (Cancer Genome Atlas Research, 2014a)	Missense	G	T		G222C	Extracellular (Cysteine-rich)	Neutral	
226	Haematopoietic (Barretina et al., 2012)	Missense	G	A		R226H	Extracellular (Cysteine-rich)	Medium	
230	Large Intestine (Barretina et al., 2012)	Missense	C	T		P230L	Extracellular (Cysteine-rich)	Medium	
238	Bladder (Kim et al., 2014)	Missense	G	C		E238Q	Extracellular (Cysteine-rich)	Medium	
238	Cervical (Cancer Genome Atlas Research)	Missense	G	A		E238K	Extracellular (Cysteine-rich)	Low	
241	Bladder (Guo et al., 2013)	Missense	C	T	722C>T	A241V	Extracellular (Cysteine-rich)	Medium	
247	Melanoma (Cancer Genome Atlas Research)	Missense	C	T		P247S	Extracellular (Cysteine-rich)	Medium	
250	Bladder (Kim et al., 2014)	Missense	C	T		S310F	Extracellular (Cysteine-rich)	Medium	
254	Lung (Ding et al., 2008)	Missense	G	A		A254_splice	Extracellular (Cysteine-rich)		
263	Colorectal (Cancer Genome Atlas, 2012a; Cancer Genome Atlas Research)	Missense	T	C		I263T	Extracellular (Cysteine-rich)	Neutral	
277	Bladder (Guo et al., 2013)	Missense	G	T	829G>T	D277Y	Extracellular (Cysteine-rich)	Low	
277	Bladder (Guo et al.,	Missense	A	G		D277G	Extracellular	Low	

	2013)						(Cysteine-rich)		
280	Cervical (Cancer Genome Atlas Research)	Missense	G	C		E280Q	Extracellular (Cysteine-rich)	Neutral	
293	Melanoma (Cancer Genome Atlas Research)	Missense	C	T		A293V	Extracellular (Cysteine-rich)	Medium	
305	Breast (Cancer Genome Atlas Research)	Missense	C	G		S305C	Extracellular (Cysteine-rich)	Neutral	
306	Breast (Cancer Genome Atlas Research)	Missense	C	T		T306M	Extracellular (Cysteine-rich)	Low	
308	Lung (Cancer Genome Atlas Research)	Missense	G	A		V308M	Extracellular (Cysteine-rich)	Low	
309	Breast(Kan et al., 2010)	Missense	G	A	926G>A	G309E	Extracellular (Cysteine-rich)		Activatng(Greulich et al., 2012)
309	Breast (Bose et al., 2013; Cancer Genome Atlas, 2012b; Cancer Genome Atlas Research)		G	A	926G>C	G309A	Extracellular (Cysteine-rich)		Activating (Bose et al., 2013)
310	Bile duct (Chmielecki et al., 2015) Bladder (Cancer Genome Atlas Research, 2014b; Chmielecki et al., 2015; Guo et al., 2013; Kim et al., 2014) Breast (Banerji et al., 2012; Eirew et al., 2015; Shah et al., 2012) Cervical (Cancer Genome Atlas Research) Colorectal (Brannon et al., 2014; Chmielecki et al., 2015) Gall bladder(Chmielecki et al., 2015) Lung (Cancer Genome Atlas Research, 2012; Chmielecki et al., 2015; Ding et al., 2008) Renal (Chmielecki et al., 2015) Stomach(Cancer Genome Atlas Research, 2014a; Chmielecki et al., 2015; Wang et al., 2011; Wang et al., 2014) Small	Missense	C	T	C929T	S310F	Extracellular (Cysteine-rich)	Medium	Activating(Banerji et al., 2012; Greulich et al., 2012)

	intestine(Chmielecki et al., 2015) Ovarian (Cancer Genome Atlas Research, 2011; Chmielecki et al., 2015) Urinary Tract (Barretina et al., 2012)								
310	Bladder (Guo et al., 2013)	Missense	C	A	929C>A	S310Y	Extracellular (Cysteine-rich)	Medium	Activating (Greulich et al., 2012)
310 +755	Breast(Shah et al., 2012)					S310F+L755S	Extracellular (Cysteine-rich)		
311	Glioblastoma (Cancer Genome Atlas Research, 2008)	Missense	T	C		C311R	Extracellular (Cysteine-rich)	High	
313	Bladder (Cancer Genome Atlas Research, 2014b) Large Intestine (Barretina et al., 2012)	Missense	C	G		L313V	Extracellular (Cysteine-rich)	Low	
319	Lung (Barretina et al., 2012)	Missense	A	T		N319Y	Extracellular (Cysteine-rich)	Neutral	
319	Glioblastoma (Cancer Genome Atlas Research, 2008)	Missense	A	G		N319D	Extracellular (Cysteine-rich)	Medium	
321	Glioblastoma (Cancer Genome Atlas Research, 2008)	Missense	A	G		E321G	Extracellular (Cysteine-rich)	Medium	Activating (Greulich et al., 2012)
326	Glioblastoma (Cancer Genome Atlas Research, 2008)	Missense	A	G		D326G	Extracellular (Cysteine-rich)	Neutral	
334	Glioblastoma (Cancer Genome Atlas Research, 2008)	Missense	T	A		C334S	Extracellular (Cysteine-rich)	High	
335	Lung (Barretina et al., 2012)	Missense	A	T		S335C	Extracellular (Cysteine-rich)	Medium	
339	Pancreas (Cancer Genome Atlas Research)	Missense	G	A		A339T	Extracellular (Cysteine-rich)	Low	
387	Renal (Cancer Genome	Frameshift	-	C		S387fs	Extracellular		

	Atlas Research, 2013)	insertion							
393	Melanoma (Hodis et al., 2012)	Nonsense	C	T		Q393*	Extracellular		
401	Cervical (Cancer Genome Atlas Research)	Missense	G	C		E401D	Extracellular	Low	
418	Lung (Imielinski et al., 2012)	Missense	G	C		S418T	Extracellular	Medium	
432	Not specified (Barretina et al., 2012)	Missense	C	T		R432W	Extracellular	High	
466	Colorectal (Cancer Genome Atlas, 2012a)	Missense	G	A		A466T	Extracellular	Low	
470	Breast (Cancer Genome Atlas Research)	Missense	T	G		H470Q	Extracellular	Neutral	
541	Lung (Barretina et al., 2012)	Missense	G	A		V541M	Extracellular	Medium	
544	Liver (Ahn et al., 2014)	Missense	G	T		C544F	Extracellular	High	
562	Medulloblastoma (Pugh et al., 2012)	Missense	C	T		P562S	Extracellular	Low	
573	Bladder (Cancer Genome Atlas Research, 2014b)	Missense	C	T		S573L	Extracellular	Low	
582	Head and neck (Stransky et al., 2011)	Missense	G	A		D582N	Extracellular	Low	
594	Melanoma (Cancer Genome Atlas Research)	Missense	C	T		P594S	Extracellular	Neutral	
609	Bladder(Kim et al., 2014)	Missense	C	T		S609F	Extracellular	Medium	
612	Stomach (Cancer Genome Atlas Research, 2014a)	Missense	C	A		P612H	Extracellular	Medium	
633	Lung (Cancer Genome Atlas Research, 2012)	Splice	A	T		S633_splice	Extracellular		
635	Bladder(Kim et al., 2014)	Frameshift del	GGACCTGGATG ACAAGGGCTGC CCCGCCGAGCA GAGAGCCA	G		V635_fs	Extracellular		
644	Lung (Cancer Genome Atlas Research, 2012)	Missense	C	T		A644V	Extracellular	Low	
646	Not specified (Barretina et al., 2012)	Missense	G	C		Q646H	Extracellular	Medium	
649	Renal (Cancer Genome Atlas Research, 2013)	Missense	G	C		S649T	Extracellular	Low	
653	Urinary Tract (Barretina et al., 2012)	Missense	C	G		S653C	Transmembrane	Medium	
654	Breast (Ehsani et al.,	Missense				V654I	Transmembrane		

	1993)								
656	Bladder (Guo et al., 2013)	Missense	G	A	1967C>T	S656F	Transmembrane	Low	
658	Endometrial (Barretina et al., 2012)	Missense	T	C		V658A	Transmembrane	Low	
659	Li-Fraumeni Syndrome (Serra et al., 2013)	Missense	TT	AA		V659E	Transmembrane		Activating (Akiyama et al., 1991)
660	Cervical (Cancer Genome Atlas Research)	Missense	G	C		G660R	Transmembrane	Medium	
663	Uterine (Cancer Genome Atlas Research et al., 2013)	Missense	T	C		L663P	Transmembrane	Medium	
678	Bladder (Guo et al., 2013) Breast (Bose et al., 2013; Cancer Genome Atlas, 2012b) Colorectal (Brannon et al., 2014; Cancer Genome Atlas, 2012a) Large Intestine (Barretina et al., 2012) Prostate (Cancer Genome Atlas Research) Stomach (Cancer Genome Atlas Research, 2014a; Kakiuchi et al., 2014; Wang et al., 2011; Wang et al., 2014) Uterine (Cancer Genome Atlas Research et al., 2013)	Missense	G	A	2033G>A	R678Q		Low	
720	Large Intestine (Barretina et al., 2012)	Missense	T	C		L720P	Extracellular	Medium	
724	Stomach(Lee et al., 2006)	Missense	G	T	2172G>T	K724N	Kinase		
733	Bladder (Guo et al., 2013; Kim et al., 2014) Stomach (Lee et al., 2006)	Missense	C	T	2198C>T	T733I	Kinase	Low	
740	Stomach (Wang et al., 2014)	Missense	T	C		I740T	Kinase	Neutral	
742	Head and neck (Cancer Genome Atlas Research)	Missense	G	C		D742H	Kinase	Medium	
744	Large Intestine	Missense	G	A		E744K	Kinase	Neutral	

	(Barretina et al., 2012)								
746	Not specified	Missense	G	T	2237G>T	G746V	Kinase		
750	Glioblastoma (Cancer Genome Atlas Research, 2008)	Missense	T	A		V750E	Kinase	High	
751	Uterine (Cancer Genome Atlas Research et al., 2013)	Missense	G	A		A751T	Kinase	High	
755	Lung (Stephens et al., 2004) Renal (Cancer Genome Atlas Research)	Insertion	TT	CC	2263TT>CC	L755P	Kinase		Activating (Stephens et al., 2004)
755	Bladder (Cancer Genome Atlas Research, 2014b) Breast (Grob et al., 2012; Lee et al., 2006; Shah et al., 2009) Central Nervous System (Barretina et al., 2012) Large Intestine (Barretina et al., 2012) Stomach (Barretina et al., 2012; Lee et al., 2006; Wang et al., 2014) Uterine (Cancer Genome Atlas Research et al., 2013)	Missense	T	C	2264T>C	L755S	Kinase	Medium	Lapatinib resistance(Bose et al., 2013)
755	Colorectal (Seshagiri et al., 2012) Breast (Cancer Genome Atlas Research) Melanoma (Cancer Genome Atlas Research)	Missense	T	A		L755M	Kinase	Medium	
755	Breast (Cancer Genome Atlas, 2012b)	Missense	T	G		L755W	Kinase	High	
755-759+ 760	Breast (Lee et al., 2006)	In-frame Deletion+Missense			2263.2278del (TTGAGGGAAA ACACAT), 2262.2263insG	Del L755-T759+S760A	Kinase		
755 -759	Breast (Shah et al., 2009)	In-frame Deletion			2264.2278del	Del L755-T759	Kinase		
776 -779	Lung (Onozato et al., 2009; Stephens et al., 2004)	In-frame insertion	-	TGT	2322 ins/dup (GCATACGTGA TG)	M774 _insAYVM	Kinase		
774 -775	Lung (Imielinski et al.,	In-frame insertion	-	GCATACGTGAT		M774_A775insAY	Kinase		Activating

	2012)			G		VM			
780 -781	Breast(Stephens et al., 2012)	In-frame Insertion	-	GGGCTCCCC	2339.2340ins	ins780(GSP)	Kinase		Activating (Bose et al., 2013) Lapatinib resistance(Bose et al., 2013)
767	Bladder (Guo et al., 2013; Kim et al., 2014) Breast (Shah et al., 2009)	Missense	C	G	2301C>G	I767M	Kinase	Neutral	No effect (Bose et al., 2013)
769	Bladder (Kim et al., 2014) Breast (Shah et al., 2009) Esophageal (Dulak et al., 2013) Stomach (Cancer Genome Atlas Research, 2014a)	Missense	G	T	2305G>T	D769Y	Kinase	Low	Activating (Bose et al., 2013)
769	Breast(Bose et al., 2013) Cervical (Cancer Genome Atlas Research) Stomach(Lee et al., 2006)	Missense	G	C	2305G>C	D769H	Kinase	Neutral	Activating(Bose et al., 2013)
769	Bladder(Cancer Genome Atlas Research; Kim et al., 2014) Breast (Cancer Genome Atlas Research)	Missense	G	A		D769N	Kinase	Neutral	
776	Gastric tumor(Stephens et al., 2004) Bladder(Kim et al., 2014)	Missense	G	A	2326G>A	G776S	Kinase	Neutral	Activating
776	Esophageal (Dulak et al., 2013) Ovarian (Barretina et al., 2012) Stomach (Cancer Genome Atlas Research, 2014a)	Missense	G	V		G776V	Kinase	Neutral	Activating
776	Lung (Stephens et al., 2004)	Insertion			2326_2327insTGT	G776>VC	Kinase		Activating (Stephens et al., 2004)
777	Breast (Cancer Genome Atlas, 2012b; Kan et al.,	Missense	G G	T C	G2329T G2329C	V777L V777L	Kinase		Activating (Bose et al., 2013)

	2010; Loi et al., 2013; Secq et al., 2014) Colorectal (Brannon et al., 2014) (Lee et al., 2006) Esophageal (Dulak et al., 2013) Stomach(Lee et al., 2006) (Wang et al., 2014)								
777	Colon(Lee et al., 2006)	Missense	G	A	2329G>A	V777M	Kinase		
777	Glioblastoma (Cancer Genome Atlas Research, 2008)	Missense	T	C		V777A	Kinase	Low	
777	Stomach (Cancer Genome Atlas Research, 2014a)	Missense	G	A		V777M	Kinase	Neutral	
779	Lung (Onozato et al., 2009; Stephens et al., 2004)	Insertion	-	CTGTGGGCT	2335_insCTGTGG GCT	S779_P780insVGS	Kinase		Activating (Stephens et al., 2004)
780	Bladder (Kim et al., 2014)	Missense	C	T		P780L	Kinase	Medium	
780		Insertion	-	GGCTCCCCA	2340_2341insGGC TCCCCA	P780_Y781insGSP	Kinase		
783	Bladder (Kim et al., 2014)	Missense	C	T		S783F	Kinase	Low	
784	Bladder (Kim et al., 2014)	Missense	C	T		R784C	Kinase	Medium	
797	Breast (Cancer Genome Atlas Research)	Missense	T	C		V979A	Kinase	Medium	
798	Endometrial (Barretina et al., 2012)	Missense	C	T		T798I	Kinase	Low	
799	Stomach (Lee et al., 2006)	Missense	A	C	2396A>C	Q799P	Kinase		
816	Renal (Cancer Genome Atlas Research, 2013)	Missense	C	T		R816H	Kinase	Neutral	
816	Renal (Cancer Genome Atlas Research, 2013)	Missense	C	T		R816C	Kinase	Medium	
821	Ovarian (Cancer Genome Atlas Research, 2011)	Missense	G	T		D821Y	Kinase	Low	
828	Bladder (Guo et al., 2013)	Synonymous-coding	G	A	2484G>A	Q828Q	Kinase		
830	Thyroid (Barretina et al.,	Missense	C	T		A830V	Kinase	Medium	

	2012)								
835	Breast(Shah et al., 2012)	Missense	A	T	A2504T	Y835F	Kinase	Low	No effect (Bose et al., 2013)
842	Breast (Bose et al., 2013; Cancer Genome Atlas, 2012b) Colorectal (Cancer Genome Atlas, 2012a; Lee et al., 2006) Endometrial (Barretina et al., 2012) Lung (Barretina et al., 2012) Stomach (Wang et al., 2014) Uterine (Cancer Genome Atlas Research)	Missense	G	A	2524G>A	V842I	Kinase	Neutral	Activating (Bose et al., 2013)
857	Ovarian tumor(Stephens et al., 2004)	Missense	A	G	2570A>G	N857S	Kinase		Activating (Stephens et al., 2004)
861	Glioblastoma (Cancer Genome Atlas Research, 2008)	Frameshift deletion	A	-		I861fs	Kinase		
862	Bladder (Cancer Genome Atlas Research, 2014b; Guo et al., 2013)	Missense	A	G	2584A>G	TAA	Kinase	Neutral	
868	Colorectal (Cancer Genome Atlas, 2012a)	Missense	C	T		R868W	Kinase	High	
869	Stomach(Lee et al., 2006)	Missense	T	A	2909T>A	L869Q	Kinase		
873	Cervical (Cancer Genome Atlas Research)	Missense	G	A		D873N	Kinase	Low	
878	Liver (Bekaii-Saab et al., 2006)	Missense			2632C>T	H878Y	Kinase		Activating(Bekaii-Saab et al., 2006)
890	Prostate (Grasso et al., 2012)	Missense	C	T		A890V	Kinase	High	
894	Prostate (Barbieri et al., 2012)	Missense	A	C		A894L	Kinase	Neutral	
896	Endometrial (Barretina et al., 2012) Ovarian (Cancer Genome Atlas Research, 2011)	Missense	G	A		R896H	Kinase	Neutral	No effect (Bose et al., 2013)
896	Breast(Lee et al., 2006)	Missense	C	T	2686C>T	R896C	Kinase		Activating (Bose et al., 2013)
897	Uterine (Cancer	Missense	G	A		R897Q	Kinase	Neutral	

	Genome Atlas Research et al., 2013)								
906	Lung (Ding et al., 2008)	Nonsense	G	A		W906*	Kinase		
914	Glioblastoma (Stephens et al., 2004)	Missense	G	A	2740G>A	E914K	Intracellular		Activating (Stephens et al., 2004)
916	Head and neck (Cancer Genome Atlas Research)	Missense	G	A		M916I	Intracellular	Neutral	
930	Head and neck (Cancer Genome Atlas Research)	Missense	G	A		E930K	Intracellular	Low	
930	Pancreas (Cancer Genome Atlas Research) Prostate (Taylor et al., 2010)	Missense	G	T		E930D	Intracellular		
937	Thyroid (Cancer Genome Atlas Research, 2014d)	Missense	A	G		K937R	Intracellular	Neutral	
943	Lung (Imielinski et al., 2012)	Nonsense	C	T		Q943*	Intracellular		
962	Bladder (Guo et al., 2013)	Missense	G	A	2884G>A	D962N	Intracellular	Low	
974	Stomach (Cancer Genome Atlas Research, 2014a)	Missense	C	T		S974F	Intracellular	Medium	
993	Melanoma (Cancer Genome Atlas Research)	Missense	G	A		D993N	Intracellular	Medium	
1006	Head and neck (Cancer Genome Atlas Research)	Missense	C	T		R1006C	Intracellular	Medium	
1021	Lung (Cancer Genome Atlas Research)	Missense	G	C		E1021Q	Intracellular	Medium	
1023	Renal (Cancer Genome Atlas Research)	Frameshift deletion	T	-		Y1023_fs	Intracellular		
1039	Esophageal (Dulak et al., 2013) Stomach (Cancer Genome Atlas Research, 2014a)	Missense	G	A		A1039T	Intracellular	Neutral	
1050	Lung (Barretina et al., 2012)	Missense	C	T		S1050L	Intracellular	Low	
1052	Bladder(Guo et al., 2013)	Synonymous coding	C	G	3156C>G	T1052T	Intracellular		
1056	Colorectal (Seshagiri et al., 2012)	Missense	G	A		G1056S	Intracellular	Low	
1057	Lung (Cancer Genome Atlas Research, 2012)	Missense	G	T		G1057V	Intracellular	Low	

1058	Breast	Missense	A	C	3173A>C	D1058A	Intracellular		
1058	Head and neck (Gaykalova et al., 2014)	Missense	A	C		D1058A	Intracellular	Neutral	
1064	Head and neck (Cancer Genome Atlas Research)	Missense	G	C		E1064Q	Intracellular	Low	
1070	Renal (Cancer Genome Atlas Research)	Missense	C	T		A1070V	Intracellular	Low	
1079	Cervical (Cancer Genome Atlas Research) Esophageal (Dulak et al., 2013)	Missense	G	A		E1079K	Intracellular	Low	
1080	Uterine (Cancer Genome Atlas Research et al., 2013)	Missense	G	T		G1080W	Intracellular	Medium	
1114	Renal (Cancer Genome Atlas Research)	Missense	A	G		E1114G	Intracellular	Low	
1115	Multiple Myeloma (Lohr et al., 2014)	Missense	G	A		D1115N	Intracellular	Medium	
1136	Liver (Ahn et al., 2014)	Missense	A	G		Q1136R	Intracellular	Low	
1146	Pancreas (Cancer Genome Atlas Research)	Missense	G	A		R1146Q	Auto-phosphorylation site	Low	
1188	Melanoma (Cancer Genome Atlas Research)	Missense	GG	AA		G1188K	Auto-phosphorylation site		
1188	Lung (Cancer Genome Atlas Research, 2012)	Missense	G	T		G1188W	Auto-phosphorylation site	Low	
1199	Glioblastoma (Cancer Genome Atlas Research, 2008)	Missense	C	A		P1199T	Auto-phosphorylation site	Neutral	
1201	Breast (Banerji et al., 2012)	Missense	G	T		G1201V	Auto-phosphorylation site	Low	
1210	Breast (Banerji et al., 2012)					G1210V	Auto-phosphorylation site		
1210	Melanoma (Krauthammer et al., 2012)	Missense	C	T		P1210S	Auto-phosphorylation site	Neutral	
1214	Endometrial (Barretina et al., 2012)	Missense	G	A		S1214N	Auto-phosphorylation site	Low	
1219	Colorectal (Cancer Genome Atlas, 2012a)	Missense	A	G		N1219S	Auto-phosphorylation	Low	

							site		
1228	Liver (Ahn et al., 2014)	Missense	C	A		P1228T	Auto-phosphorylation site	Low	
1229	Large Intestine (Barretina et al., 2012)	Nonsense	G	T		E1229*	Auto-phosphorylation site		
1230	Lung (Cancer Genome Atlas Research, 2014c)	Frameshift insertion	-	G		R1230_fs	Auto-phosphorylation site		
1234	Head and neck (Cancer Genome Atlas Research)	Missense	C	T		P1234S	Auto-phosphorylation site	Neutral	
1237	Bladder (Kim et al., 2014)	Missense	C	G		F1237L	Auto-phosphorylation site	Neutral	
1244	Bladder (Cancer Genome Atlas Research, 2014b)	Missense	G	C		E1244Q	Auto-phosphorylation site	Low	
1244	Uterine (Cancer Genome Atlas Research et al., 2013)	Missense	G	T		E1244D	Auto-phosphorylation site	Neutral	

Point Form Standard Operating Procedures

Tail Clipping Mice (GEN485.sop)

1. Anaesthetize the animal using isoflurane
2. Ear notch the animal
3. Wipe the tail with an alcohol wipe and clip the tail (~1cm) using sharp, clean scissors
4. Wait 30 seconds then blot the area with gauze
5. Use a silver nitrate stick to cauterize the end of the tail
6. Return the animal to its cage
7. Monitor the animal for bleeding and recovery from anesthetic
8. Mark that cage card that the procedure has been done

Euthanasia of Rodents by Cervical Dislocation (GEN460.sop)

1. Catch the rodent by the base of the tial and place it “abdomen down” on a flat surface
2. Place a strong cylindrical object at the back of the neck
3. Push down hard on the neck while jerking the body backwards by pulling firmly on the base of the tail to dislocate the neck
4. Check to make sure there is no pulse and bag the dead rodents
5. Dispose of the bagged animals in the appropriate CAF freezer

Euthanasia of Rodents by Carbon Dioxide (GEN755.sop)

1. Preparing the animal
    a. Move the rodents to a post mortem room cull area
    b. Take the live animals one cage at a time to the euthanasia area to minimize exposure to visual and pheromone stimuli while other animals are being euthanized
2. Preparing the euthanizing cage

a. Secure the CO2 lid on a clean holding cage without bedding material or enrichment

3. Use of the euthanizing cage a. Slow fill method

i. Place the animals in the CO2 chamber (the total number of animals should not cover more than 80% of the floor space)

ii. Slowly turn on the large high pressure valve of the regulator

iii. Slowly open the low pressure CO2 valve and gradually increase the rate to 3-5psi

iv. Continue to slowly run CO2 into the euthanizeing cage until all animals have stopped breathing

v. Confirm death with a secondary physical method (i.e. cervical dislocation or thoracotomy)

4. Completion of operation

a. When all animals have been euthanized, turn off the large valve on the tank to allow the system to drain

b. Shut off the small valve

c. Bag and dispose of the animal bodies in the appropriate freezer

DNA Isolation Kit Protocol (Norgen Kit #24700)

1. Preparing Lysate from Animal Tissues
    a. Place the tail sample in a nuclease-free microcentrifuge tube
    b. Add 250µl of homogenization solution and cut up the tissue with small scissors
    c. Add 10 KUnitz of RNase A and vortex
    d. Add 250µl of the Lysis Solution and 12µl of Proteinase K to the solution and vortex
    e. Incubate at 55°C for 1hr
2. Genomic DNA Purification

a. Binding to column

1. Add 50µl of Binding Solution to the lysate and vortex
2. Assemble a micro spin column
3. Add 500µl to the column and centrifuge at 5200xg (8000RPM) for 3min
4. Add the rest of the lysate and centrifuge at 5200xg for 3min
5. Discard the flowthrough and reassemble the spin column

b. Washing bound DNA

1. Add 500µl of Wash Solution I and centrifuge at 14000xg (14000RPM) for 1min

2. Discard the flowthrough

3. Add 500µl of Wash Solution II and centrifuge at 14000xg (14000 RPM) for 2 minutes
4. Dry spin at 14000xg for 1 minute

c. Elution

1. Assemble the spin column on the Elution tube
2. Add 200µl of Elution Buffer (or water) and centrifuge at 3000xg (6000RPM) for 1min to hydrate the DNA
3. Centrifuge at 14000xg (14000RPM) for 2min to elute the DNA
4. Repeat steps C2 and C3 to increase yield

d. Storing DNA a. Store the purified genomic DNA at 2-8°C for a few days or at -20°C for longer periods

Polymerase Chain Reaction (PCR)

1. Prepare PCR reagents (per reaction): 12.5µl H20, 2.5µl 10x buffer –MgCl2, 1.5µl MgCl2, 0.5µl dNTP (10mM), 1µl P2 (5'-CGCGCCTTAATTAAGGATCC-3'), 1µl P3 (5'- GTCGTGACAGAATGTCTTGC-3'), 1µl P4 (5'-GCATCCCAGTGACTAAGGAT-3') and 1µl Taq (5units/µl).
2. Add 4µl of DNA sample
3. Run the samples in the PCR cycler (95°C for 2:00, 95°C for 0:30, 52°C for 0:30, 72°C for 1:30 and repeat 30x then 72°C for 7:00 and hold at 4°C)
4. Run the PCR products on a 3% agarose gel
    a. Add 5µl of 6x loading dye to 25µl of reaction mix and load 17µl

b. Run with a 100bp ladder and positive (known heterozygous sample) and negative (no DNA, just ddH20) controls

c. Wildtype band shows at 330bp (P3-P4) and knockout band shows at 230bp (P2-P3)

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